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Pharmacodynamics of Non-Steroidal Anti-Inflammatory Agents in Acute Inflammation and Chronic Pain in the Horse.

Jane Granville Owens
Louisiana State University and Agricultural & Mechanical College

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Owens, Jane Granville, Ph.D.

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300 N. Zeeb Rd.
Ann Arbor, MI 48106

PHARMACODYNAMICS OF NONSTEROIDAL ANTI-INFLAMMATORY AGENTS IN ACUTE INFLAMMATION AND CHRONIC PAIN IN THE HORSE

A Dissertation

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through the Department of Veterinary Physiology, Pharmacology and Toxicology

by
Jane Granville Owens
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Dedicated to my parents,
Edwin D. and Jeannine H. Owens

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ABSTRACT

The analgesic and anti-inflammatory effects of the nonsteroidal anti-inflammatory drugs (NSAIDs) ketoprofen (2.2 and 3.63 mg/kg) and phenylbutazone (4.4 mg/kg) were compared in equine models of acute synovitis and chronic hoof pain. The eicosanoids, prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4), increased dramatically in synovial fluid after carrageenan-induced synovitis of the intercarpal joint. PGE_2 concentrations in untreated horses peaked at 9 hours while LTB_4 concentrations peaked in all horses at 3 hours. Synovial fluid concentrations of both eicosanoids returned to near baseline by 48 hours. Lameness, joint temperature, and synovial fluid volume, protein and nucleated cells increased at 3 to 12 hours with reduction to near baseline levels by 48 hours. NSAIDs when given intravenously decreased joint concentrations of PGE_2, but LTB_4 levels were unaffected by drug administration. Both drugs decreased the signs of inflammation and lameness, but phenylbutazone was more effective. These data suggest that leukotrienes are involved in equine synovitis and the development of specific leukotriene inhibitors may be of therapeutic value. The plasma half-life of ketoprofen (2.2 mg/kg) in normal horses (0.88 hours) was higher than horses with synovitis (0.55 hours). Synovial fluid levels of ketoprofen in horses with synovitis were 6.5 times higher than normal horses at one hour. The area under the synovial fluid concentration curve for horses with synovitis was greater than in normal horses. These data suggest that the inflamed joint may serve as a site of sequestration for ketoprofen. Digital vein eicosanoid levels from

horses with hoof pain from chronic laminitis were not different than those in normal horses. Although hoof pain and lameness could not be attributed to eicosanoids, both effects were reduced by the systemic administration of NSAIDs. Ketoprofen at a dose of 3.63 mg/kg (phenylbutazone equimolar dose) reduced hoof pain and lameness to a greater extent than the 2.2 mg/kg dose and phenylbutazone. These effects were still present at 24 hours for 3 of the 4 measures of hoof pain. These data suggest that phenylbutazone was more potent in alleviating acute joint inflammation whereas ketoprofen at a dosage rate of 1.65 times the therapeutic dose was more potent in alleviating chronic pain and lameness in horses.

CHAPTER 1

GENERAL INTRODUCTION

This dissertation is organized in the journal style. A global introduction and literature review precede separate chapters on specific themes, which are followed by a general conclusion chapter. The literature review (Chapter 2) encompasses three areas that were relevant to the course of this dissertation research: inflammation, pain and the use of nonsteroidal anti-inflammatory drugs to alleviate these conditions. Chapters 3 through 6 describe the development of models and analytical techniques along with the experiments used in the testing of the overall research hypothesis and specific objectives. Chapter 7 consists of an overall summary of results, conclusions and indications for future research.

This dissertation research was designed to bring further understanding to the mechanisms and alleviation of pain and inflammation in the horse. Inflammation occurs in many forms in the horse. This pathological process results in significant morbidity and often, mortality. Musculoskeletal inflammation in particular shortens the working life of the horse and results in considerable pain. Our treatment options at present are limited to a few steroidal and nonsteroidal agents that have been approved for use in the horse. Many of these products are older drugs that were originally used in humans. Researchers of nonsteroidal anti-inflammatory agents have tried to identify novel drugs that are more efficacious in relieving inflammation and pain than traditional drugs. This research has lead to the discovery of a more

complex array of inflammatory mediators and modulators. Since the discovery of the pro-inflammatory leukotrienes, emphasis has been placed on developing inhibitors of lipoxygenase and specific leukotriene antagonists.

The research hypothesis of this dissertation is that nonsteroidal anti-inflammatory drugs (NSAIDs) that inhibit the cyclooxygenase and lipoxygenase mediated breakdown of arachidonic acid are potentially more effective alleviators of pain and inflammation than NSAIDs that solely inhibit cyclooxygenase. The overall objective of this research was to compare the putative cyclooxygenase and lipoxygenase inhibitor, ketoprofen to the cyclooxygenase inhibitor, phenylbutazone in equine models of acute joint inflammation and chronic pain. Prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) were chosen as measures of cyclooxygenase and lipoxygenase activity, respectively, based on their potent inflammatory and pain mediating properties. Specific objectives were as follows:

1) Develop a self-limiting, reproducible model of acute synovitis which increases prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) concentrations in synovial fluid and produces other measurable joint inflammatory responses.

2) Compare the magnitude and time course of the anti-inflammatory and eicosanoid inhibitory effects of ketoprofen and phenylbutazone in the acute synovitis model.

3) Compare the ability of ketoprofen and phenylbutazone to reduce the clinical signs of acute synovitis.

4) Compare the time course of the anti-inflammatory effects of ketoprofen with its plasma and synovial fluid drug concentrations.

5) Determine whether digital vein eicosanoid concentrations are greater in horses with chronic laminitis than normal horses.

6) Providing that there are higher eicosanoid concentrations in laminitic versus normal horses, correlate the severity of hoof pain with the eicosanoid concentrations.

7) Determine the magnitude and time course of the eicosanoid inhibitory effects of ketoprofen and phenylbutazone in horses with chronic laminitis.

8) Objectively quantitate and compare the analgesic effects of ketoprofen and phenylbutazone in horses with chronic hoof pain associated with chronic laminitis.

CHAPTER 2

LITERATURE REVIEW

A. Introduction

This review will focus on the specific types of inflammation and pain associated with the experimental models used in this dissertation research. Emphasis will be placed on the particular drugs and eicosanoids which influence inflammation. Experimental models of joint inflammation will be discussed. In particular, carrageenan-induced joint inflammation and the clinical diseases of arthritis, synovitis and chronic laminitis will be reviewed in detail. The properties of the eicosanoids, prostaglandin E_2 and leukotriene B_4 will be discussed as well as the inhibition of these substances by the nonsteroidal anti-inflammatory agents (NSAIDs), ketoprofen and phenylbutazone. A discussion of acute and chronic pain will also be included.

B. Inflammation

Inflammation is a local, defensive process elicited by tissue insult resulting ultimately in destruction, dilution or isolation of the offending agent and injured tissues. This dynamic process in most cases restores homeostasis, but if unregulated may have deleterious effects on the affected tissue or organ. Inflammation was originally described by the first century writings of the Roman, Cornelius Celsus as: *rubor et tumor cum calore et dolore*, "redness (erythema) and swelling (edema) with heat and pain (nociception and hyperalgesia)." The founder of modern cellular pathology, Rudolph Virchow, added the fifth cardinal sign of inflammation, *functio*

laesa or loss of function [1]. Inflammation typically occurs in three phases: 1) an acute transient phase characterized by local dilation of arterioles, capillaries, and venules resulting in increased blood flow and increased vascular permeability with exudation of fluids and plasma proteins; 2) a delayed subacute phase marked by infiltration of leukocytes and phagocytic cells; and 3) the chronic proliferative phase in which tissue degeneration and fibrosis lead to healing or chronic inflammation. [2,3].

1. Stimuli

Inflammation may be elicited by a number of stimuli including: infectious agents such as microbial organisms and parasites, ischemia, antigen-antibody interactions, exposure to radiation or electrical energy, extreme temperatures, mechanical trauma and noxious chemicals [3,4]. Experimentally, chemical irritants have been used extensively to mimic the inflammatory response.

Regardless of the etiology, most forms of acute and chronic inflammation involve the cellular and humoral components of the immune system. The inciting cause is first recognized as a foreign antigen by surface antibodies on B lymphocytes or via macrophage presentation to receptors on T lymphocytes. This recognition leads to the production of pro-inflammatory substances which result in transitory vasoconstriction followed by arteriolar and precapillary vasodilation, capillary recruitment, and increased vascular permeability due to contraction of endothelial cells. Fluid and protein exudation follow as blood flow is first increased then decreased resulting in hyperemia and congestion. Leukocytes marginate within the

vessels and adhere to vascular endothelium. Ultimately, these cells migrate to the inflammatory locus and begin degranulating, leading to destruction of the inciting agent and surrounding tissue. Destruction of the antigen is the responsibility of the phagocytic cells: neutrophils; monocytes and in some cases, eosinophils. The tissue macrophages, e.g. Kupffer cells and type-A synovial lining cells, also play important local roles [2,4].

2. Mediators

a. Eicosanoids

Inflammation is mediated and in some cases modulated by various autocoids or local hormones. The eicosanoids are a family of lipid-derived autocoids that include the prostaglandins, thromboxanes, leukotrienes and the lipoxins. Eicosanoids are derived from 20-carbon polyunsaturated essential fatty acids [5]. Arachidonate is the most abundant precursor of the eicosanoids and it is derived from dietary linoleic acid. Arachidonic acid is then esterified to the phospholipids of cell membranes or other complex lipids.

i. Release of arachidonate

Arachidonic acid is released from cellular lipids *de novo* by acyl hydrolases. This release is closely regulated and occurs in response to reduced oxygen tension, thrombin generation, hormones, immunologic reactions, ultraviolet light, tumor-producing agents or other irritant stimuli that interact with membrane bound receptors coupled to guanine nucleotide-binding regulatory proteins. These G proteins then either directly activate phospholipase C and or A_2 or indirectly activate these enzymes

through an increase in cytosolic calcium. Phospholipase A_2 hydrolyzes the ester bond of membrane phospholipids such as phosphatidylcholine and phosphatidylethanolamine to release arachidonate. Phospholipase C cleaves the phosphodiester bond, resulting in the formation of a 1,2-diglyceride leading to the release of arachidonic acid [6]. Once arachidonic acid is freed it may be acted upon by the cyclooxygenase or the lipoxygenase enzyme systems to produce eicosanoids (Figure 1). In addition, arachidonic acid may be metabolized by the epoxygenase pathway in neutrophils through a cytochrome P-450-dependent mixed-function oxidase leading to the formation of unstable epoxides [7].

ii. Enzymes

The first enzyme in the production of prostaglandins is prostaglandin endoperoxide synthase or as it is more commonly called, fatty acid cyclooxygenase. This microsomal enzyme is present in all cells, except mature erythrocytes [6]. This enzyme has two activities; one is an endoperoxide synthase activity that oxygenates and cyclizes arachidonate acid to form the cyclic endoperoxide prostaglandin G_2 (PGG_2) and a peroxidase activity that converts PGG_2 to prostaglandin H_2 (PGH_2) by reducing the 15-hydroperoxy group to a 15-hydroxy. Isomerases synthesize prostaglandin E_2 (PGE_2), prostaglandin D_2 (PGD_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from PGH_2. The unstable endoperoxide PGH_2 is also metabolized to thromboxane A_2 (TXA_2) and prostacyclin (PGI_2) [8]. The specific prostaglandins produced vary with different cells or tissues depending upon the synthases and isomerases present [9]. Lipoxygenase enzymes are found in the pulmonary tissue, platelets and leukocytes [6].

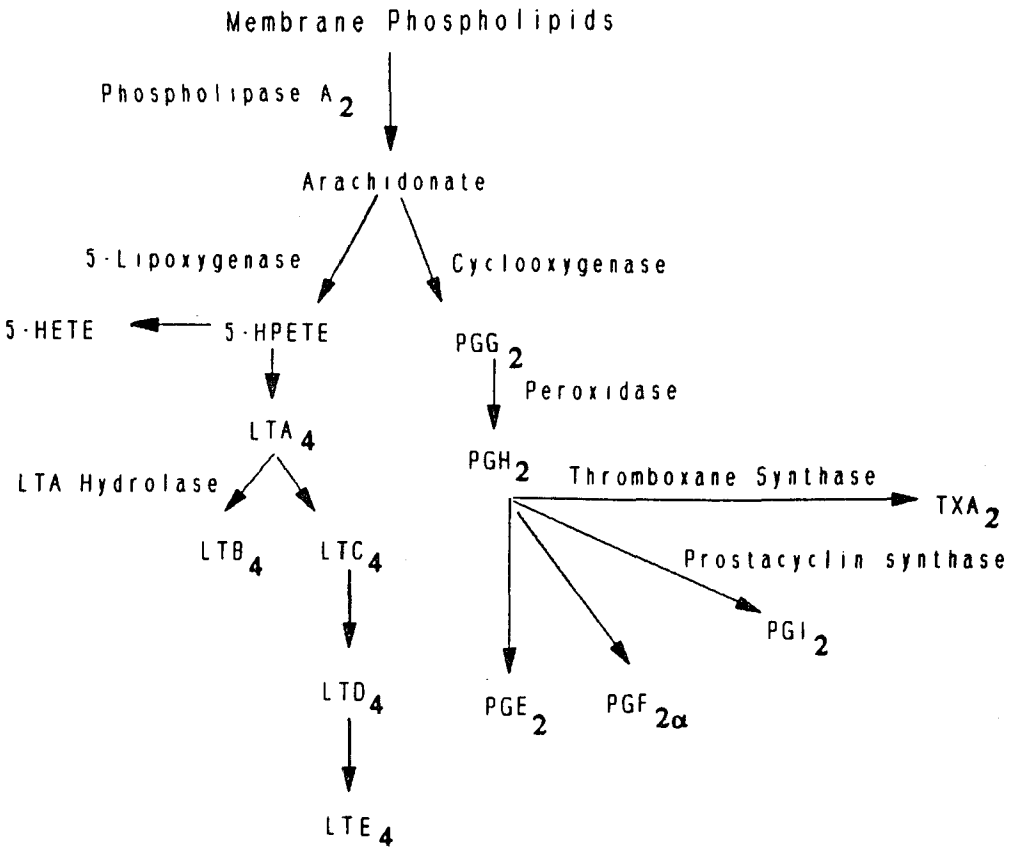


Figure 1: Enzymatic release and metabolism of arachidonate.

Lipoxygenases are a group of membrane-associated enzymes that oxygenate polyunsaturated fatty acids to lipid hydroperoxides [10]. The enzymes require a fatty acid substrate with two *cis* double bonds separated by a methylene group and are regulated by the presence of calcium [6]. The arachidonate metabolites of lipoxygenase are called hydroperoxyeicosatetraenoic acids (HPETEs). This enzyme system, like the cyclooxygenase enzyme, goes through an activation phase where it generates metabolites that act as positive and then negative feedback regulators [10,11]. Lipoxygenases differ in their specificity for placing the hydroperoxy group and tissues differ in the lipoxygenases they contain. For instance, platelets have only 12-lipoxygenase while leukocytes have 5-, 12-, and in some species, 15-lipoxygenases [12]. The HPETEs are unstable intermediates analogous to PGG_2 or PGH_2 and are further degraded by a series of enzymes. HPETEs may be converted to hydroxy fatty acids (HETEs) by a peroxidase or nonenzymatically. In a relatively newly elucidated pathway 12-HPETE can undergo a molecular rearrangement to form the hepoxillins while 15-HPETE may be converted by leukocytes to the lipoxins [6]. The 5-lipoxygenase enzyme leads to the synthesis of the pro-inflammatory leukotrienes through the metabolite, 5-HPETE. Leukotriene A synthase catalyzes the formation of the unstable 5,6-epoxide leukotriene A_4 (LTA_4) from 5-HPETE. Leukotriene B_4 (LTB_4) or (5S,12R)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid is formed from LTA_4 by LTA hydrolase. Alternatively, LTA_4 can be nonenzymatically hydrolyzed to diastereomers of 5,6-dihydroxyeicosatetraenoic acid (5,6-diHETE), and 6,8,10-*trans*-14-*cis*-diastereomers of 5,12-diHETE [13]. These hydrolysis products

have little biological importance as compared to LTB_4. In a separate microsomal pathway, LTA_4 may be conjugated with glutathione at the 6 position to form the sulfidopeptide leukotrienes: LTC_4, D_4, E_4, and F_4. LTC_4, LTD_4 and LTE_4 are also known as the slow-reacting substances of anaphylaxis [13].

iii. Prostaglandins

Prostaglandins are named according to substitutions on the cyclopentane ring and numbered according to the number of double bonds in the alkyl side chains [5]. The number 1 carbon is located at the carboxyl terminus. Prostaglandins of the subscript 2 series are the major ones in mammals and this nomenclature denotes derivation from arachidonic acid whereas the subscript 1 and 3 series may come from other polyunsaturated fatty acid precursors [5,14]. Prostaglandins of the E and D type are hydroxyketones while the F_α series are 1,3 diols [6]. Many tissues have specific prostaglandin receptors through which cell function is regulated via two second messenger systems: regulation of intracellular synthesis of cyclic AMP by activation or inhibition of adenylate cyclase and stimulation of phospholipase C resulting in the formation of inositol-1,4,5-triphosphate leading to a rise in intracellular calcium. These messenger systems in turn regulate various protein kinases which control cellular activity [6].

By most reports, the predominant stable prostaglandin product of leukocytes is PGE_2 [15]. PGE_2 produces long lasting dermal erythema and increased blood flow in cutaneous vessels and superficial vessels. PGE_2 produces little plasma exudation alone, but potentiates exudation after injection of chemical irritants, histamine and

bradykinin [16,17]. Prostaglandins increase vascular permeability in conjunction with serotonin and bradykinin by inducing vascular leakage in venules [18]. The effect of PGE_2 on plasma exudation has been studied in the skin of horses [19]. When PGE_2 was co-administered with bradykinin the volume of plasma exudate increased markedly. This synergistic effect was not seen with PGE_2 and histamine in equine skin. PGE_1 has similar activities as PGE_2, but it is less abundant [6]. In contrast to other species, PGE_2 is chemotactic for equine neutrophils at concentrations of 1 and 10 ng/ml [20].

The functions of T and B cells are modified by PGE_2 *in vitro* at concentrations similar to inflammatory exudates (10^{-8} M). Secretion of interleukin-2 is inhibited by PGE_2 in T cells accounting for inhibition of mitogen and antigen-induced T cell proliferation by PGE_2 [9]. This inhibition and the inhibitory effects of PGE_2 on B cell proliferation is associated with elevated intracellular levels of cyclic AMP [9,18]. Human rheumatoid T cells are particularly susceptible to this effect of PGE_2 which leads to a deficient production of interferon and other lymphokines [9]. Several other inflammatory modulation activities have been demonstrated by PGE_1 and PGE_2 such as: inhibition of LTB_4 production [21], inhibition of lysosomal enzyme release [22], inhibition of O_2 release and inhibition of neutrophil activation [23]. Thus, there appear to be paradoxical anti-inflammatory properties as well as pro-inflammatory effects associated with prostaglandins.

Prostanoids are not stored but are synthesized *de novo* and rapidly metabolized and excreted in the urine. TXA_2 and PGI_2 hydrolyze in less than 2 minutes to the

more stable compounds, TXB_2 and 6-keto $PGF_{1\alpha}$, respectively [9]. $PGF_{2\alpha}$ can be metabolized by a 9-keto reductase to form PGE_2 in some tissues [6]. PGE_2 is metabolized initially to 15-keto-13,14-dihydro-PGE_2 [24]. This reaction is catalyzed by 15-hydroxy prostanoate dehydrogenase and Δ^{13}-reductase which are present in most tissues, but are found in high concentrations in the liver, kidney and lung [24]. The metabolites of prostaglandins are cleared from the circulation very quickly [25] by one or two steps of β- and ω-oxidation in the lung and liver [24,26].

iv. Leukotrienes

Leukotrienes of the subscript 4 series are derived from arachidonic acid. Other polyunsaturated fatty acid precursors give rise to the 3 and 5 series [5]. Receptors for LTB_4, LTC_4 and LTD_4/LTE_4 have been identified which respond by activation of phospholipase C. LTB_4 receptors have been found on neutrophils and monocytes, while receptors for LTD_4 and E_4 have been found on smooth muscle cells [6].

LTB_4 promotes leukocyte adherence, chemotaxis and degranulation [27,28]. The chemotactic properties of LTB_4 are particularly potent, both *in vitro* and *in vivo*. This activity is dependent upon the *cis-trans-trans*-triene structure [13]. LTB_4 has been demonstrated to be a potent chemotactic agent for equine [20] and bovine neutrophils [29]. In the horse, maximum chemotactic activity is reported to be obtained at concentrations that are higher than other species [20]. LTB_4 promotes the secretion of inflammatory products by neutrophils including active oxygen molecules, hydrogen peroxide, superoxide and hydroxyl radical and other degradative enzymes [30]. Also, LTB_4 increases vascular permeability and plasma exudation in the cheek

pouch [13] and this effect is greatly potentiated by the co-administration of bradykinin or PGE_2 in the rabbit, guinea pig and rat [31,32]. LTC_4 and LTB_4 may stimulate the production of gamma interferon. LTB_4 is a potent inhibitor of human T cell mitogenesis. This inhibition may be accounted for by the ability of LTB_4 to induce maturation and enhance the activity of T-suppressor and cytotoxic cells [9]. LTA_4 can be transcellularly metabolized to LTB_4 by erythrocytes or in blood plasma [10,33]. The ω-oxidation enzymes responsible for metabolism and inactivation of these compounds are part of the cytochrome P-450 family and like the prostaglandins utilize P-450 reductase [10]. The neutrophil is primarily responsible for sequestering and metabolizing LTB_4 to 20-hydroxy-LTB_4 which is then excreted in the urine [10]. However, complete β-oxidation accounts for the major route of metabolism for LTB_4 [34].

b. Other mediators

Vasoactive amines, kinins, cytokines, plasma-derived factors, leukocytic products and phospholipid products play important roles in the pathogenesis of inflammation. Many of these mediators act synergistically with the eicosanoids in perpetuating inflammation as well as pain.

Histamine was one of the first chemicals described as an inflammatory mediator. When released by basophils, mast cells and platelets, it initiates the early vascular responses and maintains this effect for 30 to 60 minutes [4]. Intradermally administered histamine produces arteriolar dilation and also results in a flare of erythema in the surrounding tissue. The kinins, bradykinin and kallidin are liberated

by the action of kallikrein enzymes on serum kininogen [4]. Bradykinin and histamine potentiate vascular permeability in conjunction with the eicosanoids [17]. Bradykinin has been shown to stimulate phospholipase A_2 activity and PGE_2 release [35]. Serotonin and histamine increase vascular permeability exclusively at the level of the post-capillary venules [36]. Serotonin also has vasodilatory properties. However, the actions of serotonin differ among animal species. In rodents this amine is contained within tissue mast cells and has permeability increasing properties. This property is not shared by man or many other species [36].

The volume of plasma exudation following intradermally administered histamine, bradykinin, serotonin or the E-series of prostaglandins varies between species [19]. Histamine and bradykinin, but not serotonin, produced circular lesions when injected intradermally into thoracic skin of horses [19]. According to this study, histamine was more potent than bradykinin on a molar basis in producing vascular leakage and lesion formation in the horse.

Interleukin-1 is a polypeptide produced in macrophages, synovial fibroblasts and other cells after infection, injury or antigenic challenge. This cytokine has hormone-like effects systemically, but also produces distinct local effects. It acts as a pro-inflammatory substance by inducing endogenous pyrogen fever, stimulating cellular metabolism and promoting eicosanoid release [37]. Blockade of the interleukin-1 receptor inhibits PGE_2 and LTB_4 generation in human monocytes [38]. Interleukin 1 acts synergistically with many other cytokines including tumor necrosis factor [37]. Tumor necrosis factor is also associated with eicosanoid production [39].

Plasma derived factors such as complement also potentiate the release of the eicosanoids from cells [18,40]. Complement fragments and prostaglandin E_2 act synergistically to produce intradermal edema in rabbits [36,41].

Neutrophils are known to release prostaglandins of the E type during phagocytosis and release of lysosomal enzymes [18]. The lysosomal products of neutrophils are classified as: cationic proteins, acid proteases and neutral proteases. These enzymes are responsible for increased vascular permeability, chemotaxis of monocytes and immobilization of neutrophils, degradation of basement membranes under an acid pH, release of kinin from plasma kininogen and the degradation of collagen, elastin, renal basement membrane, cartilage and fibrin [1].

The tachykinin neuropeptides, substance P, neuropeptide Y, calcitonin gene-related peptide and the neurokinins A and B play important roles in the regulation of inflammation and immune response in peripheral tissues and in the central nervous system [42]. Substance P is most likely responsible for the local flare response after intradermally injected histamine. This neurokinin is also thought to mediate neurogenic inflammation as it induces increased vascular permeability after stimulation of C-fibers [36].

Platelet-activation factor, like the eicosanoids, is derived from membrane phospholipids and is synthesized *de novo*. This compound is produced by inflammatory cells, platelets, endothelial cells and renal tissues. It induces bronchospasm, hypotension, neutropenia, thrombocytopenia, increased vascular permeability and is chemotactic for leukocytes [6].

3. Models of acute inflammation

Inflammatory models used to assess the potencies and duration of action of anti-inflammatory drugs in animals include the intradermal or subcutaneous injection of carrageenan, zymosan, *Bordetella pertussis*, and immune complexes [43]. Dermal inflammation has been produced by the application of ultraviolet light [44], inflammatory mediators [41] and compound 48/80 [41,44-46]. The mammary gland of ruminants has been used as a unique acute inflammation model. Intramammarily injected lipopolysaccharides in goats produced a self-limiting inflammation that resolves within one week [47]. Many of these models are also used in pain research and in some cases they may produce a chronic inflammation.

a. Subcutaneous inflammation

Most models of subcutaneous inflammation involve the injection of carrageenan. These models were developed in laboratory rodents and have been adapted for use in the horse. Other methods of producing subcutaneous inflammation in the horse have been reported such as the subcutaneous injection of a counterirritant consisting of iodine, ether and soybean oil [48]. This method was used to test the antiinflammatory effects of the metalloprotein, orgotein.

i. Carrageenan

The sulphated mucopolysaccharide carrageenan has been used experimentally to produce local inflammation in rabbits, rats, dogs, horses and pigs [49-53]. The major source of carrageenan is the alga *Chondrus crispus* which grows abundantly in the area known as Carragheen near Waterford, Ireland. Carrageenan can also be

extracted from the seaweed *Gigartina stellata.* The extracted mucopolysaccharide can be treated with potassium chloride to form two fractions: the gel fraction, κ-carrageenan, and the non-gel forming λ-carrageenan. λ-Carrageenan is primarily composed of sulfated D-galactose residues with a molecular weight between 3.5 and 4 x 10^5. λ-Carrageenan is the more potent fraction in producing acute and chronic inflammation, anticoagulation and toxicity. Carrageenan when administered subcutaneously appears to be very poorly absorbed systemically. However, some absorption may occur in the rat [54].

The toxicity of intradermal carrageenan is thought to progress through a complicated pathway that was elucidated by Vinegar, *et al.* [55]. The inflammatory response to carrageenan is thought to be mediated by histamine, serotonin, bradykinin, the eicosanoids [54] and activation of the complement system [56]. After injection of carrageenan into the rat paw it is absorbed by mast cells resulting in cytoplasmic injury and degranulation with initiation of the arachidonic acid cascade. Hyperemia and an increase in tissue osmotic pressure ensues along with direct damage to endothelial cells by carrageenan. Within 90 to 240 minutes, neutrophils infiltrate the damaged tissue and phagocytize remaining carrageenan. Carrageenan causes degranulation of lysosomes resulting in cellular lysis and further arachidonic acid release. PGE_2 and bradykinin are thought to be the primary mediators of the exudation of fluid within the first few hours of inflammation [57]. The eicosanoids are responsible for the hyperemia, increased vascular permeability and hyperalgesia that follows. By 12 hours the monocytes infiltrate the damaged tissue to phagocytize

the cellular debris. These cells may also experience lysosomal rupture or they may retain partially degraded carrageenan for extended periods [54,58]. Fibroblast proliferation occurs after 2 to 8 days and the tissue returns to normal by 15 days post-injection [55].

Carrageenan was first used as a phlogistic agent to produce subplantar inflammation in the rat paw by Benitz and Hall in 1959 [59], and described in detail by Winter in 1962 [60]. Inhibition of the edema produced by subcutaneously administered carrageenan is commonly used by the pharmaceutical industry to evaluate NSAIDs [60]. Subplantar carrageenan in the rat hind paw model results in peak inflammatory swelling by 4 hours after injection [61]. The same authors have reported that subcutaneous carrageenan in the neck of rats results in peak swelling by 16 hours. In these models edema is measured in the rat paw by plethysmography and mercury displacement and by neck circumference [62]. Other researchers have used subcutaneous carrageenan in the rat to form an abscess that may be removed and weighed [44]. PGE_2 has been measured in the rat hindpaw after carrageenan injection. The animals were euthanized and 100 grams of tissue were then extracted and assayed for PGE_2 [63]. Carrageenan has been administered subcutaneously in the thoracic region of horses in an effort to produce a model for NSAID evaluation. There was an increase in lesion diameter and plasma extravasation for 5 hours after administration [19].

Many of these workers use a 2% solution of carrageenan in water or isotonic saline. However, the optimal strength of a solution of carrageenan in producing

inflammatory effects was 1% as determined by Gardner [49] in guinea pig skin. He noted little increase in inflammation with the 2% solution, while the higher viscosity of this solution made it difficult to inject.

These subcutaneous models are limited to the measurement of edema and pain in response to paw pressure. Other models have been developed for the measurement of white blood cells, protein, enzymes, drugs and inflammatory mediators in the exudate. Polyester sponges soaked with 2% carrageenan have been implanted subcutaneously along the ventral midline in rats [50,64-66]. The sponges are removed, immersed in heparinized saline, centrifuged and the supernatant assayed for PGE_2. This method allows for the determination of leukocyte numbers and protein concentration as well as drug concentration in the inflammatory exudate.

Higgins and Lees [67,68] describe two experimental models of non-immune inflammation in the ponies using carrageenan as the inflammatory agent that were adapted from rodent models. In one model, sterile carrageenan-impregnated polyester sponge strips were implanted into subcutaneous neck pouches in ponies. The second model involved the insertion of polypropylene tissue cages subcutaneously into the necks of ponies. Carrageenan was injected intracaveally into the cages. Sponges and exudate from the tissue cages were removed in a serial fashion and assayed for eicosanoids, protein and cellularity. PGE_2, TXB_2 and 6-keto-$PGF_{1\alpha}$ were detected in inflamed fluid obtained from both models [69-71]. LTB_4 was also detected in the inflammatory exudates of both models [69,72,73]. Mean total leukocyte counts and total protein were increased in both models after carrageenan stimulus. PGE_2 and

LTB_4 have been identified and quantified by radioimmunoassay (RIA) in carrageenan-induced inflammatory exudates from several species as seen in Tables 1 and 2.

b. Synovitis

i. Joint anatomy

Diarthrodial joints consist of articulating surfaces of bone covered by hyaline cartilage, a synovial membrane, synovial fluid within the joint cavity, a joint capsule, and surrounding ligamentous structures. The equine synovial membrane is more villous than other animals and the villi may project considerably into the joint space [74]. The synovial membrane is a modified mesenchymal tissue consisting of two layers [75]. The intima is made up of an incomplete cell layer that overlies the subintimal connective tissue layer. These layers constitute a selective barrier in the joint that allows for the passage of molecules of less than approximately 12,000 daltons in molecular weight [76]. The synoviocytes of the intimal layer are usually 1 to 4 cells thick and are not connected by desmosomes or supported by a basement membrane [77]. The synovial lining cell layer is often incomplete in the horse [74] and human [77]. Synoviocytes are pleomorphic, but have been classified into two types in many species, including the horse [78,79]. Type A synoviocytes resemble macrophages structurally and functionally and may be part of the mononuclear macrophage system [80]. Type B cells appear to be more like fibroblasts in that they contain large amounts of endoplasmic reticulum [79] and may secrete hyaluronic acid into the joint fluid [77]. These cell types most likely represent distinct cell populations rather than different functional states of the same cell line as the type A

Table 1: Mean peak PGE_2 concentrations from carrageenan-induced inflammation models.

METHOD/ SPECIES	TIME OF PEAK CONCENTRATION	PEAK CONCENTRATION (ng/ml)	ANALYTICAL METHOD	REF.
Tissue cage/horse	12 hrs.	197.0	RIA	[70]
Tissue cage/horse	8 hrs.	84.8	RIA	[69]
Tissue cage/horse	12 hrs.	66.4	RIA	[71]
Sponge/horse	4 hrs.	12.2	RIA	[73]
Sponge/horse	12 hrs.	12.8	RIA	[70]
Sponge/rat	8 hrs.	20-30	RIA	[65]
Sponge/rat	24 hrs.	65.0	RIA	[66]

Table 2: Mean peak LTB_4 concentrations from carrageenan-induced inflammation models.

METHOD/ SPECIES	TIME OF PEAK CONCENTRATION	AVG. PEAK CONCENTRATION (ng/ml)	ANALYTICAL METHOD	REF.
Tissue cage/horse	8 hrs.	1.74	RIA	[72]
Tissue cage/horse	4 hrs.	2.50	RIA	[69]
Sponge/horse	4 hrs.	9.60	RIA	[73]
Sponge/rat	6 hrs.	6.90	RIA	[65]

cell, unlike the B cell, may be derived from the bone marrow [77]. Unlike epithelial cells, the rate of cell division in synovial lining cells from normal joints is low [77]. Blood vessels, lymphatics and myelinated and nonmyelinated nerve fibers are found in the subintimal layer. The majority of the nerve endings have been described in the fibrous joint capsule with some free nerve endings in the membrane [75]. The nerve supply to the membrane appears to be primarily vasomotor in nature. However, nociceptive fibers have been demonstrated in the synovial membrane of humans [81]. Further, the equine synovial membrane has evidence of sensory innervation with neuropeptide transmitters located perivascularly [82].

ii. Pathophysiology

Synovitis is characterized by inflammation of the synovium without gross disturbance of the articular cartilage or disruption of major supporting structures. Non-infectious synovitis results from physical or chemical damage to the soft tissue, such as by repetitive overextension, subluxation or intra-articular injection of chemicals [83]. Synovitis involves the classic mediators and modulators of inflammation: histamine, serotonin, prostaglandins, leukotrienes and lysosomal products along with the activation of the kinin, complement and clotting systems [84].

During synovitis, hypertrophy and hyperplasia of the synovium occur, and an increased number of synovial lining cells are found in the synovial fluid [79,85,86]. An increase in vascularity of the synovial villi occurs and inflammatory cells infiltrate the subintimal layer. Degenerative changes in equine synoviocytes that occur during synovitis include: increase in organelles including lysosomes; dilation and vesiculation

of the rough endoplasmic reticulum; mitochondrial condensation; dilation of the nuclear envelope; and ultimately, loss of plasma membranes [79]. Lysosomal enzymes and collagenase produced by synoviocytes, chondrocytes and inflammatory cells perpetuate the synovitis and may degrade the cartilage matrix leading to arthritis [80]. Also, these lysosomal enzymes, along with hyaluronidase and oxygen-derived free radicals produced by neutrophils and macrophages are capable of degrading hyaluronic acid [80,83,84]. Interleukin-1 produced by macrophages and synoviocytes induces the production of PGE_2, neutral proteases and collagenase by synoviocytes and chondrocytes [87-89]. PGE_2 inhibits proteoglycan synthesis in articular cartilage [80] and is a potent vasodilator in the synovial microcirculation [90]. Substance P also stimulates the production of PGE_2 and collagenase by rheumatoid synoviocytes [91]. This neuropeptide has been demonstrated in perivascular neural filaments from normal equine synovia and in synovial fluid. Elevated concentrations were found in arthritic middle (intercarpal) joints as compared to normal joints [92].

iii. Clinical signs

Clinical signs of acute synovial inflammation include: synovial effusion with distension of the joint capsule; increased skin temperature over the joint; hypertrophy and hyperplasia of the synovia resulting in a palpable thickening of the membrane; a decrease in the range of motion of the joint; and lameness in the affected limb. Synovial effusion develops as the result of an imbalance between production and removal of synovial fluid [75]. Inflammatory mediators such as bradykinin, prostaglandins and histamine increase synovial membrane vascular permeability [17]

and the metabolic rate of synoviocytes [37]. As the inflammation progresses, the permeability of the synovial membrane increases and proteins accumulate in the joint resulting in an increase in fluid osmotic pressure. Local production of thromboxanes from platelet aggregation cause congestion of the microvascular bed leading to congestion and heat production [93]. As fluid accumulates within the joint, the synovial pressure rises leading to joint instability and a reduction in the effective blood supply to the articular cartilage. Hypoxic acidosis and lowered glucose concentration in the fluid may result in a decreased nutritional state of the cartilage [83].

A decrease in the range of motion in these joints results from the edema, hypertrophy and hyperplasia of the synovial membrane [83]. The effusion of synovial fluid often results in pain and overt lameness [94]. In humans there is a positive linear correlation between intra-articular pressure and joint pain [95]. The inflammatory mediators released by local tissue destruction in the joint activate nociceptors and also sensitize these receptors through lowering of activation thresholds [96,97]. The neuromediators such as substance P from primary afferent neurons and the interaction of norepinephrine with sympathetic postganglionic neurons in the synovium further result in hyperalgesia and potentiation of the inflammatory response [98]. These factors contribute to the process known as neurogenic inflammation which is an important component of rheumatoid arthritis and other forms of joint inflammation [99].

iv. Experimental induction

Synovitis has been experimentally induced in horses, ponies, calves, dogs and rats by the intra-articular injection of a variety of chemical substances. The intra-articular injection of monosodium urate crystals in dogs [100,101] and calves [102,103] has been used as a model of the synovitis that occurs during gouty arthritis. Autogenous hyaline cartilage has been used to produce acute synovial inflammation in dogs [104].

The metalloprotein orgotein, which is often administered intra-articularly as an anti-inflammatory agent, has been shown to produce a marked inflammatory response in horses characterized by an increase in leukocytes and protein within 24 hours [86]. This inflammatory reaction lasted for up to one week. Sodium monoiodoacetate has been used to induce synovitis in immature horses [105]. Inflammatory changes were seen 12 hours after injection. No articular cartilage damage was seen after 14 days. Synovitis has been induced in horses [39] and ponies by the intra-articular injection of *E. coli* lipopolysaccharide into the intercarpal joint [106,107]. These authors suggested that this model mimics acute bacterial infection. The horses with endotoxin-induced synovitis had peak PGE_2 and tumor necrosis factor levels two hours after injection. The ponies in the study by Firth *et al.* became lame within 2 hours and the synovial fluid protein, leukocyte and alkaline phosphatase levels increased significantly by this time post-injection [106]. Synovial fluid analyses showed abnormalities even at 6 days post-injection. In addition, there were marked

changes in attitude, temperature, appetite, arterial pressure, pulse and respiration. These abnormalities persisted for up to 20 hours for some parameters.

In a series of experiments by Lowther and Gillard [108-110], carrageenan was injected into rabbit stifles. A single injection of sterile carrageenan (0.3 ml of a 1% solution of carrageenan in water) produced synovitis with an increase in synovial tissue levels of cathepsin D and acid phosphatase, two lysosomal enzymes. When more than two injections were administered into the same joint, visible cartilage erosion was seen. The single injection produced a decrease in the rate of proteoglycan synthesis corresponding to a 40% loss in glycosaminoglycan content in the articular cartilage for 3 to 7 days after injection. The rate of synthesis of proteoglycan increased significantly in the following days resulting in a net replacement of proteoglycans lost during the early phase of inflammation. The authors also established by autoradiographic studies that very little carrageenan penetrated the cartilage matrix. They concluded that the inflammatory response in the synovium affects the synthetic ability of the articular cartilage. It has been postulated that the pathophysiology of carrageenan-induced joint inflammation includes a component of neurogenic inflammation as the pretreatment of carrageenan injected joints with capsaicin or a substance P antagonist resulted in a significant suppression in inflammation [111].

Carrageenan was used to induce synovitis in the intercarpal joint of the horse in order to evaluate the superoxide production by stimulated neutrophils and the inhibition of this effect by NSAIDs *in vitro* [52]. The authors described a dramatic

increase in synovial fluid leukocyte numbers which peaked at 4 hours and an increase in synovial fluid lysosome concentration which peaked at 24 hours. They did not describe the severity or time course of the clinical effects of carrageenan-induced synovitis such as lameness, heat production and joint effusion. Further, the quality and quantity of the synovial fluid was not discussed.

The above described substances used to produce synovitis vary markedly in the severity and duration of inflammation they caused. Some of these compounds have the potential to produce cartilage damage (see arthritis section) and in some cases result in systemic alterations.

c. Arthritis

i. Pathophysiology

Some degree of synovitis occurs in most types of equine joint disease. In the majority of these diseases, the synovial inflammation varies only in intensity [75]. McIlwraith [112] has suggested that the inciting cause of degenerative joint disease is synovitis rather than direct damage to the cartilage. The relationship between the synovium and articular cartilage is complex and *in vitro* and *in vivo* evidence suggests that structural and function alteration in one tissue intimately affects the other [83].

Traumatic arthritis or osteoarthritis occurs as a result of inherent instability or trauma to the articular cartilage or subchondral bone [83]. Osteoarthritis is associated with degeneration of the articular cartilage resulting in splitting and fragmentation (fibrillation) with resorption and sclerosis of bone (eburnation in severe cases) and synovitis [75,113]. In horses, the term degenerative joint disease is used to

distinguish chronic inflammation resulting in degenerative changes from acute joint inflammation [114].

One consistent feature of the articular cartilage in equine degenerative joint disease is the decrease in glycosaminoglycans [76]. The loss of these proteoglycan aggregates may be mediated by interleukin-1 and PGE_2 from the synovia and chondrocytes which activate neutral metalloproteoglycanases [80]. The inflamed synovial membrane is also a source for the degradative lysosomal enzymes and reactive oxygen species which lead ultimately to cartilage degeneration [76].

ii. Experimental induction

Experimental arthritis has been induced in laboratory animals by the intra-articular injection of the polyene antibiotic filipin [115,116], fibrin complexes [117], polycations [118], various bacterial agents, distilled water, papain, histamine, mucopolysaccharides, physiologic saline, carbolic acid, hydrochloric acid, tincture of iodine, xylene, turpentine, formaldehyde, and formalin [119]. Intradermal *Mycobacterium butyricum* induces a polyarthritis in rats that also serves as a model of chronic pain (see section on models of chronic pain) [120].

Serial intra-articular injections of carrageenan have been used to induce articular damage in dogs [51,121-123] and rabbits [49,108]. In contrast to the other species, the specific-pathogen free pig did not experience articular cartilage damage after three separate weekly injections of carrageenan [53]. The reason for this difference is not clear, although only two pigs were given repeated injections. Arthritis has been induced in horses by surgical means [124], injection of bacteria

[125], amphotericin [126], serial injections of the polyene antibiotic filipin [112,127], repeated injections of sodium monoiodoacetate [128] and combinations of the above [129].

PGE_2 [130-134] and LTB_4 [133,135-138] have been identified in synovial fluid from human patients with various forms of arthritis. Monocytes from patients with rheumatoid arthritis have been shown to produce large amounts of LTB_4 [139]. Further, plasma PGE_2 [140] and serum LTB_4 [141] levels of patients with rheumatoid arthritis are reportedly higher than normal controls and the time course of changes of PGE_2 in blood plasma levels reflects the disease dynamics [140]. *In vitro* research on cells from humans with rheumatoid arthritis indicates that activated synovial lining cells produce PGE_2 whereas LTB_4 originates mainly from synovial fluid neutrophils [142]. Studies in other inflammatory exudates have also shown that the primary arachidonic acid products of neutrophils are leukotrienes [7]. PGE_2 has been quantitated by radioimmunoassay (RIA) in synovial fluid of normal horses (26.5 pg/ml +/- 3.33) and those with various forms of clinical arthropathy (144.9 pg/ml +/- 22.19). In the arthritic horses, PGE_2 concentrations were reduced after treatment with corticosteroids and antibiotics [143].

Eicosanoids have also been quantified in synovial fluid during experimental arthritis. Synovial fluid from dogs with carrageenan-induced arthritis contained PGE_2 (peak 9.5 ng/joint) and LTB_4 (peak 279.2 pg/joint) as measured by reversed phase high performance liquid chromatography (RP-HPLC) [51]. Additional data is presented in Tables 3 and 4.

Table 3: Mean peak synovial fluid PGE_2 concentrations from various species.

METHOD/ SPECIES	TIME OF PEAK CONCENTRATION	PEAK CONCENTRATION (ng/ml)	ANALYTICAL METHOD	REF.
Polycation-induced arthritis in rabbits	2 days	12.5	RIA	[118]
Clinical arthritis/horse	N/A	0.144	RIA	[143]
Rheumatoid arthritis/human	N/A	0.119	RIA	[134]

N/A = Data not available

Table 4: Mean peak synovial fluid LTB_4 concentrations from various species.

METHOD/ SPECIES	TIME OF PEAK CONCENTRATION	PEAK CONCENTRATION (ng/ml)	ANALYTICAL METHOD	REF.
Carrageenan arthritis/dog (10 weekly inj.)	10 weeks	3.14	RP-HPLC	[123]
Rheumatoid arthritis/human	N/A	0.34	RP-HPLC	[135]
Osteoarthritis/ human	N/A	0.81	Bioassay	[136]

N/A = Data not available
RP-HPLC = Reversed phase high performance liquid chromatography

C. Pain

Pain is defined by the International Association for the Study of Pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage" [144]. Pain in animals has been described as an aversive sensory and emotional experience which elicits protective motor actions, results in learned avoidance and may modify species-specific traits of behavior, including social behavior [145]. Pain is an important homeostatic mechanism that serves to warn the organism away from the inciting cause or to inform it of a existing pathology. Nociception, the response to the application of a noxious stimulus, involves peripheral receptors and the transmission of the signal along neural pathways leading to the perception of pain [145]. Nociceptors are a group of undifferentiated terminals lacking a specialized receptor apparatus [146]. Nociceptive impulses are carried by small myelinated afferent A-delta fibers which are associated with sharp, stabbing, well localized pain, and unmyelinated C-fibers which are responsible for dull, burning, diffusely localized pain [147]. These C-fibers conduct pain very slowly and are responsible for second pain. They make up 70% of all afferents [41]. The C-fiber group also contains efferent fibers of the sympathetic nervous system which release substance P and in this capacity they may mediate chronic pain and neurogenic inflammation [147,148].

Two general types of central neurons are involved in nociception, nociceptive specific neurons that respond only to painful stimuli and polymodal neurons that respond to noxious and non-noxious stimuli. These neurons lie in the dorsal horn of

the spinal cord and have input from A-Delta, C-fibers and in the case of polymodal neurons, low threshold mechanosensitive Aß-fibers [147]. Impulses are carried from these neurons to central terminals in the thalamus via axons in the spinothalamic and spinocervicothalamic tracts [149]. The thalamus is connected to the cerebral cortex by both ascending and descending projections which are responsible for recognition and modification of nociceptive input [150].

1. Acute pain

Nocifensive reflexes from acute pain are manifested by the peripheral nervous system in flexion and withdrawal responses and changes in the autonomic nervous system such as vasospasm and inhibition of the gastrointestinal and genitourinary tracts. These autonomic responses usually result in an increase in ventilation and hypothalamic activity with an increase in cardiovascular and endocrine function [151]. The autonomic changes in response to painful stimuli also include increased plasma concentrations of epinephrine and norepinephrine [152]. Cortical responses are more complex and result in avoidance behavior.

In animals and man the threshold at which peripheral nociceptors start to discharge does not always coincide with the pain reaction threshold. The pain reaction threshold is usually higher than the nociceptive threshold of the peripheral neuron as central control centers affect the pain reaction threshold. Care must be taken to distinguish between pain reaction and simple spinal reflexes. In animals with complete spinal transection, pain reaction is absent but the animal may have exaggerated spinal reflexes. Pain reaction in animals may also be manifested by

turning the head towards the stimulus, respiratory changes, pupillary dilation, aggression towards the stimulus and vocalization [145]. The assessment of pain in animals is difficult without knowledge of the previous condition and behavior of the animal. Animals may also manifest acute pain by alterations in normal behavior including: restlessness, guarding, vocalization, self-mutilation, reluctance to move or recumbency, abnormal posture, altered feeding or sleep cycles and aggression or agitation [153].

2. Chronic pain

Chronic pain in humans is the most frequent cause of suffering and disability that seriously impairs the quality of life [154,155]. Chronic pain is defined as that which persists a month beyond the usual course of an acute disease or reasonable time for an injury to heal, or pain that recurs at intervals for months or years [155]. Chronic pain is predominantly due to prolonged excitation of nociceptors such as occurs in arthritis, peripheral vascular disease and chronic musculoskeletal disorders [155]. This peripheral mechanism of chronic pain is associated with the release of various algogenic substances in the tissue such as bradykinin, serotonin, histamine, substance P and the prostaglandins. Central mechanisms are involved in chronic pain syndromes associated with neuropathy [154]. In contrast to acute pain, the autonomic responses usually decrease progressively during the course of chronic pain and may disappear [151].

Behavioral changes in animals associated with chronic pain include: reluctance to move or recumbency, anorexia, grooming abnormalities, depressed attitude,

constant licking, rubbing or scratching of an area and altered feeding or sleep cycles [153].

3. Mediators

Numerous algogenic substances are responsible for the various expressions of pain seen in acute and chronic conditions such as: allodynia-pain resulting from a non-noxious stimulus to normal skin; hyperalgesia-excessive sensitivity of polymodal nociceptors; and hyperpathia-abnormally exaggerated subjective response to painful stimuli [155]. Hydrogen ions, serotonin, histamine, bradykinin and prostaglandins have excitatory effects on nociceptors as application of these substances to skin blisters in humans results in pain. These compounds have dramatic effects on microcirculation as discussed in the inflammation section. These effects may further add to the excitability of nociceptors [147].

Prostaglandins and leukotrienes are considered mediators of hyperalgesia rather than pain. When administered alone they do not produce substantial pain except when administered in high doses [156]. Prostaglandins facilitate pain evoked by physical stimuli [157] and potentiate pain induced by histamine and bradykinin [147,156]. PGE_2 does not directly alter the resting discharge of nociceptive afferents, but it produces a dose-dependent increase in the firing rate after stimulation [157]. PGE_2 and PGI_2 have been used to produce hyperalgesia to mechanical stimulation in the rat hind paw. In this model, PGI_2 produces immediate hyperalgesia while PGE_2 produces more potent effects lasting for more than 6 hours [158]. PGE_2 when injected intra-articularly in dogs produces incapacitation of the injected joint for more than four

hours [158]. The hyperalgesic effect of prostaglandins in the inflamed rat paw can be completely blocked by the administration of indomethacin [98]. Prostaglandins are involved in the hyperalgesia that is produced by the local injection of bradykinin and in norepinephrine-potentiated hyperalgesia [98,159]. It is known that prostaglandins of the E series are released upon sympathetic nerve stimulation where they may represent a trans-synaptic feedback process [160,161]. Prostaglandins are also released from cholinergic synapses after stimulation of the phrenic nerve [162].

In addition to these peripheral algogenic effects, evidence exists for a central role in the facilitation of the pain message by prostaglandins. Prostaglandins are thought to be released centrally in response to nociceptive input. High intensity stimulation of peripheral nerves in the cat [163] and frog [164] result in the release of prostaglandins in the spinal cord. Intrathecal injections of PGE_2 produce hyperalgesia and block endogenous opioid-mediated analgesia. These effects are dose-dependent and are reduced by the intrathecal administration of NSAIDs [165]. These researchers concluded that prostaglandins inhibit the bulbospinal noradrenergic component of the endogenous pain control pathway by inhibiting the spinal release of norepinephrine. It is clear from the literature that although prostaglandins are not essential for nociception, when released peripherally they facilitate transmission of nociceptive impulses and within the central nervous system they inhibit tonic modulatory systems [163].

LTB_4 produces hyperalgesia in the rat paw that is independent of cyclooxygenase and bradykinin activity [166]. LTB_4 was approximately three times

more potent on a molar basis than bradykinin in this model. This effect was shown to be dependent on neutrophils as the depletion of these cells prevented the hyperalgesic action of LTB_4 [166] Similar concentrations of PGE_2 produced hyperalgesia, but this effect was independent of neutrophils [166] and was not synergistic with LTB_4 [32]. Further studies have shown that neutrophils in response to chemotactic factors such as LTB_4, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), or C_{5a} produce hyperalgesia by generating hyperalgesic 15-lipoxygenase products [167,168].

The nonapeptide bradykinin is one of the most potent pain-producing substances. Bradykinin produces pain in man when applied intradermally, intraarterially or intraperitoneally. However, intramuscular administration of bradykinin did not produce pain in man whereas hypertonic saline and histamine did [169]. Other researchers have reported that bradykinin excites small afferent fibers of cutaneous and muscular origin. [170]. The hyperalgesia induced by bradykinin is dependent on norepinephrine as the effect is not seen after the intradermal injection of bradykinin in sympathectomized rats [98]. Bradykinin also facilitates the release of the prostaglandins through phospholipase A_2[159]. Administration of NSAIDs attenuates the hyperalgesic effect of bradykinin in the rat paw [166].

Substance P from sensory nerve fibers participates in neurogenic inflammation. Orthodromic or antidromic excitation of substance P containing neurons results in release of the mediator. This results in an increase in capillary permeability,

vasodilation and edema [36,147]. However, this peptide when administered locally is not directly algogenic and does not excite nociceptors [157].

Norepinephrine is thought to mediate pain in damaged tissue (hyperalgesia) but not in normal tissue. In rats made hyperalgesic by the application of irritants to the hindpaw, the injection of norepinephrine significantly reduced the nociceptive threshold, presumably through prostaglandin release [98].

4. Models of acute pain

There are several methods described in the literature for the production of stimuli and quantification of responses in various models of pain. These methodological differences make it difficult to compare nociceptive thresholds from differing pain modalities [150]. It is known that the potency of analgesics differ depending on the model and type of stimuli employed [171]. Furthermore, different analgesic systems may be activated by noxious stimulation of different body regions [172]. Therefore, caution should be used when attempting to compare data from different pain models and regions of the body.

The ideal nociception model should distinguish between responses to noxious and non-noxious stimuli. The nociceptive stimulus should be quantifiable, repeatable and precise in order to minimize variability. Nociceptive responses associated with the stimulus should be reduced dose dependently by analgesics. Further, there should be no lasting tissue damage [144,173]. As repeated presentation of the painful stimulus often results in response modification such as conditioning, the stimulus should be applied in a limited fashion [144]. Since animals are not able to verbally

express pain, measurable nociceptive responses often include autonomic changes in blood pressure, heart rate, respiratory rate, etc. and somatomotor responses such as the tail flick, writhing, vocalization and limb withdrawal.

Acute pain models can be classified according to the type of stimulus into chemical, electrical, mechanical and thermal methods. Various chemicals have been used to excite peripheral nociceptors. Formalin when injected into the footpad of a rat and cat causes measurable pain responses such as paw licking lasting for approximately one hour [155,173]. Chemicals such as acetic acid, acetylcholine, alloxan, bradykinin, hydrochloric acid, hypertonic saline, lipoxidase, $MgSO_4$, oxytocin, phenylquinone, serotonin, epinephrine, tryptamine and ATP have been injected into the peritoneal cavity of mice and rats to produce a writhing response. Analgesic efficacies are determined by measuring the latency to the first writhe and the frequency of writhing [144,174]. ATP and acetylcholine are considered inducers of inflammatory-type pain whereas $MgSO_4$ produces a non-inflammatory pain [175]. Lactic acid has been injected into the paravertebral muscles of horses as a model of acute myositis. Pain responses such as flinching and kicking were graded after digital pressure on the injected area [176].

Electrical stimulation of the rat tail produces three types of pain responses according to the intensity of the stimulus. Low intensity stimulation produces a tail withdrawal. Moderate stimulation produces a brief vocalization and higher voltages produce vocalization after termination of stimulus [144,174]. Electrical stimulation of the tooth pulp has been used in rats [144] and horses [177]. The tooth pulp is

thought to be innervated exclusively by A delta and C-fibers which are nociceptive fibers. However, non-nocifensive Aß-fibers are also stimulated in this model [178]. The animal responds by lifting the head or by the jaw-opening reflex.

Selective stimulation of the mechanoceptors is accomplished by applying high pressure. These techniques stimulate low pressure mechanoceptors in addition to the high pressure receptors. Some techniques use hair or nylon strings of different diameters and lengths (Von Frey's hairs) as stimuli [144]. Artery clips have been applied to the tails, ears, paws, and toes of rodents and the resulting biting behavior quantitated. Similarly, the compression of the tail or toe of the animal results in flexor reflexes or vocalization. These methods lack precision in the amount of mechanical pressure applied [174]. A more precise model of mechanical pain has been produced in sheep using a device that presses a blunt pin at a set pressure against the dorsum of the radius [179]. A similar model has been developed for the horse [180]. The rat paw yeast test of Randall and Selitto is used to induce hyperalgesia and test NSAIDs [181]. The drug is administered one hour after yeast challenge and pressure is applied to the inflamed paw until the animal struggles or vocalizes [174].

The classic thermal model is the tail-flick method in rats and mice. This model is used as a standard screening procedure for analgesics and is highly correlated with their effectiveness in relieving pain in humans. A radiant heat stimulus is applied to the blackened area of the tail and the latency to tail flick is measured [173]. A similar test involves placing the rodent's tail in water kept at 45 to 65° C [144]. Another model of thermal pain in the rodent involves placing the animal in a closed

container with heat applied to the floor. The responses noted include agitation, rapid withdrawal of the paw, licking of the paws and jumping [144]. Thermal thresholds have been measured in sheep using a heated device applied to the pinna. The sheep responds by flicking the ear or shaking the head [179]. In the horse, thermal stimulation of the withers and lateral fetlock has been used to evaluate pain thresholds. The horse responds by twitching the skin over the withers or by lifting the forefoot after stimulation of each region [182,183]. Thermal devices have been implanted over the radial periosteum of ponies in an attempt to model deep pain. The response to this stimulus was noted as slow and unreliable [184-186].

Reflex behaviors such as the tail-flick, toe pinch and the jaw-opening reflex are not measures of pain reaction, but are nociceptive reflexes. Some of these simple reflexes are considered spinal reflexes as they may be elicited in animals with spinal transection [173].

5. Models of chronic pain

Chronic pain in humans and animals is frequently encountered in a clinical setting and is often extremely difficult to alleviate. Most of the experiments in pain research utilize acute rather than chronic paradigms. However, chronic pain in not simply a prolongation of acute pain. There are distinct central changes associated with chronic pain [41]. Therefore, knowledge gained from these acute pain models is often not applicable to the alleviation of the chronic pain state. A few chronic pain models have been developed in animals. The paucity of models of chronic pain in animals is in part due to the important ethical considerations in using animals that may

suffer needlessly. Some of models of chronic pain have been described previously under models of acute inflammation. Most models of arthritis produce lasting tissue damage resulting in a perpetuation of inflammation into the chronic state. They may then be used for the study of chronic pain [173]. In addition several models of neuropathic pain have been developed by severing peripheral nerves [187].

a. Adjuvant models

Freund's adjuvant models of cutaneous inflammation and polyarthritis involve the inoculation of a paraffin oil suspension of heat-killed *Mycobacterium butyricum* into the footpad or dermal tissue at the base of the tail of rats. The cutaneous model results in inflammation within 4 hours and peaks within 1 to 2 days. The affected limb is hyperalgesic and edematous for 1 week to 10 days [173]. In the adjuvant-induced arthritis model, animals experience a temporary polyarthritis with an increased severity in the hindlimbs. Inflammation of the base of the tail, periorbital region, snout, ears and penis occur as well. Behaviorally, the animals exhibit an increased stress reaction, show frequent scratching activity and are more aggressive. Weight loss and a decrease in spontaneous locomotion are also evident. This condition is fully developed 22 to 28 days post-induction and is usually associated with a slow recovery to normal by the 55th day. Pain is inferred from the increased scratching behavior in these animals as it is alleviated by morphine and other analgesics [120]. Other authors have used this model to evaluate nociception by applying pressure to the inflamed hind paw and determining the vocalization threshold

[188] and to demonstrate self-administration of NSAIDs and narcotic analgesics [189,190].

Several investigators have demonstrated that joints from adjuvant-induced rats are hyperalgesic in that there is a lower threshold to mechanical stimuli and the areas known to be involved in nociception from the peripheral receptor to the cortex are markedly altered. A-delta and C-nociceptor afferents, which are normally sensitive only to noxious stimuli, were stimulated by non-noxious mechanical means in this model of rat arthritis [191]. The mechanosensitivity of the myelinated and unmyelinated units of the medial articular nerve of the knee was also increased in this model [192]. Similar results were found in sheep with chronic pain from foot rot. These sheep had lowered mechanical thresholds in the affected limb. It is likely that these effects are brought about by peripheral changes as the local injection of lidocaine returned the thresholds to control values [179].

Central neuronal changes have been demonstrated in animals with chronic pain. In decerebrate rats with adjuvant-induced arthritis there was an increased activity of dorsal horn cells and a high degree of responsivity to light mechanical stimuli. The superficial dorsal horn cells driven from inflamed areas converted from responding only to noxious stimulation to responding to mild mechanical stimuli [193]. Dorsal horn neurons in decerebrate rats showed an increase in cutaneous receptive field after repeated excitation of C-fiber afferents. These neurons also began responding to low threshold stimuli such as brush and touch [194]. Recordings from the ventrobasal complex of the thalamus in arthritic rats indicate that there is an increased proportion

of the neurons responding to the light pressure or flexion and extension of the diseased joints as compared to normal animals [195]. These responses were reduced by 50% after the injection of the NSAID, diclofenac, indicating possible prostaglandin involvement [196]. The analgesic effect of diclofenac was more potent in arthritic rats as compared to normal animals.

The somatosensory cortex also is altered during states of chronic pain. Central somatosensory neurons of animals with chronic inflammatory pain show a marked increased sensitivity to moderate mechanical stimulation of inflamed tissues and surrounding areas as compared to normal animals [197].

b. Laminitis

Laminitis is one of the major causes of chronic hoof pain in the horse [198]. Horses afflicted with the acute form of this disease experience extreme pain and are often euthanized. The chronic form is associated with debilitation, decreased fertility, lameness and pain which may last for the life of the animal. Equine laminitis has multiple etiologies and the pathophysiology is complex and incompletely understood [199]. This disease is not simply defined as inflammation of the laminae of the foot as was once described, but is peripheral vascular disease with ischemic necrosis of the laminae associated with hoof pain [200]. The pathology within the foot is a manifestation of a systemic metabolic disorder that affects the cardiovascular, endocrine and renal systems.

i. Hoof anatomy

The distal phalanx or coffin bone is covered by intermeshing dermal and epidermal laminae which support the bone within the hoof capsule. The insensitive laminae are composed of the epidermis except for the stratum germinativum which with the dermis makes up the sensitive laminae [200]. The circulation of the hoof is complex and unique owing to its role in thermoregulation and nutrition to the proliferating epidermis. The laminar microvasculature arises from distal branches of the digital arteries so that laminar blood flow is in a distal to proximal direction with the dorsal laminae being the last to receive blood [201]. Numerous arteriovenous anastomosis are found within the foot which open to decrease vascular resistance and increase the blood flow to the limbs in order to cool the body [202]. The veins of the hoof and lower limbs are thick muscular structures and there are no venous valves in the foot [203].

Sensory receptors in the equine foot include lamellated corpuscles in the dermis of the heel similar to Pacinian corpuscles and free nerve endings containing calcitonin gene related peptide-like immunoreactivity in the solar dermis and epidermis [204]. The myelinated corpuscles in the heel are likely to be involved in relaying proprioceptive information during locomotion. In contrast, the free nerve endings in the solar dermis are thought to be involved in nociception and regulation of blood flow [204].

ii. Etiology

Numerous etiologies have been identified that produce laminitis: ingestion of toxic quantities of wheat, corn and barley grains resulting in carbohydrate overload; ingestion of large quantities of water; ingestion of high quality, rapidly growing, succulent grasses or legumes by obese animals; excessive weight bearing or application of severe concussive forces to the foot; severe bacterial infection or viral respiratory disease; exposure to black walnut shavings; ingestion of beet tops [200]; and high doses of corticosteroids [205,206]. Experimental laminitis has been produced by the administration by aqueous extract of black walnut (*Juglans nigra*) [207,208] and more commonly by the oral administration of large amounts of carbohydrates [209].

iii. Pathophysiology

It has been hypothesized that a laminar ischemia develops acutely due to decreased capillary perfusion from opening of arteriovenous shunts. This results in a lowering of vascular resistance and an increase in total hoof blood flow as may be noted clinically by an increase in hoof temperature and a bounding digital pulse that persists through the chronic phase of the disease. Several groups have measured an increase in total digital blood flow [210] and have demonstrated regions of hypoperfusion within the dorsal laminae [208,211,212]. Other workers have not supported the hypothesis that lamellar ischemia is the primary cause of acute laminitis [213], while others have noted a decrease in total digital blood flow [208]. These discrepancies are due primarily to the techniques used to determine blood flow and

these results should be interpreted with caution [214]. Allen *et al.*, concluded that profound increases in postcapillary resistance, and capillary pressures predispose the foot to interstitial fluid volume accumulation and increased tissue pressure in the prodromal stages of laminitis [215]. The increased tissue pressure in this noncompliant compartment results in hoof pain [214] which accounts for the increased plasma catecholamine concentration seen in horses developing laminitis [199,216]. These catecholamines further exacerbate ischemia through vasoconstriction of digital vessels [217]. Systemic hypertension occurs in the developing phase, but it is secondary to hoof pain and is also mediated through the sympathetic nervous system [199]. This hypertension is thought to be a positive feedback mechanism from the digital vasoconstriction and is often present in the chronic forms of the disease [218].

Evidence for disseminated intravascular coagulopathy exists in horses developing laminitis as platelet numbers drop before the development of lameness along with the development of other coagulation abnormalities [199]. Other workers have not detected significant changes in platelet number or function in horses with experimental laminitis [219].

Bacterial endotoxin has been incriminated as a mediator or inciting cause in laminitis as many horses with endotoxemia develop laminitis [220] and horses with carbohydrate overload laminitis have a increase in plasma endotoxin that is associated with lameness [221]. Endotoxemia is associated with increase plasma levels of LTB_4 [222], PGE_2, TXB_2 and $PGF_{2\alpha}$ and the administration of NSAIDs prevents many of the effects of experimentally induced endotoxin [223]. The association between

endotoxin-induced eicosanoid production and the development of laminitis is theorized but has yet to be proven due to the inability to produce laminitis with experimentally-induced endotoxemia. Further the role of eicosanoids in equine laminitis has not been evaluated [220].

Early histological changes in the foot include endothelial cell swelling and edema, laminar distortion, epithelial hyperplasia, microvascular thrombosis, congestion and hemorrhage [220,224-226]. There is no evidence of inflammatory cell influx, but edema and vacuolation of the dermal tissue with necrosis and atrophy of the epidermal laminae occurs [220,224]. In severe cases of laminitis, degeneration of the laminar interdigitation occurs causing the distal phalanx to separate from this supporting structure and rotate within the hoof capsule [201]. Ventral deviation or distal displacement of the distal phalanx is due to the biomechanical forces exerted on the foot including the downward load of the weight of the horse exerted on the bony column and the proximal pull of the deep digital flexor from its insertion on the ventral surface of the distal phalanx [220]. The tearing force exerted against the toe during midstride also acts to separate the distal phalanx from the hoof wall [227]. The rotation forces on the distal phalanx may cause penetration of the bone through the sole just dorsal to the apex of the frog. The degree of rotation as determined radiographically can be used as a prognostic indicator [228]. In extremely acute cases, the laminae surrounding the distal phalanx die resulting in a total separation from the bone [229]. In these obviously terminal cases no rotation occurs as the bony column sinks in the hoof capsule.

Chronic laminitis is classified by greater than 48 hours of continual pain from a laminitic episode or when rotation of the distal phalanx occurs [200]. Angiographic studies in horses with chronic laminitis have shown irregular digital vascular patterns with areas of avascularity in the corium [230]. Hyperplasia of the dermal and keratinizing epidermal laminae [224] occurs dorsally in the hoof wall which creates a wedge of tissue that forces the epidermal and dermal laminae apart. This is evidenced on the sole as 'seedy toe' or an abnormally large white line. This wedge is thought to perpetuate the rotation of the distal phalanx [200]. Diverging hoof growth rings that are wider at the heels are often present giving the hoof a concave dorsal surface. Due to the ischemic necrosis within the laminae and large white line, these horses have an increased tendency for abscessation and cracks which may also cause hoof pain and lameness [231]. The gait of these horses is characteristic as the foot strikes the ground in a heel-toe fashion. The solar surface of the foot is particularly sensitive to the pressure of the hoof tester over the sole, midway between the apex of the frog and the toe [231]. Horses with severe chronic laminitis often have difficulty remaining in the standing position for any substantial length of time due to the pain associated with weight bearing. If pain is severe, prolonged periods of recumbency may occur which lead to the formation of decubital ulcers, reduced body condition and impaired ventilation. Furthermore, since weight bearing by the foot is essential for normal circulation, prolonged recumbency may lead to thrombus formation within the digital vascular system further exacerbating laminitis [231,232].

Analgesics temporarily reduce the pain and suffering associated with laminitis and allow the animal to more comfortably stand and ambulate, thereby promoting blood flow to the foot and reducing the adverse effects of long term recumbency. In addition, some analgesics lower plasma catecholamine concentrations that are associated with nociception and stress [152]. When released, these endogenous amines are thought to produce vasoconstriction of the digital vasculature, resulting in ischemia which further exacerbates laminitis [200]. According to Hood, horses that are treated early with systemic analgesics have a lower incidence of rotation than those treated late in the course of the disease [199]. Phenylbutazone (4.4 mg/kg) is regarded as the single most important therapeutic agent in treating laminitis [231,233]. NSAIDs are thought to be particularly useful in treating the coagulopathy because of their inhibitory effects on eicosanoid formation and platelet function [199,234]. $PGF_{2\alpha}$ and thromboxane are known to be potent constrictors of digital arteries and veins with the veins being more responsive than the arteries [217]. Although it is known as a vasodilator, PGE_2 was less potent in producing digital artery dilation as compared to acetylcholine, acepromazine, isoxsuprine and prostacyclin [235]. During experimental laminitis, the administration of phenylbutazone returned hypertensive horses to normotension. These authors found that most horses (85%) recovered from the laminitic episode without rotation following the administration of phenylbutazone while housed in soft sand stalls [199].

D. Nonsteroidal anti-inflammatory agents

1. Mechanism of action

a. Cyclooxygenase inhibition

The medicinal effect of the bark of the willow tree has been known for centuries. The active ingredient in the bark is salicin, a bitter glycoside. It was first used for the treatment of rheumatic fever and gout which led to the development of the first NSAID, aspirin or acetylsalicylic acid, that was then commercially prepared and marketed by Bayer. The NSAIDs are a chemically diverse group of compounds, although most are weak organic acids (pK_a 3.0 to 5.5 for carboxylic or enolic acids, pK_a 9-10 for phenolic acids) [236]. These drugs share common features in that most are antipyretic, anti-inflammatory and analgesic.

In 1971 Vane [237] demonstrated that aspirin and indomethacin inhibited prostaglandin formation in guinea pig lung in a dose dependent manner. In that same year, Smith and Willis [238] demonstrated that the administration of two tablets of aspirin to human volunteers resulted in deficient prostaglandin production in platelets one hour later. These experiments supported the increasing evidence that prostaglandins participated in the pathogenesis of fever and inflammation. Further work showed there to be an overall positive correlation between the concentrations of NSAIDs that block prostaglandin synthesis *in vitro* and doses in which they exert analgesic, anti-inflammatory, and antipyretic activities in animals [239,240]. Inhibition of platelet function and prolongation of bleeding times with some NSAIDs have also been shown to be a result of cyclooxygenase inhibition [238].

Aspirin acetylates and irreversibly inactivates the cyclooxygenase in platelets [241] and in human synovial microsomes [242] through covalent bonding. Other NSAIDs such as indomethacin, meclofenamic acid and flurbiprofen inhibit cyclooxygenase reversibly at first and then essentially irreversibly by non-covalent binding in a stereospecific and time-dependent manner to subunits of the prostaglandin endoperoxide synthase enzyme [243]. This time-dependent effect appears to depend on the presence of a halogen and a carboxyl group [244]. Other drugs such as ibuprofen and mefenamic acid are not able to inactivate the enzyme and are considered reversible inhibitors [244] Some of these drugs such as phenylbutazone inhibit the enzyme more efficiently when the concentration of lipid hydroperoxides is reduced [245].

There is good correlation between the rank order of potencies in reducing edema and prostaglandin concentrations *in vivo*, although the doses required to prevent edema are usually much higher (Table 5) [64]. Inhibition of lysosomal release by NSAIDs has been documented for several compounds but the rank order of potency of these drugs does not correlate with their anti-inflammatory activity [8].

b. Leukocyte migration

NSAIDs have been shown to inhibit leukocyte migration in carrageenan-induced inflammation in the rat [50,246]. However, the doses producing this effect are generally much higher than those which prevent erythema and hyperalgesia [8]. However, therapeutic doses of indomethacin, piroxicam and ibuprofen reduce the function of neutrophils in human subjects [247]. Further, doses approximating the

Table 5: Rank order of potency of NSAIDs in inhibiting carrageenan-induced edema and prostaglandin synthesis (as determined by bioassay) *in vivo* in the rat. Adapted from Higgs *et al.*, 1976 and 1983 [8,64].

DRUG	RELATIVE POTENCY EDEMA INHIBITION	RELATIVE POTENCY PG SYNTHESIS INHIBITION
Aspirin	0.10	0.02
Phenylbutazone	0.73	0.39
Ibuprofen	0.85	0.20
Naproxen	1.00	1.00
Indomethacin	5.54	7.30
Ketoprofen	8.50	64.70

therapeutic ranges of flunixin and phenylbutazone decreased the LTB_4-induced chemotaxis of canine neutrophils *ex vivo* [248]. The inhibitory effects of NSAIDs on neutrophils are not thought to be mediated by cyclooxygenase inhibition but by uncoupling protein interactions within the plasmalemma [247]. In contrast, low doses of phenylbutazone (0.05 mg/kg), indomethacin [50], aspirin and flurbiprofen [15] stimulate leukocyte counts in the rat carrageenan model. This modulation of leukocyte migration was not correlated with cyclooxygenase inhibition. There may be some potentiation of lipoxygenase at low doses of some NSAIDs which could account for the increased number of leukocytes. Or perhaps NSAIDS modify leukocyte migration by another mechanism. NSAIDs have been shown to inhibit several enzyme systems independently of cyclooxygenase such as: superoxide anion generation by a NADPH oxidase system in neutrophils; mononuclear cell phospholipase C activity; and the conversion of 12-HETE from 12-HPETE in platelets [247]. Further, many of these drugs uncouple oxidative phosphorylation; alter the uptake and membrane incorporation of arachidonate; and inhibit anion transport in the human erythrocyte, rabbit choroid plexus and renal tubular epithelium [247].

c. Lipoxygenase inhibition

At therapeutic doses most NSAIDs do not inhibit the formation of leukotrienes. However, the pyrazolone derivative phenidone [249], the propionic acid derivative benoxaprofen [250], hydroxamic acids [251] and the phenylpyrazoline analogues of phenidone, BW755C and BW540C [252] have been shown to be both cyclooxygenase

and lipoxygenase inhibitors. These analogues are effective in reducing inflammation in the horse but are not available clinically [253].

It has been shown that several NSAIDs may actually potentiate *in vitro* lipoxygenase product formation through shunting of the arachidonic acid substrate into the leukotriene pathway following cyclooxygenase inhibition [250,254]. Cyclooxygenase blockade during acute anaphylaxis in guinea pigs augments the production of the slow-reacting substances of anaphylaxis and LTB_4 [255]. In contrast, some prostaglandins inhibit the formation of lipoxygenase products from activated neutrophils [21].

d. Distribution into inflamed tissues

NSAIDs bind extensively to albumin (90 to 99%) and may achieve high concentrations in an inflammatory site due to the increase in vascular permeability and exudation of plasma proteins including albumin. The clinical anti-inflammatory effect of NSAIDs in human arthritics correlates directly with the degree of plasma protein binding [256]. NSAIDs bound to albumin are thought to be released by the degradation of albumin by lysosomal enzymes at the site of inflammation [257]. This one way flow of albumin serves to increase the tissue concentration of the drug [258]. Also the lowered pH of the inflamed environment serves to keep these planar, anionic molecules unionized and thus increasing membrane permeability [247]. Ion trapping is postulated to occur intracellularly as the environment inside the cell is relatively alkaline compared to the acidic inflamed tissue [259-261]. The effect may also occur in gastric and renal tubular epithelium cells which border acidic extracellular fluids

[258,262]. The optimal pK_a for this effect is reported to be between 4 and 5 [263]. In autoradiographic studies in rats, acidic NSAIDs were found to reach high concentration in the stomach wall, liver, blood and bone marrow, kidney cortex and in inflamed tissue. The non-acidic NSAIDs were equally distributed throughout the body [264]. Further, acidic salicylates reached levels in inflamed chicken joints that were three times higher than in control joints. The inflamed joint levels approximated the corresponding plasma levels at 2 hours post-dose [265].

e. Pain relieving effects

NSAIDs do not produce analgesia in normal tissues and in most cases they do not elevate simple pain thresholds in experimental models in which thermal, electrical or mechanical stimuli are applied to normal tissues. However, these drugs are particularly effective in situations where an inflammatory response is present and the area is hypersensitive to mechanical stimuli. In these situations, NSAIDs usually do not completely block the painful response, but only reverse the hyperalgesia [163]. In electrophysiologic experiments in rats made hyperalgesic by the induction of adjuvant arthritis, systemically administered aspirin reduced the afferent discharge from affected joints within 20 to 30 minutes after injection [197]. Similar results were obtained from the local application of aspirin and lysine acetylsalicylate [266]. These experiments further add to the evidence that prostaglandins are involved in the mediation of pain and inflammation.

Early studies in the dog spleen demonstrated that NSAIDs produce analgesia peripherally [267,268]. Local injection of small amounts of NSAIDs into

inflammatory lesions confirmed this peripheral site of action [269]. However, prostaglandins are released from the spinal cord during noxious stimulation [270] and intrathecal administration of prostaglandins lower nociceptive thresholds [269,271]. The intrathecal administration of NSAIDs reduces nociception and the simultaneous intrathecal and peripheral administration of NSAIDs produce synergistic effects [269]. A central component of the analgesia seen with NSAIDs has been demonstrated since intraventricular administration of these drugs reduced the hyperalgesia caused by carrageenan in the rat hind paw [272] and in the adjuvant-induced arthritis rat model [273]. A thalamic site of action is also likely as the administration of NSAIDs are found to reduce the nociceptive impulses from stimulation of the sural nerve in arthritic rats [148]. There are three possible central sites of action that have been identified: the hypothalamus [274], the periaqueductal grey area and the thalamus [148]. The mode of action of these drugs has been attributed to prostaglandin inhibition in neural tissues. However, there may be other yet unknown mechanisms as several NSAIDs such as salicylic acid, paracetamol and phenazone possess potent analgesic and antipyretic properties but are less efficacious anti-inflammatory agents or prostaglandin inhibitors in some models [275]. In a review of the literature by McCormack and Brune in 1991, they were unable to find a correlation between antinociceptive activity and inhibition of prostaglandin synthesis for the antipyretic analgesics [258]. Further work with enantiomers of flurbiprofen have shown that the *S*-enantiomer, which is almost solely active in cyclooxygenase inhibition, is

approximately equal in antinociceptive activity as the *R*-enantiomer which has almost no cyclooxygenase inhibitory properties [276].

2. Ketoprofen

Ketoprofen [(±)-2(3-benzoylphenyl)propionic acid] was synthesized in France by Rhone-Poulenc chemists in 1967, three years after the prototype ibuprofen [277]. This drug is approved in humans for the treatment of rheumatoid arthritis, osteoarthritis, and mild-to-moderate pain [278]. Ketoprofen was approved for the alleviation of musculoskeletal pain and inflammation in the horse in 1990. Few studies have been published on the anti-inflammatory and analgesic effects in the horse. Kinetic and metabolism studies in the horse are just now being published.

a. Pharmacokinetics

The kinetics of ketoprofen in man are well established. The terminal half-life is 1.8 hours after an intravenous dose. The volumes of distribution in man are low, approximately 4 L for the volume of the central compartment and 10 L for the steady state volume [278]. The drug like most NSAIDs is highly bound (> 90%), presumably to albumin primarily [279]. In human patients with various forms of arthritis, the area under the curve (AUC) for total (unbound and bound ketoprofen) was greater in serum than synovial fluid. However, the free fraction (unbound drug) AUC was similar for both. Further, the mean residence time in the joint was about three times that in serum which helps to explain the discrepancy of a longer duration of therapeutic effect than serum half-life [280]. The drug is conjugated and excreted primarily as an inactive metabolite in the urine. Further, a small percentage of the

drug is excreted in the urine as an hydroxylated metabolite [278]. Ketoprofen has been shown to have extensive tissue penetration into human tonsils [281] and crosses the blood-brain barrier quite rapidly [282]. This is due to the high lipid solubility of ketoprofen which has a heptane/water partition coefficient of 3.5 at pH 7 [282].

Sams *et al.* have reported plasma kinetics of ketoprofen in the horse [283]. This group determined that the half life is short in the horse (90 minutes). Protein binding was extensive with 92.8% bound at 0.5 ng/μl and 91.6% at 10 μg/ml. Renal elimination of unchanged ketoprofen and a base-labile conjugate accounted for 22.4% of total clearance and was due to both tubular secretion and filtration. Little tubular resorption occurred as horses have alkaline urine which serves to keep the drug ionized in the urine. In the Sams study, approximately 42.8% of the dose was not recovered from the urine. This was possibly due to the formation of a metabolite that was not recovered by the extraction method. The conjugate may be a glucuronide that has undergone intramolecular acyl migration rendering the compound resistant to β-glucuronidase hydrolysis [283]

The 2-arylpropionic acids contain a chiral center and are usually administered as a racemate. In man, approximately 10% of the drug was found to invert from the *R* to the *S* enantiomer upon oral administration [284]. In the horse, the ratio of the *S* to *R* enantiomer increased over time and attained a ratio of 70:30 less than one hour after intravenous administration [285,286]. Cyclooxygenase inhibition is accomplished by the *S*-enantiomer primarily [287,288]. Metabolic chiral inversion of the *R*- to the *S*-enantiomer occurs after administration through the probable mediation

of coenzyme A [289]. However, ketoprofen exhibits little stereoselectivity in its pharmacokinetics as the enantiomers have similar plasma time-courses and do not appear to interact with one another in man [284]. Some degree of stereoselective binding to albumin may occur but this binding does not appear to affect the disposition or kinetics of total (unbound and bound) enantiomer concentration in the therapeutic range in man [284].

b. Anti-inflammatory effects

Ketoprofen has been shown to be a potent reversible inhibitor of prostaglandin synthase in several cyclooxygenase enzyme preparations at concentrations within the human therapeutic range [277,278,290,291] (Table 6). Values are expressed as the inhibitory concentration (IC_{50}), which is defined as the concentration of inhibitor necessary for 50 percent inhibition of the enzyme reaction. In addition, ketoprofen is a potent inhibitor of inflammation in several models (Table 7). Values are expressed as effective dose (ED_{50}) which is defined as the dosage which gives a pharmacological effect equal to 50% of the maximum possible effect or which gives the expected pharmacological effect in 50% of the animals tested. As can be seen from the following tables ketoprofen demonstrates lower effective doses and inhibitory concentrations than many of the other commonly used NSAIDs.

There is equivocal evidence in the literature concerning the effect of ketoprofen on the lipoxygenase pathway as can be seen in Table 8. Ketoprofen has been shown to increase the SRS-A release in guinea pig lung but inhibited the release from human lung [250,292,293]. The production of 5-HETE and 5,12-diHETE, a diasteromer of

Table 6: Inhibitory concentrations [IC_{50} (μM)] of various NSAIDs on cyclooxygenase enzymes.

METHOD	KETO-PROFEN	PHENYL-BUTAZONE	IBU-PROFEN	INDO-METHACIN	BW755C	REF.
Sheep seminal vesicles*	6.92	4.89	5.82	6.40	N/A	[291]
Mouse peritoneal macro-phage*	7.65	5.26	6.26	8.77	6.53	[291]
Rat platelet	1.45	79.30	N/A	5.90	21.6	[290]
Rat neutrophil	0.58	15.70	N/A	4.78	11.6	[290]
Rat renal medulla	2.20	20.90	N/A	5.07	12.1	[290]

N/A = Data not available
*IC_{50} data expressed as -log mol/l

Table 7: Effective doses [ED_{50} (mg/kg)] of various NSAIDs on inhibition of acute inflammation and pain.

METHOD	KETOPROFEN	PHENYLBUTAZONE	IBUPROFEN	INDOMETHACIN	REF.
INFLAMMATION MODELS:					
Carrageenan rat hind paw edema	1.89	97.55	N/A	8.17	[63]
Carrageenan rat hind paw edema	9.00	N/A	N/A	9.00	[44]
Carrageenan abscess in rat	1.40	110.00	29.00	1.30	[44]
Inhibition of leukocyte migration	2.50	52.00	45.00	5.70	[15]
Contraction of rat colon	0.002	2.00	1.50	0.016	[296]
PAIN MODELS:					
U.V. erythema in guinea pig	7.50	N/A	N/A	10.20	[44]
Rat paw pressure	2.40	37.00	14.00	3.50	[44]
Writhing response in mouse	2.30	N/A	N/A	2.20	[44]

N/A = Data not available

Table 8: Inhibitory concentrations [IC_{50} (μM)] of various NSAIDs on lipoxygenase enzymes.

METHOD	KETOPROFEN	PHENYLBUTAZONE	IBUPROFEN	INDOMETHACIN	BW755C	REF.
Soybean 15-LO	225.00	123	575.00	87.50	65.50	[294]
Soybean 15-LO	No effect	N/A	N/A	No effect	38.50	[290]
Rabbit PMN 5-LO	Approx. 11.81	N/A	N/A	Potentiated	N/A	[250]
Rat platelet 12-LO	No effect	No effect	N/A	916.00	66.20	[290]
Rat PMN 5-LO	No effect	N/A	N/A	332.00	55.70	[290]

N/A = Data not available

LTB_4, from rabbit neutrophils was inhibited by ketoprofen [250]. The lipoxygenase inhibition appeared to be dose-related in humans [292], but not in the rabbit model [293]. Ketoprofen and several other NSAIDs have been shown to inhibit soybean lipoxygenase [294] while other researchers report no effect in the enzyme system [290]. The concentrations required to inhibit lipoxygenase are generally higher than those that inhibit prostaglandin synthase activity. These studies underscore the variability that other researchers have noted in the drug's effect on lipoxygenase activity. Such data varies depending on the source of the lipoxygenase enzyme and the species [293,295].

Ketoprofen reduced the PGE tissue concentration in carrageenan-induced hind paw inflammation in the rat [63]. Prostaglandin synthesis was also inhibited in the guinea pig lung preparation as determined by the perfusion of lung effluent over rat colon strips and measurement of the resultant contraction [296]. In human patients with rheumatoid arthritis, ketoprofen reduced the synovial fluid concentration of PGE_2 and $PGF_{2\alpha}$ three hours after intravenous administration of 100 mg of ketoprofen [297]. Ketoprofen (10 mg/kg *per os*) in the rat carrageenan pleurisy model decreased the total number of monocytes and slightly increased the number of neutrophils in the inflammatory exudate [298]. Ketoprofen (1, 3 or 9 mg/kg) when administered one hour before induction of carrageenan-induced pleurisy reduced the volume of exudate and leukocyte count in a dose-dependent manner. This effect was greater than that produced by the same doses of indomethacin [299]. Alleviation of inflammation has been demonstrated when ketoprofen was injected locally into carrageenan abscesses

in the rat and intra-articularly in dogs with urate-induced arthritis [44]. Ketoprofen has demonstrated anti-bradykinin activity in the guinea pig that was 8 times more potent than indomethacin [44]. *In vitro* and *in vivo* evidence suggests that ketoprofen stabilizes lysosomes in the rat. Interestingly, this effect was more marked in rats with adjuvant induced arthritis than in normal animals [300].

c. Pain relieving effects

Ketoprofen has demonstrated analgesic and anti-inflammatory effects in rheumatoid arthritis, osteoarthritis, ankylosing spondylitis and acute gout in humans [301]. Clinical trials with ketoprofen in humans have shown that it is therapeutically equivalent to aspirin, indomethacin and ibuprofen in rheumatoid arthritis and with aspirin in osteoarthritis [277]. Ketoprofen when evaluated in human dental pain models was more effective than ibuprofen and aspirin [302]. Compared to aspirin, ketoprofen was superior in controlling osteoarthritis [277] and postpartum pain [303]. This drug provided analgesia similar to that of morphine [303] and produced a longer duration of action than acetaminophen plus codeine [304] in clinical cases of post-operative pain. Ketoprofen is thought to have supraspinal analgesic properties [305]. Experiments with normal human volunteers and paraplegic patients that were stimulated by electrical impulses applied to the sural nerve have shown that intravenous ketoprofen has rapid inhibitory effects on spinal nociceptive reflexes in normal subjects while no significant effects were seen in the patients with chronic spinal section [306]. The injection of ketoprofen into the cerebral ventricles in rats with adjuvant-induced arthritis reduced the discharge of thalamic neurons after ankle

mobilization. In addition, ketoprofen reduced the spontaneous firing rate in a dose-dependent manner [307]. These data provide good evidence for the direct central antinociceptive actions of NSAIDs.

d. Clinical studies

Ketoprofen has been evaluated in experimentally-induced endotoxemia in neonatal calves [308]. Ketoprofen inhibited the formation of the stable metabolites of TXA_2 and prostacyclin, TXB_2 and 6-keto-$PGF_{1\alpha}$, respectively, in plasma. However, it failed to significantly alter the degree of leukopenia and hypoglycemia associated with the endotoxemia in calves.

Ketoprofen was evaluated in a model of arthritis using Freund's complete adjuvant injected into the carpal joint of horses. Horses became lame and experienced swelling, heat and joint pain 5 to 7 days after induction. Horses then received drugs once daily and were evaluated for 5 days for maximum joint flexion, stride length and lameness. Ketoprofen (2.2 mg/kg intravenously or intramuscularly) was therapeutically equivalent to flunixin meglumine (1.1 mg/kg) [309]. The same group of investigators evaluated ketoprofen in clinical cases of non-infectious musculoskeletal inflammation and the following were scored: lameness; pain on palpation or compression; pain on flexion, extension or rotation; swelling; heat; reaction after injection; and side effects. Cases included arthritis, laminitis, myositis, cellulitis, soft tissue inflammation and minor fractures. Statistical level of significance was set liberally at $P < 0.10$. There were no differences between ketoprofen and flunixin meglumine and there were no side effects noted [309].

In clinical colic studies in horses, significant pain relief occurred at 15 minutes after intravenous ketoprofen administration (2.2 mg/kg) and continued for 60 minutes after drug administration [310]. This effect lasted up to four hours in some cases [311,312]. No difference was seen between ketoprofen (2.2 mg/kg) and flunixin meglumine (1.1 mg/kg) in any of the parameters measured [313]. In mares with experimentally-induced endotoxemia, ketoprofen (0.5 mg/kg) was equally as effective as flunixin meglumine (0.25 mg/kg) in suppressing the effects of the endotoxin. These investigators attempted to assay LTB_4 in plasma during endotoxemia, but were not successful [314].

e. Adverse effects

The safety and toxicity of this drug in the horse has been compared to several of the currently used NSAIDs. Ketoprofen was administered intramuscularly in the neck and gluteal regions without any noted injection site reaction [315]. Therapeutic intravenous doses of ketoprofen (2.2 mg/kg), flunixin meglumine (1.1 mg/kg) and phenylbutazone (4.4 mg/kg) were administered every 8 hours for 12 days and compared in normal adult horses. Phenylbutazone caused: 1) a decrease in serum total protein and albumin concentrations; 2) edema of the small intestine; and 3) erosions and ulcers of the large colon. Some horses in the flunixin group and the phenylbutazone group developed renal crest necrosis. All NSAIDs including ketoprofen caused erosions of the glandular mucosa of the stomach. However, only flunixin and phenylbutazone treated horses developed ulcerations of the glandular mucosa [316]. Ketoprofen was evaluated in a subacute safety study after the

intravenous administration of five times the therapeutic dose (11.0 mg/kg). No evidence of toxicity was reported in any horses after this 15 day regime. However, in drug tolerance studies using 25 times the therapeutic dose for 5 days, horses experienced depression, inappetence, icterus, nephritis, hepatitis, and hemorrhagic necrosis of the adrenal glands [309].

3. Phenylbutazone

Phenylbutazone is approved for the alleviation of musculoskeletal inflammation in the horse and dog. Phenylbutazone is a pyrazolone derivative with a pK_a of 4.8 [162]. In man, this group causes hypersensitivity reactions, diarrhea, vomiting, mucosal ulceration, hepatitis, nephritis, sweating, aplastic anemia and agranulocytosis [3]. The half-life in man is 72 hours owing to the extensive enterohepatic recirculation and renal tubular resorption [162], whereas in horses the reported values range from 4.8 [317] to 8.6 [19] hours. The reported half-life in man, like the horse varies considerably among subjects [162]. The half life is concentration dependent and for this reason it should not be used outside of the therapeutic range [318]. Phenylbutazone causes dose-dependent inhibition of superoxide generation at concentrations approximating therapeutic levels in humans [319]. This property adds to the anti-inflammatory potency of this drug [318]. It also enhances the excretion of uric acid which is beneficial in the treatment of gout in man [3]. In ponies, therapeutic doses decrease urinary sodium and chloride excretion with a concurrent decrease in plasma pH, bicarbonate and carbon dioxide [320]. Phenylbutazone reduces platelet function in ponies through both thromboxane/prostaglandin dependent and independent

pathways [321]. However, unlike aspirin in man, phenylbutazone does not prolong bleeding times [321].

a. Anti-inflammatory effects

Oral phenylbutazone in dogs with experimentally-induced urate arthritis significantly reduced the symptoms associated with the synovitis [101]. Phenylbutazone (4.4 mg/kg, intravenously) when administered to ponies with subcutaneously implanted carrageenan-soaked sponges significantly reduced the PGE_2 production in inflammatory exudates for at least 24 hours but it did not have any effect on leukocyte migration [322,323]. The same dose of phenylbutazone significantly reduced serum TXB_2 concentrations for 24 hours [324]. Pre-treatment with phenylbutazone also attenuated the endotoxin-induced rise in the stable metabolite of prostacyclin in equine plasma [223].

Oral administration of the therapeutic dose of phenylbutazone in ponies with carrageenan-induced inflammation also reduced prostaglandin concentrations and reduced skin temperatures below the incision line where the sponges were implanted [325]. The maximum depression of prostaglandin synthesis occurred at times of peak drug exudate concentration [325]. Also in ponies, the tissue cage fluid, peritoneal fluid and synovial fluid concentrations of phenylbutazone and the active metabolite, oxyphenbutazone were one third to two thirds of the corresponding plasma concentrations 6 and 12 hours after intravenous and oral doses of 4.4 mg/kg [323]. In another study by the same group, the level of phenylbutazone in inflammatory exudates from carrageenan soaked sponges in ponies approximated plasma levels from

4 to 12 hours after intravenous administration [326]. Administration of ^{14}C-labelled phenylbutazone in rats with carrageenan-induced inflammation in the hind paw and neck resulted in high concentrations in the inflamed areas and also the stomach, small intestine and kidney. This effect was attributed to ion-trapping of the acidic drug intracellularly in environments with a low pH. Further, in chickens with urate-induced arthritis, the synovial fluid concentration of phenylbutazone was higher in inflamed joints as compared to normal joints and the concentration in these joints was higher than corresponding plasma values [263].

b. Pain relieving effects

Phenylbutazone failed to alter normal pain thresholds in horses as tested by the thermal evoked hoof withdrawal and skin twitch reflexes [183]. These results are in agreement with the belief that NSAIDs do not produce a true analgesia but inhibit the hyperalgesia associated with the sensitization of the peripheral afferent [163,271]. Drugs of the pyrazolone class are reported to be less potent analgesics than other NSAIDs in animals [318]. In clinical studies phenylbutazone was equivalent to naproxen in alleviating pain and joint stiffness in human patients with ankylosing spondylitis [327]. In a clinical study involving several species of domestic and exotic animals, phenylbutazone treatment resulted in an overall recovery rate from lameness of 68.97% [328].

c. Adverse effects

Toxic doses of phenylbutazone produce central nervous system depression, anorexia, diarrhea, hypoproteinemia, mucosal ulceration [329] and phlebitis [330].

The ulcerogenic properties of this drug are more pronounced in the pony owing probably to less efficient absorption of oral doses in the gut [318]. Inhibition of prostaglandin plays an important role in the pathogenesis of ulcers as the co-administration of PGE_2 with the toxic doses of phenylbutazone resulted in no clinical abnormalities [329].

CHAPTER 3

QUANTIFICATION OF EICOSANOIDS IN EQUINE PLASMA AND SYNOVIAL FLUID

A. Introduction

The quantitation of eicosanoids in inflammatory, allergic and cardiovascular conditions has been performed most commonly using radioimmunoassay (RIA) [65,66,70,72,331-333]. Other quantitation techniques have been applied such as bioassay [136,334], high performance liquid chromatography (HPLC) [335-337], gas chromatography coupled with mass spectrometry (GC/MS) [338,339] and more recently, enzyme-linked immunosorbent assay (ELISA) [340-343]. The ELISA procedure offers advantages over RIA in that it is easier, faster, less expensive and produces no radioactive waste [341,344].

The detection of eicosanoids is complicated by the fact that these substances are very potent, having endogenous concentrations of a few pg/ml of plasma. Many have short biological half-lives [24]. For example, the *in vivo* half-life of leukotriene B_4 (LTB_4) in carrageenan exudate was reported as approximately 45 minutes [345]. Circulating prostaglandins are metabolized within minutes in the lung and liver [24].

Eicosanoid assays must be specific as both the prostaglandin and leukotriene series contain many similar compounds that are produced concurrently from polyunsaturated fatty acid precursors, mainly arachidonic acid. Many eicosanoids and their metabolites have minor structural differences in functional groups, such as

hydroxy or keto groups and double bonds, that may make isolation by extraction and chromatography difficult [346]. Some eicosanoids are unstable and can undergo non-enzymatic degradation depending upon pH conditions or in the presence of albumin [24]. For example, the plasma metabolite of prostaglandin E_2 (PGE_2), 15-keto-13,14-dihydro-PGE_2 forms a bicyclic product by dehydration and an internal addition reaction [347].

Further complicating analyses are sampling methods such as venipuncture and tissue extraction which can activate phospholipase A_2 resulting in *ex vivo* formation of eicosanoids [348]. The coagulation process routinely produces prostaglandins while cells such as neutrophils are capable of eicosanoid formation in whole blood. Therefore, it is recommended that biological samples be collected into chilled tubes containing inhibitors of prostaglandin and leukotriene synthesis and an anticoagulant with expedient removal of the cell fraction [348]. One group of investigators determined that collection of samples into tubes with ethylenediamine tetraacetate (EDTA) resulted in lower prostaglandin levels than those collected with heparin [349]. This same group also determined that centrifugation of samples at room temperature gave higher thromboxane and prostaglandin levels than samples centrifuged at 4° C. Subtle differences in these sampling techniques make comparison of eicosanoid concentrations obtained from different laboratories difficult.

Extraction of biological fluids before assaying eicosanoids is necessary to remove other lipids that may interfere with the analysis or cause erroneously calculated concentrations. Reports of assays without prior extraction show higher

levels of these compounds than those found when extraction preceded the assay [350]. Several techniques for extraction have been published including various liquid/liquid techniques using organic solvents [333] and, more recently, solid phase extraction procedures [351-353]. Most of these methods involve acidification of the biological sample in order to protonate the eicosanoid and increase solubility in the organic solvents. In some cases purification by normal or reversed phase HPLC after extraction is necessary to isolate the compound(s) of interest before assaying by other means.

The purpose of this study was to evaluate methods of eicosanoid extraction and quantitation in equine plasma and synovial fluid. Specifically, two methods for extracting PGE_2 and LTB_4, and an ELISA method for quantitation of these eicosanoids in equine synovial fluid and plasma were examined. As the PGE_2 ELISA was reported to have cross reactivity with PGE_1, a method of validation and confirmation of this assay by mass spectrometry was developed.

B. Materials and methods

1. Sample collection

a. Plasma from digital veins

Blood samples were collected from the digital veins of normal and laminitic horses (see also Chapter 6) using a 20 gauge, 1" needle. After 1-2 ml of blood were discarded, 10 ml were collected into chilled evacuated siliconized glass tubes (Vacutainer, Becton Dickinson, Rutherford, NJ) containing 3.3 μg/ml of BW755C

(Wellcome Research Laboratories, Beckenham, Kent, England) as an inhibitor of *ex vivo* eicosanoid synthesis [70] and 10 mg/ml of the anticoagulant, disodium ethylenediamine tetraacetate (EDTA) [348] purchased from Sigma Chemical Co., St. Louis, MO. Samples were centrifuged at 2,500 rpm for 15 minutes at 4° C (Beckman J21B, Palo Alto, CA). The platelet poor plasma was stored in polypropylene centrifuge tubes (Sarstedt, Newton, NC) at -20 ° C for 2 days then at -70° C until assayed [350].

b. Synovial fluid

Synovial fluid was collected from the left intercarpal joints of 24 horses with experimentally-induced synovitis (see also Chapter 4). Synovial fluid was aseptically withdrawn from each joint using a 20 gauge, 1" needle and 12 or 20 ml polypropylene syringes. The fluid was immediately transferred into tubes containing the inhibitors of eicosanoid synthesis. The use of BW755C in this project was discontinued due to limited availability. Instead, BWA4C (Wellcome Research Laboratories, Beckenham, Kent, England) was used as an inhibitor of *ex vivo* leukotriene synthesis along with indomethacin (Biomol, Plymouth Meeting, PA) as inhibitor of *ex vivo* prostaglandin synthesis. The concentration of BWA4C used was based on the studies on the *in vitro* inhibition of LTB_4 in guinea pig blood [343]. Indomethacin was used at a concentration recommended by Benedetto [348]. Stock solutions containing 8.64 mg/ml of BWA4C in dimethylsulfoxide (DMSO) and 6.6 mg/ml indomethacin in ethanol were prepared and stored at 4° C. Each chilled evacuated tube (Vacutainer, Becton Dickinson, Rutherford, NJ) contained 6.48 μg/ml of BWA4C, 5 μg/ml of

indomethacin, 1.5 mg/ml of EDTA (K_3) and 57 μl saline. As DMSO concentrations above 1.5% have been shown to cause hemolysis [354], saline was added to dilute the DMSO concentration in each 2 ml tube to 1.5%, with a final concentration in 2 ml of synovial fluid of 0.075% [354]. Tubes were then centrifuged at 2,500 rpm for 15 minutes at 4° C (Eppendorf 5415C, Brinkman Instruments, Westbury, NJ). The cell-free synovial fluid was stored in polypropylene micro-centrifuge tubes (Dot Scientific, Inc., Flint, MI) at -20° C for 2 days and then at -70° C until assayed.

2. Extraction

Two extraction procedures were evaluated, a liquid/liquid extraction technique and a solid phase extraction method. For the digital vein eicosanoid concentration study, (see also Chapter 6) and for the mass spectrometry quantitation of PGE_2 in inflamed synovial fluid, a liquid/liquid extraction technique was used. Synovial fluid samples described in Chapter 4 were extracted via a solid phase extraction method. All reagents were HPLC grade and were purchased from commercial sources. Water was triple distilled and filtered (Modulab Polisher I™, Continental Water Systems Corp., San Antonio, TX).

a. Liquid/liquid extraction

For plasma samples, 4 ml were extracted for both the LTB_4 and PGE_2 ELISA assays. For the synovial fluid assays, 0.25 ml was used for determination of PGE_2 by ELISA and 0.575 ml was used for samples to be confirmed by mass spectrometry. One-half ml of synovial fluid was extracted for LTB_4 determination by ELISA. Both plasma and synovial fluid were acidified to pH 3.5 with 1 N HCl in polypropylene

centrifuge tubes. Three volumes of ethyl acetate were added to extract the eicosanoids. After vortexing and centrifugation at 2,500 rpm for 15 minutes (Dynac, Becton Dickenson and Company, Parsippany, NJ), the aqueous and organic layers were separated. The ethyl acetate was evaporated under nitrogen.

b. Solid phase extraction

i. Preparation

Synovial fluid (0.5 ml) was extracted for LTB_4 determination by ELISA. Samples processed using this technique contained high concentrations of PGE_2 (Chapter 4) which necessitated reduced volumes of synovial fluid or dilution in order to stay within the limits of PGE_2 quantitation (10-5000 pg/ml). Volumes of 0.25, 0.125, 0.0625 or 0.01 ml were extracted depending on the anticipated concentration of PGE_2. For synovial fluid obtained from horses with severe inflammation, serial dilutions of synovial fluid were made with water acidified to pH 3 with 1 N HCl (1:10 and 1:100) and the diluted sample was then extracted. Sep-Pak Plus™ cartridges containing 360 mg of C_{18} were purchased from the Millipore Corporation, Milford, MA. Pre-washed 20 ml syringe barrels (Sherwood Medical, St. Louis, MO) were attached to the cartridges and served as reservoirs for the synovial fluid and reagents. A modified polypropylene pipet tip was attached to the cartridge in order to direct effluent flow. The new cartridges and syringes were washed with 20 ml of hexane followed by 20 ml of ethyl acetate and 20 ml of methanol under vacuum from a manifold device (Amicon, Lexington, MA) to remove impurities and immunoreactive substances according to a modified method of Jubiz [355]. Previously used cartridges

were washed with 40 ml of hexane, ethyl acetate and methanol before each extraction according to a modified method of Powell [351]. Cartridges were used for extraction a maximum of three times. Every cartridge was washed immediately before use with 20 ml of methanol and pH 3 water according to a modified method of Powell [351].

ii. Synovial fluid extraction

The synovial fluid was acidified to pH 3.5 using 1 N HCl in a polypropylene centrifuge tube. Water (0.5 ml) acidified to pH 3 was added and the sample was vortexed for 15 seconds before being transferred to the cartridge reservoir. Care was taken not to transfer the precipitated protein as it was shown to clog the cartridge. The sample was allowed to flow through the cartridge slowly under slight vacuum pressure (<5 inches of mercury). After the sample was evacuated, 6 ml of pH 3 water was added under vacuum to remove water soluble inorganic salts, amino acids, proteins and other polar compounds in the sample [350,351]. This flush was followed by 6 ml of hexane under vacuum to remove the neutral lipids in the sample. Finally, the cartridges were removed from the vacuum manifold and the eicosanoids were eluted from the cartridge with 6 ml of ethyl acetate using gentle pressure applied with a syringe plunger. The effluent was collected into polypropylene centrifuge tubes. The ethyl acetate was then evaporated under a stream of nitrogen gas.

c. Recovery studies

Extraction efficiency for plasma and synovial fluid samples was determined using 5,6,8,11,12,14,15-^{3}H-PGE_2 (200 Ci/mmole) purchased from Advanced Magnetics (Cambridge, MA). Radioactivity in disintegrations per minute (dpm) of

spiked samples and standards was counted on a scintillation counter (Packard Tri Carb™, Packard Instrument Co., Downers Grove, IL). Absolute recovery of ^{3}H-PGE_2 and ^{3}H-LTB_4 was calculated by expressing the radioactivity (dpm) of 3 extracted standards in synovial fluid or plasma as a percent of the mean radioactivity of 3 non-extracted controls. Recovery was corrected for background radioactivity. Percent recovery was determined for both eicosanoids in plasma using the liquid/liquid technique and synovial fluid using liquid/liquid extraction, solid phase extraction, and also for solid phase extraction using methyl formate as the final eluting agent. The coefficient of variation (CV) of each technique was calculated from the mean and standard deviation (SD) of the percent recovery.

3. ELISA

Quantitation of plasma and synovial fluid eicosanoids was achieved by commercially available PGE_2 and LTB_4 ELISA kits (Advanced Magnetics, Cambridge, MA). All reagents, standards and 96-well precoated micro-titer plates were provided in the kits. These assays were based on the principle of a competitive ELISA where PGE_2 or LTB_4 in the sample (reconstituted after extraction in buffer solution) competed with fixed amounts of alkaline phosphatase labelled PGE_2 or LTB_4 for binding to a limited number of sites of the specific rabbit antibody (anti-PGE_2 or LTB_4) bound to the microtiter well. Unbound material was removed by washing with a buffered saline solution. *p*-Nitrophenyl phosphate was added to each well as substrate for alkaline phosphatase. Color development was terminated following incubation at 37° C by the addition of 0.2 N sodium hydroxide. The intensity of the

yellow colored product formed was inversely proportional to the amount of unlabelled eicosanoid bound to the well. Absorbance (optical density) was read at 410 nm by a microtiter plate reader (Dynatech MR5000, Dynatech Laboratories, Alexandria, VA). Optical density (OD) results were normalized by subtracting the mean absorbance of 2 blank wells (containing only *p*-Nitrophenyl phosphate and sodium hydroxide) from sample ODs. Samples with the greatest color development or optical density values (ODs) contained the least PGE_2 or LTB_4. Absorbance was correlated with concentration by means of a standard curve ranging from 10 to 5000 pg/ml (Figure 2). Quantitation of unknown samples processed in duplicate was achieved by averaging the absorbance of sample duplicates and calculation of the concentration from the standard curve. All sample concentrations were then corrected for extraction efficiency.

a. Intra-assay variation

An estimate of the within run precision or the variation between duplicate wells of the same sample run in the same assay under identical conditions was determined for PGE_2 and LTB_4. This represents the variability inherent in the assay, *e.g.*, antibody binding, reagent stability, pipet accuracy, or plate reader accuracy. It was defined as the CV of duplicate wells and was calculated from the mean optical density and SD for each of 5 non-extracted standard concentrations. The CV values were averaged for 9 PGE_2 assays and 4 LTB_4 assays at each concentration and overall $\pm$ SD.

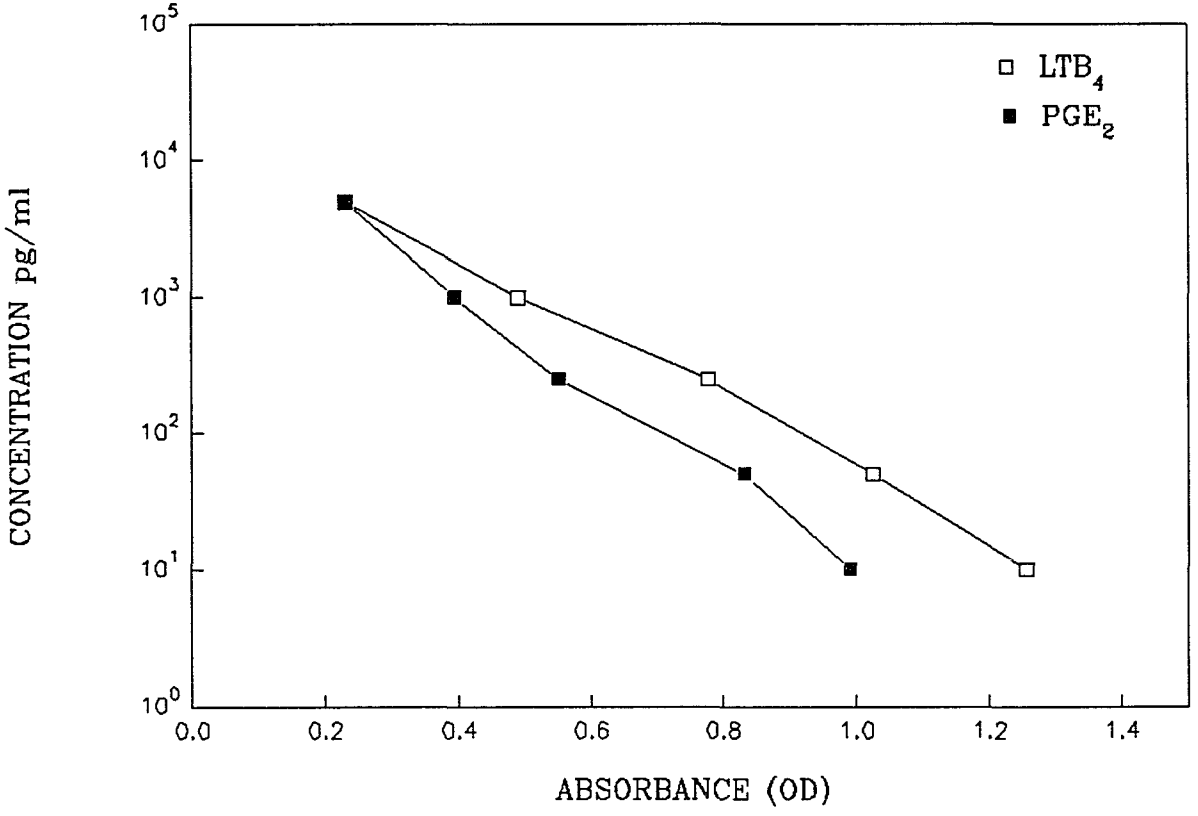


Figure 2: Standard curves from PGE_2 and LTB_4 ELISAs.

b. Inter-assay variation

An estimate of the variation of non-extracted standards of PGE_2 and LTB_4 assayed in different kits was determined. This value includes variations in incubation times, operator technique, manufacturing of kits and intra-assay variance. Inter-assay variation was defined as the CVs of 5 standard concentrations run in 9 PGE_2 assays and 4 LTB_4 assays. An overall CV was determined by averaging the individual concentration CVs to get an overall CV $\pm$ SD. In addition, the correlation coefficient of the standard curve was calculated for each assay (SigmaPlot, Jandel Corp., San Rafael, CA). The mean correlation coefficient $\pm$ SD was calculated for 9 PGE_2 assays and 4 LTB_4 assays.

4. PGE_2 confirmation

Synovial fluid obtained from the intercarpal joint of one horse 12 hours after induction of carrageenan synovitis was extracted using the liquid/liquid technique and assayed for PGE_2 by the ELISA procedure outlined above. A separate aliquot was also extracted using the liquid/liquid technique, subjected to purification by a HPLC technique and then assayed for PGE_2 by GC/MS [356]. The results were then compared in an effort to confirm and validate the PGE_2 ELISA. Before extraction, PGD_2 (16 ng/sample or 1.92 ng on the GC column) was added to the synovial fluid and served as an internal standard for quantitation by GC/MS.

a. Eicosanoid standards

PGD_2 [(5Z,9α,13E,15S)-9,15-Dihydroxy-11-oxoprosta-5,13-dien-1-oic acid] was used as the internal standard for PGE_2 [(5Z,11α,13E,15S)-11,15-Dihydroxy-9-oxoprosta-5,13-dienoic acid]. Both were purchased from Cayman Chemical Co., Ann Arbor, MI and were stored in ethanol at -70° C.

b. Solvents

Liquid chromatography and mass spectrometry grade solvents were obtained from commercial sources. HPLC mobile phase A consisted of water, acetonitrile, and trifluoroacetic acid in a ratio of 75:25:0.0008 (pH approximately 6). Mobile phase B consisted of methanol, acetonitrile and trifluoroacetic acid at a ratio of 60:40:0.002. Each mobile phase was filtered through a 0.45 μm membrane filter (FP Vericel™, Gelman Sciences, Inc., Ann Arbor, MI).

c. High performance liquid chromatography (HPLC)

Synovial fluid was subjected to HPLC in order to isolate and collect PGE_2. Samples were analyzed with a Hewlett Packard 1090 High Performance Liquid Chromatograph equipped with a variable volume auto-injector and a photodiode array detector set at 192 nm (4 nm bandwidth) with a reference spectrum of 450 nm (80 nm bandwidth). Instrument control, data acquisition, and peak integration were accomplished with Hewlett-Packard HPLC ChemStation software (Waldbronn, Germany). A 4 mm x 30 cm reversed phase column packed with 10 μm octadecylsilyl derivatized silica particles (Varian, Walnut Creek, CA) maintained at ambient temperature was used. Solvents were run using a gradient between 100%

mobile phase A and 100% mobile phase B over 40 minutes at a flow rate of 1 ml/minute. After extraction by the liquid/liquid technique, the synovial fluid samples were dissolved in 120 μl of Mobile Phase A and transferred to a 100 μl HPLC autosampler vial insert (American Scientific Products, McGraw Park, IL) that allowed for complete injection of the sample volume.

Due to the low sensitivity of the diode array detector for PGE_2, a radio-chromatography detector (Series A-500, Radiomatic Instruments and Chemical Co., Meriden, CT) was connected in series to the HPLC in order to establish the retention time of tritiated PGE_2. The scintillation fluid, Flo-Scint II™ (Radiomatic Instrument and Chemical Co., Meriden, CT), was pumped through the detector at 4 ml/minute. The radioactivity of ^{3}H-PGE_2 spiked synovial fluid samples was determined in counts per minute (cpm). These samples were run immediately before incurred (experimental) samples to determine the retention time of PGE_2.

Samples for PGE_2 confirmation were injected onto the HPLC and collected within $\pm$ 0.75 minutes of the predetermined retention time of PGE_2. This fraction was placed under a stream of nitrogen gas to evaporate the mobile phase and then stored at -20° C overnight.

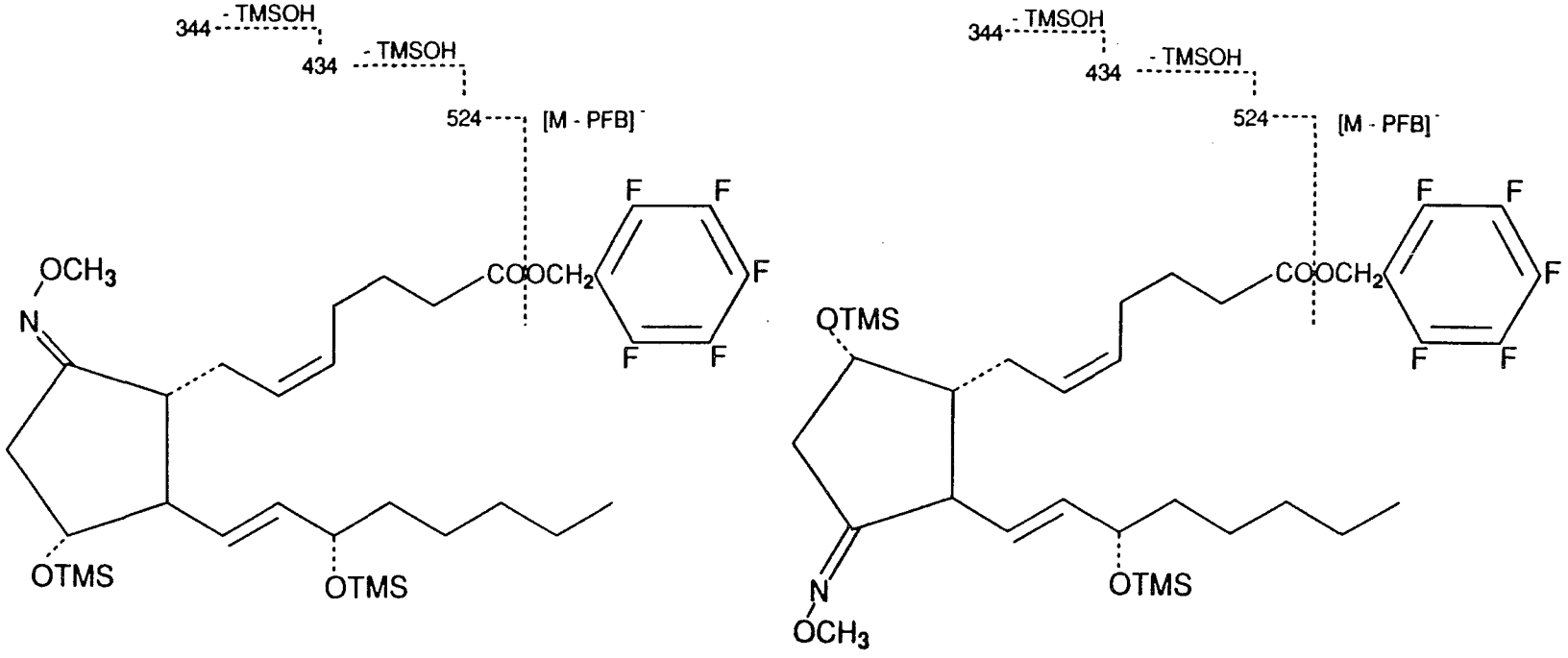
d. Gas chromatography and mass spectrometry (GC/MS)

Standards for PGE_2 were prepared using concentrations of 39.6, 79.0, 158.5, 634.0, 1268.3, and 2536.0 ng/ml. These values represented 118.9, 237.0, 475.6 pg, and 1.9, 3.8 and 7.6 ng of PGE_2 injected on the GC column. PGD_2 was added to each standard as an internal standard at a concentration of 640 ng/ml (1.92 ng on

column). These standards were derivatized and analyzed immediately before incurred synovial samples in order to establish a standard curve for quantitation.

Prior to GC/MS analysis, all samples and standards were derivatized to increase molecule volatility and thermal stability [346]. PGE_2 and PGD_2 were esterified at the carboxyl terminus with pentafluorobenzyl bromide (PFB), the keto group was oximated (MO) and then the trimethylsilyl ether (TMS) derivative was formed at both hydroxy groups (Figure 3). Derivatization was accomplished by adding 10 μl of methanol, 50 μl of acetonitrile, 2 μl of pentafluorobenzyl bromide and 1.5 μl of diisopropylethylamine to dried samples in glass vials with teflon-lined caps. The samples were vortexed and heated at 60° C for 15 minutes [357]. A 2% solution of methoxyamine hydrochloride in pyridine (25 μl) was added and samples were kept at room temperature overnight. After drying under nitrogen, 20 μl of water were added and the samples were extracted twice with 50 μl of ethyl acetate [358]. After drying again under nitrogen, 25 μl of N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) were added and samples were heated for 15 minutes at 60° C according to a modified method of the manufacturer. All derivatizing reagents were purchased from Pierce Chemical Co., Rockford, IL.

Immediately following derivatization, 3 μl of each sample were injected into a Finnigan MAT 9610 Gas Chromatograph (Sunnyvale CA) using a modified method of Waddel *et al.*, 1983 [359]. A 30 M DB-5 column (J & W Scientific, Fulsom, CA) with 0.25 μm of polymer coating and an internal diameter of 0.22 mm was used in the splitless injector mode with a helium purge of 0.75 seconds. The initial



PGE_2

(as the MO, PFB-ester, bis-TMS derivative)

PGD_2

(as the MO, PFB-ester, bis-TMS derivative)

Figure 3: Structure of derivatized PGE_2 and PGD_2. Dotted lines indicate fragmentation sites that produced masses (m/z) monitored by MID.

temperature was 200° C. This was increased by 30° C per minute to a final temperature of 300° C. The injector and transfer line temperatures were set at 220 and 300° C, respectively. The column was plumbed directly into a Finnigan MAT TSQ 4500 Mass Spectrometer (Sunnyvale, CA). The method of ionization was negative ion chemical ionization (NICI) using methane as the reagent gas. The ionizer source pressure was 0.5 Torr and the ionizer temperature was set at 150° C. Electron energy was 70 eV and the emission current was 0.30 mA. The electron multiplier was set at 2500 volts.

Data was acquired using multiple ion detection (MID) scanning quadrapole 3 (Q3) for masses of 524, 434, 344 and 196. This type of selective ion monitoring enabled pg detection. The second isomer of PGE_2 and PGD_2 was used for quantitation. The base peak of the second isomer of PGE_2 was 524 m/z and 434 m/z for PGD_2. The abundance of each base peak was obtained from the mass spectrum by the computerized summing of scans from the initiation to the termination of individual isomer peaks. Baseline noise was subtracted from the summed abundances of each peak. Standard curves for quantitation of samples for PGE_2 confirmation were prepared using the ratio of base peak abundances of PGE_2 and PGD_2.

C. Results

1. Sample collection

Synovial fluid samples from normal equine joints were very viscous. To insure that the synovial fluid supernatants were free of cells that could contribute to

ex vivo eicosanoid formation, 3 samples were evaluated for cellularity by microscopic examination of Wright's stained direct smears. No white blood cells or platelets were found and slides contained scant numbers of red blood cells.

Several samples of synovial fluid from horses with acute synovitis were subjected to two freeze/thaw cycles before extraction and ELISA in order to determine if previously thawed samples could be refrozen and assayed at a later date. Both PGE_2 and LTB_4 levels increased markedly (485.40% and 393.77%, respectively) after two freeze thaw cycles.

2. Extraction

Percent recoveries of LTB_4 and PGE_2 from plasma using liquid/liquid extraction and synovial fluid using liquid/liquid extraction, solid phase extraction, and solid phase extraction with methyl formate as the final eluting agent are reported in Table 9. Recoveries of PGE_2 from synovial fluid and plasma using the liquid/liquid technique modified from Coker *et al.*, [333] were 67.95 and 43.82%, respectively. Recovery of LTB_4 from synovial fluid and plasma using this method were 98.39 and 39.13%, respectively.

Attempts were made to duplicate the solid phase extraction procedure of Powell [351,360] using aqueous ethanol as a sample diluent and initial wash followed by a water wash, a petroleum ether wash and elution using methyl formate. The reported recovery of PGE_2 from urine and plasma using this method is $> 90\%$. However, in the present study this method demonstrated that recoveries of PGE_2 and LTB_4 were poor, in large part due the loss of eicosanoids from the Sep-Pak upon

Table 9: Percent Recovery of PGE_2 and LTB_4 in synovial fluid and plasma using various techniques.

		SYNOVIAL FLUID			PLASMA
		LIQUID/LIQUID	SOLID PHASE-ETHYL ACETATE	SOLID PHASE-METHYL FORMATE	LIQUID/LIQUID
PGE_2	% RECOVERY	67.95	61.04	48.97	43.82
	CV	14.11	2.97	8.64	9.55
LTB_4	% RECOVERY	98.39	57.67	65.99	39.13
	CV	7.37	12.25	11.70	10.06

(n=3)

application of the diluted sample fraction and the initial aqueous ethanol wash. It was determined that higher recoveries were obtained using water at pH 3 as the sample diluent and initial wash. Further, hexane was substituted for petroleum ether as it was found to produce less damage to the plastic syringe plungers. Ethyl acetate was found to give approximately equal recoveries (PGE_2- 61.04%; LTB_4- 57.67%) as the highly flammable and irritating methyl formate (PGE_2- 48.97%; LTB_4- 65.99%) when used with this modified method.

The cartridges and reservoirs were washed extensively before use to reduce the immunoreactive substances that were shown to interfere with the PGE_2 assay. Other researchers have also reported immunoreactive substances or other impurities from Sep-Paks that were not thoroughly washed before use [361,362]. According to Powell, cartridges can be re-used [351]. It was determined that cartridges could be re-used a maximum of three times without contributing any carryover immunoreactivity if they were washed with 40 ml each of hexane, ethyl acetate and methanol before each re-use.

3. ELISA

The limit of quantitation based on the lowest concentration of the standard curve was 10 pg/ml for both assays. According to performance characteristics supplied by the manufacturer, the sensitivity of the LTB_4 ELISA was 8.9 pg/ml with minimal cross reactivity with other leukotrienes, prostaglandins and arachidonic acid metabolites (Table 10). The reported sensitivity of PGE_2 ELISA was 1.5 pg/ml. The reactivity of this kit was low for other eicosanoids and metabolites tested except for

Table 10: Percent cross reactivity of PGE_2 and LTB_4 ELISAs as determined at 50% B/B_0. Standards were spiked into assay buffer (data supplied by Advanced Magnetics, Cambridge, MA).

PGE_2		LTB_4	
PGE_2	100.0%	LTB_4	100.0%
PGE_1	50.0%	5(S),12(R)-DiHETE	6.7%
PGA_1	6.0%	5(S),12(S)-Di-HETE	2.0%
PGA_2	1.9%	LTC_4	<1.0%
PGB_1	<1.8%	LTD_4	<1.0%
PGB_2	<1.8%	LTE_4	<1.0%
6-keto-PGE_1	<1.8%	5-HETE	<1.0%
15-keto-13,14-dihydro-PGE_2	<1.8%	12-HETE	<1.0%
$PGF_{2\alpha}$	<1.8%	15-HETE	<1.0%
5-HETE	<1.0%	PGD_2	<1.0%
12-HETE	<1.0%	PGE_1	<1.0%
6-keto-$PGE_{1\alpha}$	<1.0%	PGE_2	<1.0%
PGD_2	<1.0%	Thromboxane B_2	<1.0%
Arachidonic acid	<1.0%	20-hydroxy-LTB_4	<1.0%
Thromboxane B_2	<1.0%	Arachidonic acid	<1.0%

PGE_1 which had 50% reactivity with the PGE_2 antibody at 50% B/B_0 (sample OD/blank OD).

a. Intra-assay variation

The intra-assay variance or the within assay precision of the PGE_2 and LTB_4 ELISA as expressed as the coefficient of variation are presented in Table 11. Individual concentration and overall CVs were less than 10% as recommended by Feldcamp and Smith [363].

b. Inter-assay variation

The between assay variability based on the coefficient of variation of 9 PGE_2 assays and 4 LTB_4 assays was greater than the intra-assay variation (Table 11). The overall repeatability of these assays was 14.32% for PGE_2 and 17.28% for LTB_4. These values are in the range reported for ELISAs [342,364]. The mean correlation coefficient $\pm$ SD for the standard curves was 0.9869 $\pm$ 0.0102 for PGE_2 and 0.9850 $\pm$ 0.0140 for LTB_4.

3. PGE_2 Confirmation

A gradient of the 2 mobile phases reported by Powell [335] provided good chromatography of PGE_2 with minor column and flow rate modifications. A representative chromatogram of ^3H-PGE_2 spiked synovial fluid is shown in Figure 4. Figure 5 shows liquid chromatograms of 10 μg of PGE_2 and PGD_2 obtained with photodiode array detection as well as a radio-chromatogram of ^3H-PGE_2. Photodiode array chromatograms are expressed as milliabsorbance units (mAU) versus time, whereas radio-chromatograms are expressed as dpm versus time. The chromatograms

Table 11: Inter- and intra-assay variance (CV) of PGE_2 and LTB_4 ELISAs.

	PGE_2		LTB_4	
CONCENTRATION (pg/ml)	**INTER-ASSAY**	**INTRA-ASSAY**	**INTER-ASSAY**	**INTRA-ASSAY**
10	16.89	3.00	14.50	3.50
50	15.46	2.38	9.78	3.44
250	10.77	5.32	14.73	3.44
1000	12.52	2.79	18.40	3.04
5000	15.95	4.12	28.99	6.69
OVERALL CV (mean ± SD)	14.32 ± 2.57	3.52 ± 1.19	17.28 ± 7.23	4.02 ± 1.50

PGE_2 n=9
LTB_4 n=4

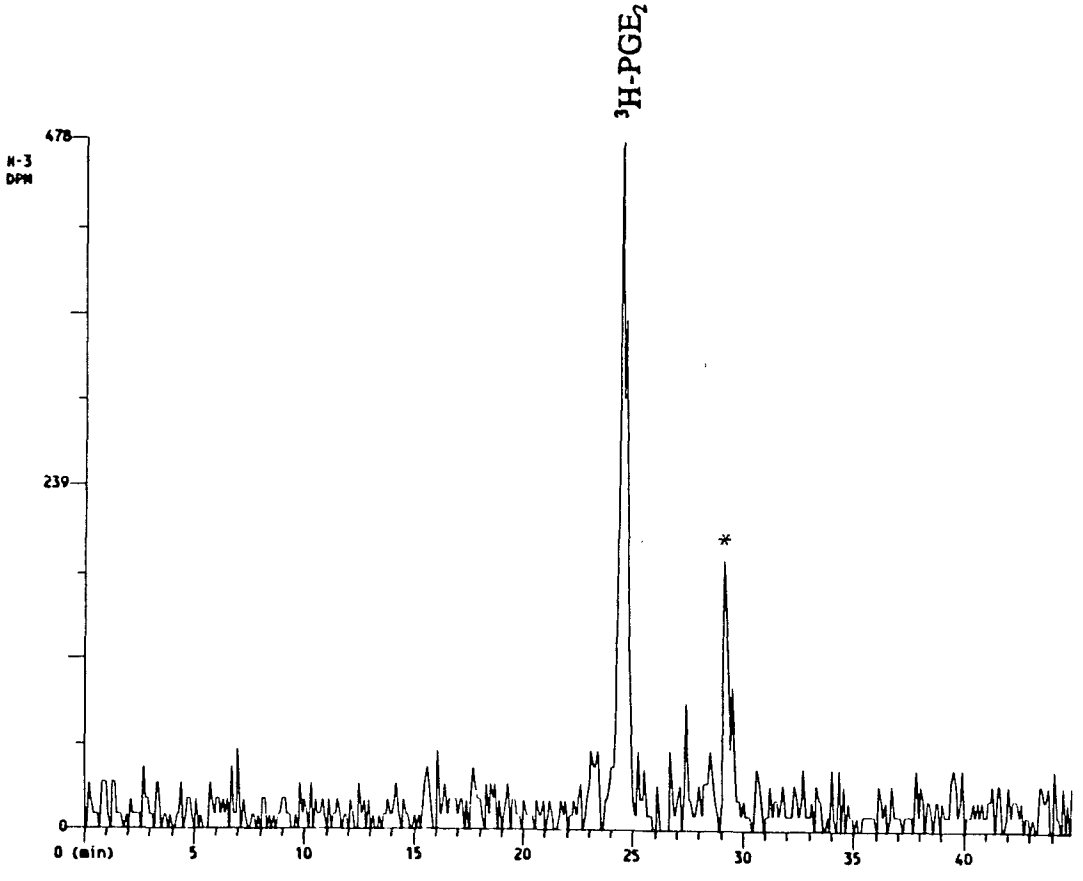


Figure 4: Liquid chromatogram with radio-chromatography detection of ^{3}H-PGE$_2$ spiked synovial fluid. The small peak at 29 to 30 minutes (*) is likely a degradation product of PGE$_2$.

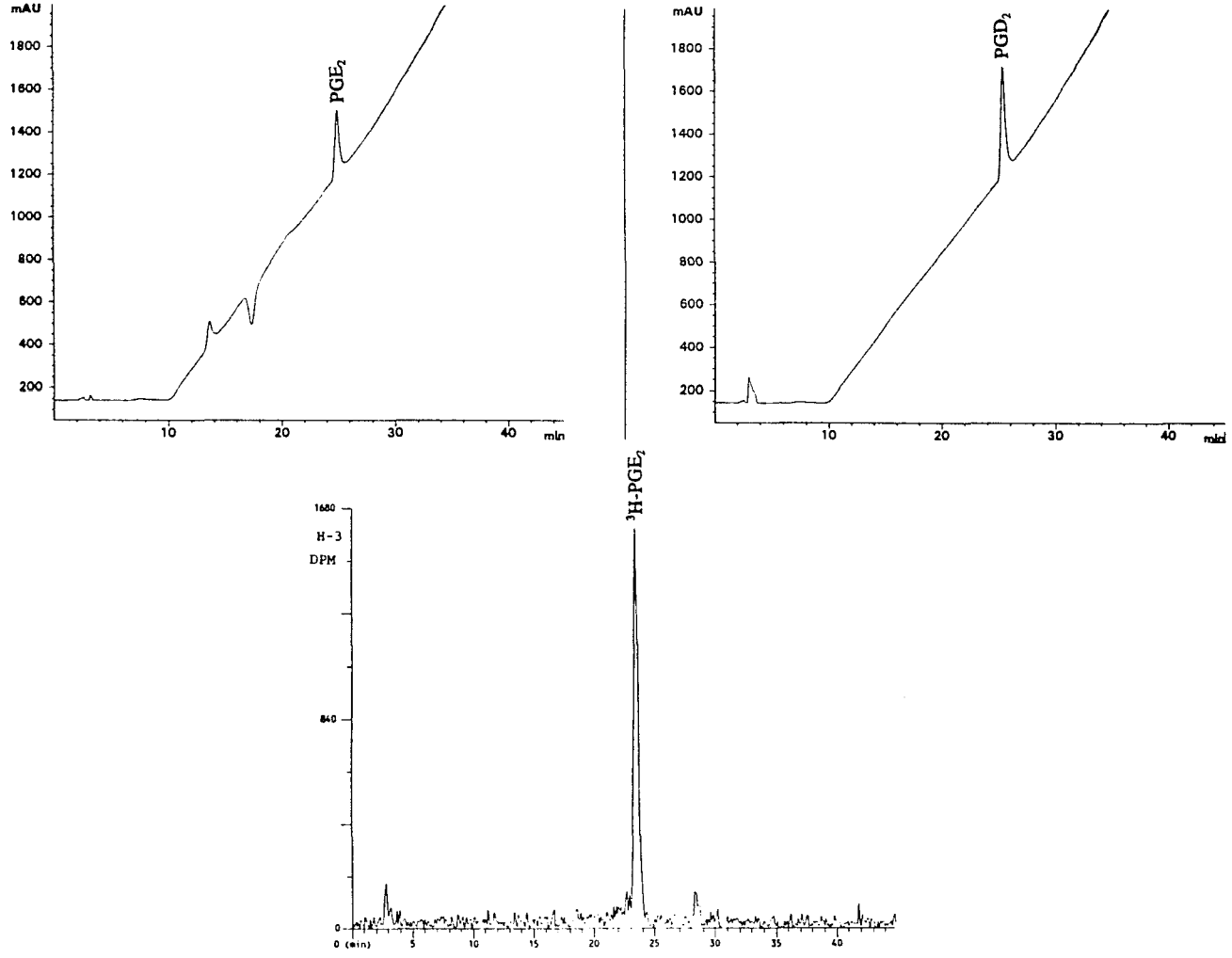


Figure 5: Liquid chromatograms of PGE_2 and PGD_2 obtained with photodiode array detection at 192 nm and ^3H-PGE_2 obtained with radio-chromatography detection. The slow increase in absorbance over time in the chromatograms of PGE_2 and PGD_2 is due to the solvent gradient.

obtained with photodiode array detection at a wavelength of 192 nm show interference due to the solvent gradient, making detection of small quantities of PGE_2 difficult. Thus, the retention time of PGE_2 was confirmed before collection of incurred samples using ^3H-PGE_2 with a radio-chromatography detector. ^3H-PGE_2 eluted between 0.4 and 0.12 minutes before PGE_2 due to the increase in hydrophilicity associated with the ^3H-labelled compound [365]. The retention times of PGE_2 and PGD_2 were 24.89 and 25.26 minutes, respectively. PGE_2 and PGD_2 differ only in the position of the hydroxy and keto groups (PGE_2- 11-hydroxy-9-keto; PGD_2- 9-hydroxy-11-keto). This difference accounts for the subtle difference in HPLC and GC/MS retention times.

The limit of detection for PGE_2 on GC/MS was 118 pg on column (39.6 ng/ml). A representative chromatogram of a standard solution of PGE_2 (237 pg on column) and PGD_2 (1.92 ng on column) is shown in Figure 6. PGE_2 and PGD_2 formed *syn* and *anti* methoxime isomers [366,367]. The first isomer of PGE_2 and PGD_2 coeluted but their second isomers separated adequately with the PGD_2 isomer eluting before PGE_2, as reported previously [359]. The second isomers were used for quantitation by expressing the abundance of each base peak of PGE_2 to PGD_2 as a ratio. NICI of these eicosanoids resulted in fragmentation of the molecular ion $(M)^-$ of the compound of interest (parent compound with derivatized functional groups, total molecular weight, 705). Cleavage of PFB from the molecular ion $(M\text{-}PFB)^-$ at the ester linkage resulted in the 524 m/z ion. Loss of one TMS as an alcohol resulted in the 434 m/z ion. The 344 m/z ion was produced following cleavage of the second TMS alcohol. The 196 m/z ion represented PFB with an oxygen molecule cleaved

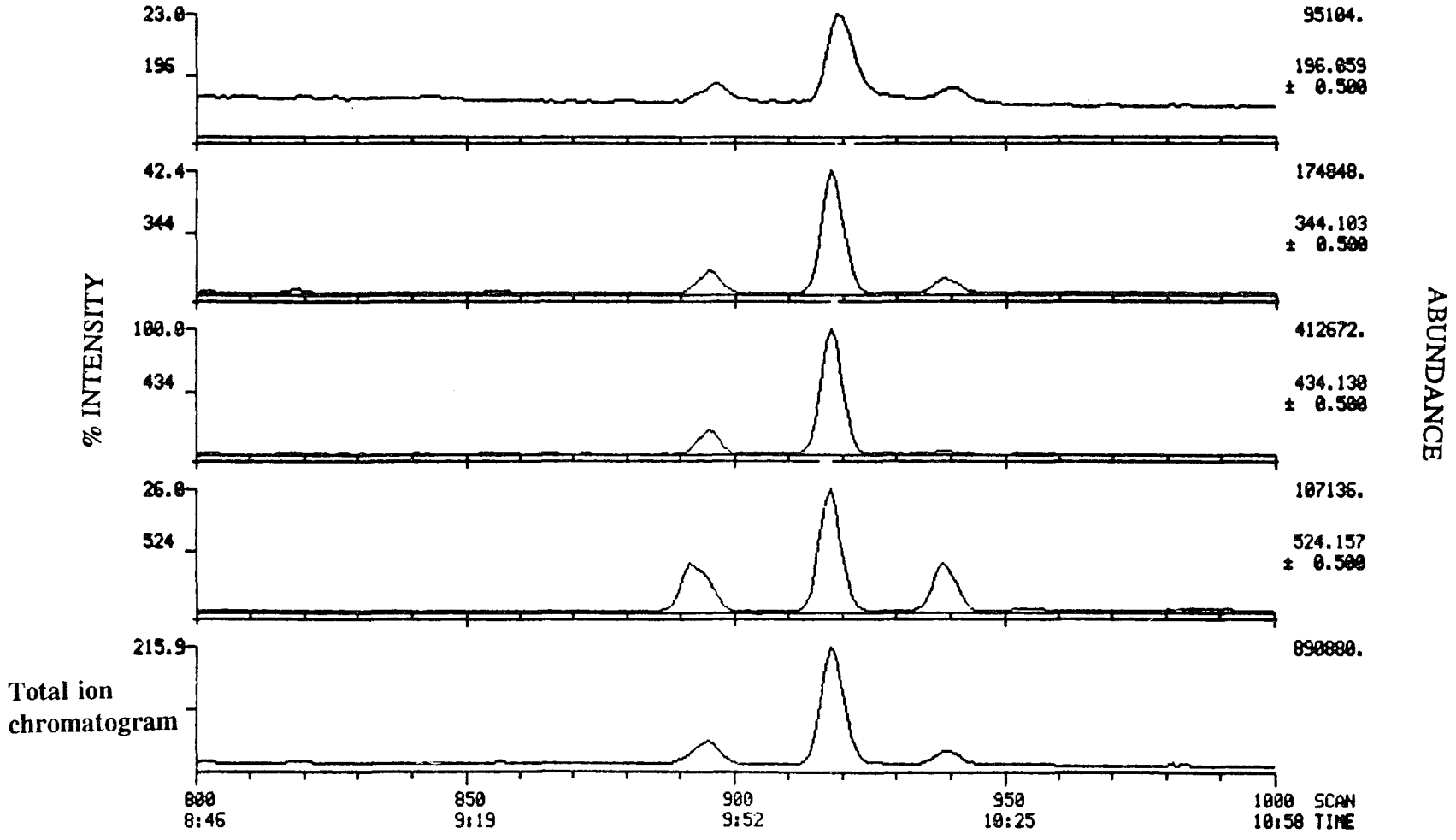


Figure 6: GC/MS chromatogram of a standard solution of PGE_2 (237 pg on column) and PGD_2 (1.92 ng on column). The first peak contains the first isomers, while the second and third peaks are the second isomers of PGD_2 and PGE_2, respectively.

from the prostaglandin. MID scans showing the intensity of the base peak of the second isomer of PGE_2 (524 m/z) and the base peak of the second isomer of PGD_2 (434 m/z) are shown in Figure 7. The standard curve using concentrations of PGE_2 ranging from 118.9 pg to 7.61 ng on column was linear with a coefficient of determination of 0.9981 (Figure 8).

The GC/MS chromatogram of synovial fluid obtained from a horse with experimentally-induced synovitis for PGE_2 confirmation is shown in Figure 9. Scans showing base peak abundances of PGE_2 and PGD_2 are shown in Figure 10. GC/MS analysis detected 1.07 ng of PGE_2 (on column) which was calculated to be 15.49 ng/ml in the original synovial fluid sample. The ELISA assay of a separate aliquot of the same synovial fluid resulted in 20.79 ng/ml of PGE_2.

D. Discussion

According to initial studies, PGE_2 and LTB_4 levels in synovial fluid of horses with acute synovitis increased after repeated freeze/thaw cycles. This was not due to cellular metabolism of arachidonic acid as these samples contained no cells capable of eicosanoid production. The reason for this effect is not readily apparent, although it has been seen previously in plasma but not serum [333,349]. Perhaps the subcellular particles containing cyclooxygenase and lipoxygenase enzymes metabolized the arachidonic acid present in membrane fragments in the synovial fluid. Or perhaps eicosanoids bound to proteins and glycosaminoglycans in the synovial fluid are released upon damage caused to these molecules by repeated freeze/thaw cycles.

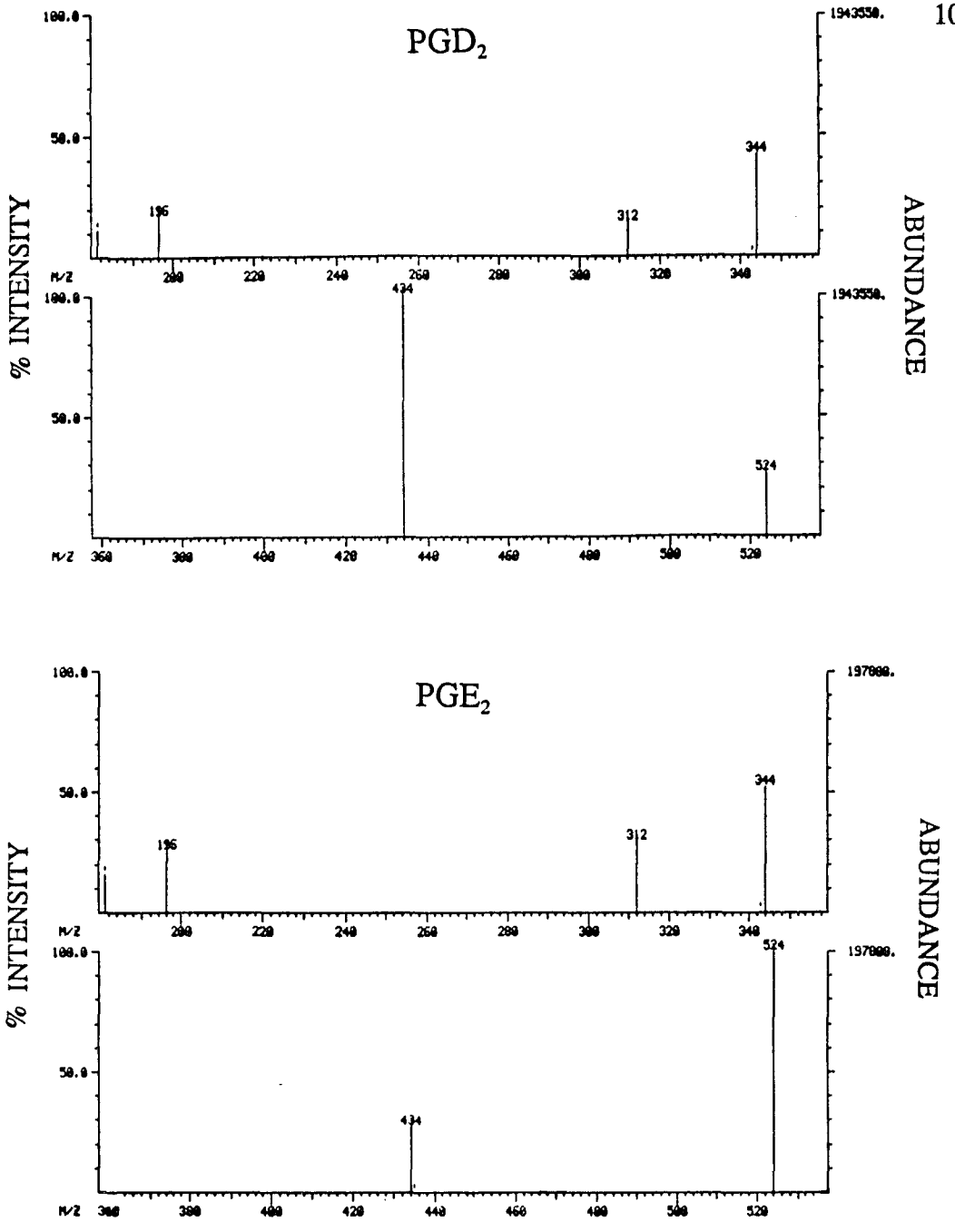


Figure 7: MID scans from the chromatogram in Figure 6. The intensity of the base peaks of the second isomer of PGD_2 (top) and PGE_2 (bottom) are shown.

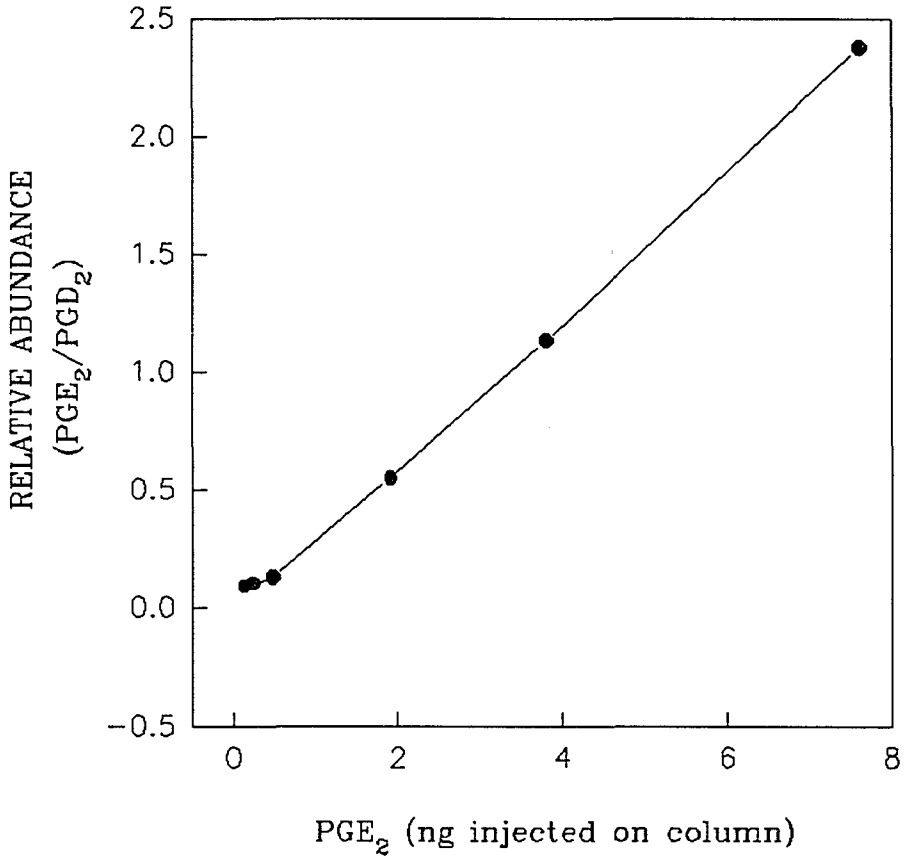


Figure 8: GC/MS standard curve of PGE_2 using PGD_2 as an internal standard.

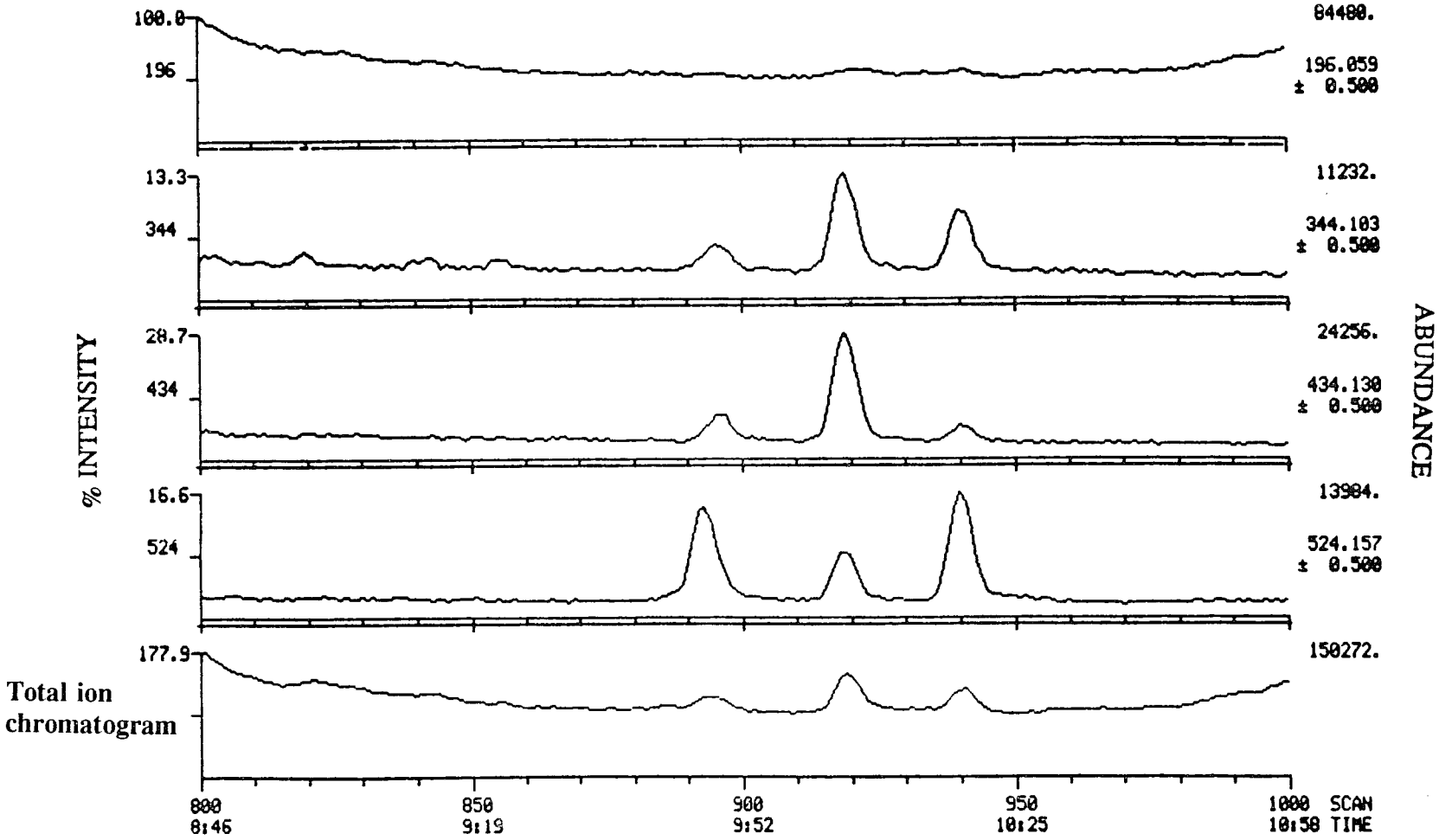


Figure 9: GC/MS chromatogram of a synovial fluid sample from a horse with experimentally-induced synovitis. PGD_2 (1.92 ng on column) was added as an internal standard. The calculated value of PGE_2 on column was 1.07 ng.

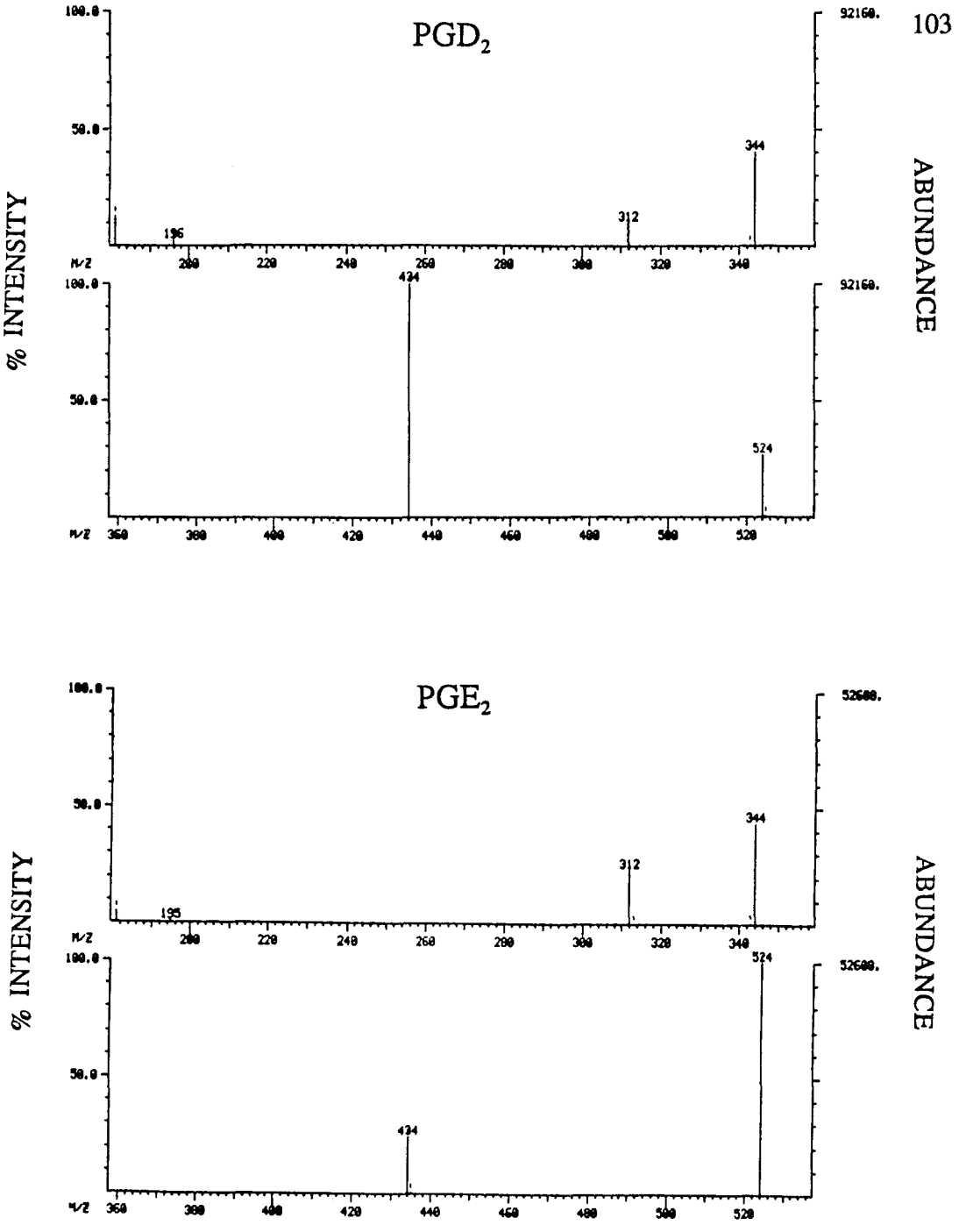


Figure 10: MID scans from the chromatogram in Figure 9. The concentration of PGE_2 in synovial fluid was 15.49 ng/ml as calculated from the standard curve of the relative abundances of PGE_2 (bottom) and PGD_2 (top).

Owing to this effect, all samples reported in the present studies were thawed immediately prior to extraction and assay. Results from other laboratories have demonstrated that eicosanoids are stable when thoroughly frozen (-20° C) for prolonged periods of time [333].

Results from the liquid/liquid extraction technique indicated that this method gave good recovery from small volumes of synovial fluid, whereas larger volumes of plasma resulted in lower recovery. Solid phase extraction of synovial fluid resulted in lower recovery of LTB_4 than the liquid/liquid procedure, although the solid phase recovery was in the range of reported extraction efficiencies. Recovery of leukotrienes using Sep-Paks with modified Powell methods were reported to be 73% [368] and 79% for LTC_4 [369] and 57% [362], 73% [345], 89% [355] and 96% [370] for LTB_4. Recovery of PGE_2 using solid phase extraction has been reported to be > 90% [351,368,371] which is higher than the recovery determined in this study. Recoveries calculated from serum [368], plasma, urine [351] and tissue culture medium [362] may not be comparable to equine synovial fluid due to the viscosity of this matrix which could bind or trap eicosanoids.

Solid phase extraction of eicosanoids from biological matrices has largely replaced liquid/liquid extraction owing to the specificity, efficiency and rapid processing of samples [346]. The technique as originally described by Powell [372] is based on the affinity of the eicosanoids for the non-polar octadecylsilyl (ODS) packing material of the cartridge. The cartridges function as reversed phase chromatography columns [351]. The application of strong organic solvents such as

hexane or petroleum ether allows for the removal of very non-polar compounds such as triglycerides and other lipids. The application of more polar solvents such as methyl formate or ethyl acetate elutes the eicosanoids. In the present study, this approach allowed for a more selective extraction procedure than the liquid/liquid extraction techniques.

Most eicosanoids are small haptens that have only one epitope and are unsuitable for the original "sandwich" ELISA where two molecules of antibody recognized the same antigen [344]. Most eicosanoid ELISA procedures, including the ones described in this study, are of the competitive type where labelled antigen competes with sample antigen for binding on the specific antibody. In immunoassay procedures the degree of displacement of the antigen from the antigen-antibody complex is quantified. The binding of the labelled antigen is inhibited by the antigen of interest (PGE_2 or LTB_4 in the sample). However, cross-reacting substances of similar structure and excessive amounts of unrelated compounds may interfere with binding. Although too expensive and complicated to be used for routine analysis, GC/MS has been used as a validation procedure for immunoassays [373]. GC/MS is regarded as the most specific and sensitive method for analysis of eicosanoids. Specificity is based on the gas chromatographic retention time of the compound and detection of the characteristic fragment ions by mass spectrometry [339].

The HPLC technique used for isolation and collection of PGE_2 before GC/MS has been shown to separate many cyclooxygenase and lipoxygenase metabolites that may then be subjected to GC/MS [335,336]. However, in the present study, the use

of radio-chromatography of tritium labelled standard was needed to confirm the retention time of PGE_2 due to the relative insensitivity of the photodiode array detector. This technique proved less expensive than using μg quantities of PGE_2 on the photodiode array detector as the radio-chromatography detector was sensitive to ng quantities of tritiated PGE_2.

Quantitation of eicosanoids is accomplished most effectively by the highly sensitive NICI GC/MS technique [346]. The analysis of PFB derivatives of eicosanoids by NICI GC/MS has the advantage of producing a high yield of stable ionized products and a low fragmentation rate [346]. The sensitivity of this method is due to fragmentation that is primarily directed away from the prostaglandin molecule thereby leaving the carbon skeleton intact [366]. Quantitation of eicosanoids by GC/MS using stable isotope dilution techniques is most often accomplished using deuterated analogues of the compound of interest acting as internal standards. These techniques are advantageous in GC/MS due to the similar structure and properties of the isotope and native compound [374]. The technique employed in this study, whereby a structurally similar compound is used as an internal standard, represents a simple and sensitive alternative to the use of deuterated compounds.

Analytical problems have been an obstacle in eicosanoid research. Thus, progress in this field is dependent on the advancement and validation of analytical techniques [338]. The ELISAs used in these experiments to quantitate PGE_2 and LTB_4 in equine synovial fluid and plasma had not been described previously for use in the horse. Cross reactivity information obtained from the manufacturer indicated

that the LTB_4 assay was highly specific and sensitive. As unique interfering substances from equine synovial fluid are unlikely, validation procedures were not performed for LTB_4. The PGE_2 assay although sensitive, had 50% cross reactivity with PGE_1. The validation procedures used in the present study confirmed the results of the PGE_2 ELISA on inflamed equine synovial fluid. GC/MS analysis detected 15.49 ng/ml of PGE_2 in the synovial fluid while the ELISA performed on a separate aliquot of the same sample resulted in 20.79 ng/ml. These results are in good agreement taking into account minor losses of sample during purification by HPLC and derivatization before GC/MS. Therefore, the PGE_2 ELISA appears to specific for this compound in equine synovial fluid, indicating the absence of interferences from this matrix.

CHAPTER 4

EFFECT OF KETOPROFEN AND PHENYLBUTAZONE ON ACUTE SYNOVITIS

A. Introduction

Synovitis accompanies most forms of equine joint disease [75]. Synovitis is characterized by inflammation of the synovium without gross disturbance of the articular cartilage or disruption of major supporting structures [83]. Clinical signs of acute synovial inflammation include: synovial effusion with distension of the joint capsule; increased skin temperature over the joint; hypertrophy and hyperplasia of the synovia resulting in a palpable thickening of the membrane; a decrease in the range of motion of the joint; and lameness in the affected limb. Eicosanoids and other inflammatory mediators contribute to the inflammatory response by increasing synovial vascular permeability [17], increasing the metabolic rate of synoviocytes [37], and causing congestion of the microvascular bed [93]. As the permeability of the synovial membrane increases, proteins accumulate in the joint resulting in an increase in fluid osmotic pressure. The resulting effusion of synovial fluid often results in pain and overt lameness [94]. In humans there is a positive linear correlation between intra-articular pressure and joint pain [95]. The inflammatory mediators released by local tissue destruction in the joint activate nociceptors and also sensitize these receptors through lowering of activation thresholds [96,97]. Further,

the release of substance P and other neuropeptides [98] may potentiate the inflammatory response and result in neurogenic inflammation [99].

Experimentally, synovitis has been induced in horses, ponies, calves, dogs, rabbits and rats by the intra-articular injection of a variety of chemical substances. Models of joint inflammation have been used for screening and evaluation of anti-inflammatory agents [101] The equine joint is a unique site for an inflammatory model due to the structure and function of the synovial membrane and the sheer size of the joint space. The synovial membrane acts as a selective barrier in the joint in that it allows for the passage of molecules of less than approximately 12,000 daltons in molecular weight [76]. Transfer of small molecules to and from the synovial fluid occurs readily due to specialized structures within the membrane such as: a synovial lining cell layer that is 1 to 4 cells thick and often incomplete [74]; lack of desmosomes or a basement membrane [77]; and the presence of fenestrated capillaries, secretory and macrophagic cells, surface pores and large numbers of lymphatics [74].

Nonsteroidal anti-inflammatory drugs (NSAIDs) are considered the mainstay of treatment for non-septic joint disease in animals [76,375]. It is well documented that these agents alleviate the pain and lameness associated with joint inflammation [101] through reduction in the peripheral nociceptive impulses from the inflamed joint [197] and through central analgesic mechanisms [148,273]. However, few studies have described the anti-inflammatory effects of these agents in detail in experimentally induced joint inflammation [297].

The purpose of this study was: 1) to develop a self-limiting, reproducible model of acute synovitis which increased prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) synovial fluid concentrations along with other measurable joint inflammatory responses; 2) compare the magnitude and time course of the anti-inflammatory and eicosanoid inhibitory effects of ketoprofen and phenylbutazone in the acute synovitis model; and 3) compare the ability of ketoprofen and phenylbutazone to reduce the clinical signs of acute synovitis. The approved therapeutic doses of ketoprofen (2.2 mg/kg) and phenylbutazone (4.4 mg/kg) were used. In addition, the phenylbutazone molar equivalent dose of ketoprofen (3.63 mg/kg) was used in order to compare the potency of ketoprofen and phenylbutazone. NSAIDs and saline were administered at the same time as carrageenan induction in order to determine the anti-inflammatory effects of the NSAIDs on developing and acute inflammation.

The intercarpal joint was chosen for induction of synovitis because of its large volume of synovial fluid [376] as it communicates with the carpometacarpal joint in most (90%) horses [74]. Further, this joint has three access sites: medial and lateral to the extensor carpi radialis tendon and at the palmarolateral reflection of the intercarpal joint capsule, distal to the accessory carpal bone [377]. As most of the horses used in this study were retired racing Thoroughbreds, the left carpus was used because of the increased incidence of joint pathology in the right carpus [378,379]. Inflammation was induced in each horse only once due to the potential for permanent cartilage damage with successive carrageenan injections [108]. Further, it has been

documented that the prior injection of carrageenan in the rat paw causes hyperalgesia to develop much faster when the contralateral paw is injected [272].

B. Materials and methods

1. Experimental animals

Twenty-four mares and geldings (5 American Quarter Horses and 19 Thoroughbreds) weighing from 410 to 554 kg and ranging from 6 to 13 years of age were used. Prior to induction of synovitis, horses were judged to be healthy and sound based on physical examination, lameness evaluation, complete blood count, left intercarpal synovial fluid clinical pathology analysis and radiographic examination of the left carpus. Horses with radiographic or synovial fluid pathology of the intercarpal joint were excluded.

All horses were vaccinated at least three weeks prior against eastern and western equine encephalomyelitis, influenza, tetanus (Equi-Flu EWT™, Coopers, Mundelein, IL) and rhinopneumonitis (Rhinomune™, SmithKline Beecham Animal Health, Exton, PA). The horses were dewormed with oxibendazole (Equipar™, Coopers, Mundelein, IL). Horses were housed in 10.9 x 12.5 feet stalls and were maintained on 5 pounds of a pelleted ration (Purina Horse Chow 100™, Purina Mills Inc., St. Louis, MO) containing: a minimum of 10% protein; 2% fat; a maximum of 25% fiber; and a vitamin/trace mineral supplement twice daily with mixed grass hay (11% protein, 1.4% fat, 24% fiber) and water provided *ad libitum.*

2. Drugs and reagents

a. NSAID solutions for administration

Ketoprofen (Ketofen™) was obtained commercially from Aveco Company, Inc., Fort Dodge, IA. Each ml of the sterile solution contained: 100 mg ketoprofen; L-arginine, 70 mg; citric acid to adjust pH to approximately 7; and benzyl alcohol, 0.25 ml as a preservative. Phenylbutazone (Butazolidin™) was obtained commercially from Coopers Animal Health, Inc., Kansas City, KS. Each ml of the sterile solution contained: phenylbutazone, 200 mg; sodium hydroxide to adjust pH to between 9.5 and 10.0; and benzyl alcohol, 10.45 mg as a preservative.

b. Carrageenan solution

Carrageenan (type IV; molecular weight, approximately 300,000) was purchased from Sigma Chemical Co., St. Louis, MO. This type of non-gelling carrageenan was isolated from two seaweed species, *Gigartina aciclulaire* and *Gigartina pistillata*. It was sterilized in powder form using ethylene oxide (H.W. Andersen Products, Haw River, NC) and then mixed to a suspension with sterile 0.9% saline according to a modified method of Higgins and Lees to produce a 1% solution [70]. All horses were induced using the same batch of carrageenan as different batches are known to vary in their inflammatory potency [380]. Cultures of the 1% solution were made in duplicate on blood agar (aerobic culture medium) and in thioglycollate broth (anaerobic culture medium) weekly for the duration of this study [381]. These cultures were maintained at 37° C for three days and were examined for microbial growth daily. The solution was thoroughly mixed and (0.3

ml) was drawn up in a sterile 1 ml syringe using a 25 gauge, 1 inch needle on the morning of each experimental day.

3. Experimental protocol

a. Overview

In the beginning of each experimental session, horses were evaluated for lameness and then brought into the environmentally controlled laboratory and restrained in an equine stanchion. The left carpal joint was radiographed and measured. The horse was then sedated and after 5 minutes, blood was collected and heart rate, respiratory rate and rectal temperature were determined. A thermography apparatus was applied to the joint and then it was scrubbed in preparation for arthrocentesis. In baseline sessions, synovial fluid was collected. Then at time 0, carrageenan was injected into the joint and ketoprofen (2.2 mg/kg and 3.63 mg/kg), phenylbutazone (4.4 mg/kg) or saline was administered intravenously. Horses were subsequently evaluated according to this protocol at 1, 3, 6, 9, 12, 24 and 48 hours after administration.

b. Horse preparation

Each horse was washed and shaved over the entire carpal region 24 hours prior to drug administration. Indwelling catheters (14 gauge, 5.7 cm) (Quick-Cath™, Baxter Healthcare Corp., Deerfield, IL) were aseptically placed bilaterally in the jugular veins of each horse before each session.

c. Synovial fluid collection and induction of synovitis

Horses were sedated with intravenous detomidine HCl (Dormosedan™, Norden Laboratories, Lincoln, NE) at doses of 10 μg/kg before the baseline collection, and 5.0 μg/kg before each post-treatment arthrocentesis to reduce the risk of iatrogenic joint hemorrhage and trauma. In addition, all horses were nose twitched during arthrocentesis. Before each arthrocentesis the entire left carpal area was scrubbed with Hibiclens™ (Stuart Pharmaceuticals, Wilmington, DE) and alcohol at least three times with a final scrub directly over the site for arthrocentesis. Sterile needles (20 gauge, 1 inch), syringes (12 ml or 20 ml) and gloves were used to collect fluid from the intercarpal joint while the limb was held in the flexed position by another examiner. Immediately following the collection of the baseline synovial fluid sample, 0.3 ml of 1% solution of sterile carrageenan was injected into the left intercarpal joint (dorsolateral aspect) using sterile technique [52]. An independent examiner administered the NSAIDs or saline via the left jugular catheter immediately following carrageenan injection.

Synovial fluid was then collected from the intercarpal joint at 1, 3, 6, 9, 12, and 24 hours after drug and carrageenan administration. Fluid was collected alternatingly from the dorsolateral and dorsomedial aspect of the intercarpal joints. In addition, the palmarolateral access site was used intermittently when the joint become effused. Sites for arthrocentesis were rotated in order to minimize iatrogenic hemorrhage into the joint capsule. At each time point, the maximum volume of

synovial fluid available was withdrawn in order to reduce the degree of discomfort in the horse caused by joint effusion.

All synovial fluid samples were aliquoted for ketoprofen determination, bacterial culture, clinical pathology analysis and eicosanoid determination. Fluid (at least 1.5 ml) for the PGE_2 and LTB_4 assay was placed immediately into chilled evacuated tubes containing 1.5 mg/ml of ethylenediamine tetraacetate K_3 (EDTA) (Vacutainer, Becton Dickinson, Rutherford, NJ) with 6.48 μg/ml of BWA4C, 5 μg/ml of indomethacin, and 57 μl saline. Tubes were then centrifuged at 2,500 rpm for 15 minutes at 4° C (Eppendorf 5415C, Brinkman Instruments, Westbury, NJ). The cell-free synovial fluid was stored in polypropylene micro-centrifuge tubes (Dot Scientific, Inc., Flint, MI) at -20° C for 2 days, then at -70° C until assayed. Fluid for clinical pathology analysis (at least 0.75 ml) was placed into 2 ml evacuated tubes containing 3 mg/ml of EDTA (Vacutainer, Becton Dickinson, Rutherford, NJ). Fluid for ketoprofen determination was processed according to methods described in Chapter 5.

Synovial fluid volume was recorded and the fluid was graded for hemorrhage based on the following scale:

Grade 1: A slight amount of blood contamination, often obtained upon exiting the joint.

Grade 2: Moderate blood contamination upon exiting the joint or fluid that had a moderate red or brownish-red haze indicating formation of unconjugated bilirubin from the breakdown of erythrocytes.

Grade 3: Very hazy, opaque, red or brownish-red fluid.

Grade 4: Fluid containing a large quantity of frank blood or evidence of clotting.

d. Blood collection

All NSAIDS and detomidine were administered via the left jugular catheter. The right jugular catheter was used for blood collection before drug and carrageenan administration (baseline) and at 1, 3, 6, 9, 12, 24 and 48 hours after drug injection for determination of complete blood counts. Blood was also collected for determination of plasma ketoprofen concentrations as outlined in Chapter 5. The patency of each catheter was maintained after each blood collection with 3 ml of 100 IU/ml of heparinized saline. Before blood was collected, 2-3 ml was discarded to remove any residual heparin from the catheter. Blood (7 ml) was collected with a 18 gauge, 1.5 inch needle and a 20 ml syringe and then placed immediately into 7 ml evacuated glass tubes containing 10.5 mg of EDTA as an anticoagulant (Vacutainer, Becton Dickinson, Rutherford, NJ) and stored at 4° C until analysis.

e. Lameness evaluation

Horses were evaluated at a walk and trot on a concrete surface and graded for lameness as follows [377]:

Grade 1: The horse exhibited a normal gait at a walk. The trot showed a slightly shortened weight bearing phase for the left forelimb with an audible cadence abnormality. There was even head and neck lifting for each foot.

Grade 2: The walk showed stride changes with no abnormal head or neck lifting. The trot showed obvious lameness with uneven head and neck lifting.

Grade 3: The lameness was obvious at a walk and trot with prominent head and neck lifting as the left forelimb was weight bearing.

Grade 4: The horse experienced difficulty bearing weight on the left forelimb.

f. Radiography

The left carpus was radiographed (Min X-Ray 300, Evanston, IL) laterally while held in rigid flexion by the examiner, in order to obtain the angle of maximum flexion of the joint, using 20.3 x 25.4 cm film cassettes and TML diagnostic film (T-Mat™, Eastman Kodak Co., Rochester, NY). After development of the radiographic film (RP X-Omat Processor, Eastman Kodak Co., Rochester, NY) lines were drawn on the radiographic image along the long axis of the radius and third metacarpal bones at the measured midpoint of each diaphysis. The angle formed by the intersection of these lines dorsal to the carpus was measured with a standard protractor to provide an estimate of the range of motion of the carpus.

g. Joint circumference and effusion grade

The circumference of the intercarpal joint was measured in cm using a standard tape measure. To ensure measurement consistency, the joint was marked with fingernail polish in three places (medial, lateral and palmar) in the beginning of each experimental session. Each horse was subjectively evaluated for carpal effusion based on the following scale:

Grade 1: Slight effusion around the site of carrageenan injection.

Grade 2: Moderate effusion of the intercarpal joint only without distention of the palmarolateral joint pouch.

Grade 3: Marked effusion of the intercarpal joint with distension of the palmarolateral joint pouch. Some swelling proximal and distal to the intercarpal joint.

Grade 4: Severe swelling of the entire carpal region.

h. Temperature, heart and respiratory rates

Rectal temperature was obtained from a rectal probe (Model RET-1, Sensortek, Clifton, NJ) and digital thermometer (Model TH-6D, Sensortek, Clifton, NJ). Heart and respiratory rates (bpm) were obtained by auscultation.

i. Carpal thermography

A contact thermography system (Novatherm, Med Tech Products, Inc., Dayton, OH) was used to detect thermal emissions from the dorsal surface of the left carpus. The detector consisted of a liquid crystalline latex membrane wrapped around a pressurized frame with a viewing window. Eight membranes were used with mean detection temperatures of 22, 24, 26, 28, 30, 32, 34 and 36° C. Each membrane had an approximate temperature range of 4° C. Color changes in the membrane were correlated with temperatures by color calibration scales on each membrane (approximately 0.8° C change per color). Membranes were held against the dorsal surface of the carpus for 60 seconds, withdrawn and immediately photographed (Polaroid auto-focus single lens camera with Polaroid instant film (779, ASA 640), Polaroid Corp., Cambridge, MA). Room temperature in the laboratory was

maintained between 23 and 26° C. Isothermic areas on the photographic images were calculated using a digitizing tablet and computerized area integration program (SigmaScan, Jandel Corp., San Rafael, CA). Each isothermic area was expressed as a percent of the total photographed area and the overall temperature of the joint was calculated. For each horse, post-injection data were expressed as a percent change from baseline in order to minimize variance between horses in basal joint temperatures.

4. Synovial fluid analysis

a. Clinical pathology

Fluid was kept at 4° C and warmed to room temperature (20-22° C) before analysis. Fluid was analyzed for cellularity, protein and quality of hyaluronic acid. The total number of nucleated cells (neutrophils, large mononuclear cells, lymphocytes, eosinophils and basophils) and red blood cells per μl were determined by a multispecies automated hematology analyzer (Baker 9000, Baker Instrument Co., Allentown, PA). Samples with total nucleated cell counts of less than 500 cells/μl were reanalyzed by the direct hemocytometer method [382]. The overall quality of the hyaluronic acid in the synovial fluid was determined by the mucinous precipitate quality test (MPQ). This serves as a reliable qualitative measure of the concentration and polymerization of synovial fluid hyaluronic acid [376]. Synovial fluid (0.5 ml) was added to 5 ml of 5% acetic acid and mixed thoroughly. The resultant precipitate was subjectively graded as: 1) good- tight ropy mass in clear solution; 2) fair- soft mass with some shreds; or 3) poor- shredded, small, soft masses in a turbid solution

[75]. Synovial fluid protein concentration was analyzed after centrifugation (Hemofuge™, American Scientific, manufactured by Heraeus-Christ, Osterod, West Germany) at 12,000 rpm for 4 minutes and was determined by a hand held refractometer (TS Meter, American Optical, Keene, NH). Values below 2.5 g/dl were not determined. These determinations were performed by the Louisiana State University, School of Veterinary Medicine, Clinical Pathology Laboratory.

Differential leukocyte counts were made from direct and concentrated synovial fluid smears. For samples with total nucleated cell counts less than 7,500 cells/μl, 5 drops of synovial fluid were concentrated on a glass slide by centrifugation at 1,000 rpm for 6 minutes (Cytospin 2, Shandon Southern Products, Astmoor Runcorn, Chesire, England). Samples with elevated nucleated cell counts (>7,500 cells/μl) were examined directly on glass slide smears. Slides were air dried and stained with modified Wright's stain (Hema-Tek™, Miles Laboratory, Inc., Elkhart, IN) using an automatic stainer (Hema-Tek™ slide stainer, model 4480, Ames Co.; Miles Laboratory, Inc., Elkhart, IN). A standard light microscope with 10X, 40X and 100X oil immersion objective lenses was used for all synovial fluid cell counts (Ernst Leitz Wetzlar, Germany). Slides were examined for neutrophils, lymphocytes, large mononuclear cells (monocytes and macrophages [382]), eosinophils and basophils. 100 cells were counted for every 30,000 nucleated cells/μl.

b. Bacterial culture

Synovial fluid remaining in the collecting syringe after aliquoting for the other assays was diluted with sterile 0.9% saline and cultured in duplicate on blood agar

plates and in thioglycollate broth tubes. Cultures were incubated at 37° C for 3 days and were examined visually for bacterial or fungal growth every 24 hours.

c. Eicosanoid determination

i. Extraction

Synovial fluid (0.5 ml) was extracted before LTB_4 determination by ELISA. In order to stay within the range of ELISA quantitation for PGE_2 (10-5000 pg/ml), synovial fluid volumes of 0.25, 0.125, 0.0625 or 0.01 ml were extracted depending on the anticipated concentration of PGE_2. Further, synovial fluid obtained from some horses at times 6, 9 and 12 hours was diluted with water acidified to pH 3 with 1 N HCl (1:10 and 1:100) and the diluted sample was then extracted. Sep-Pak Plus™ cartridges containing 360 mg of C_{18} (Millipore Corporation, Milford, MA) were washed and cleaned according to methods described in Chapter 3. The synovial fluid was acidified to pH 3.5 using 1 N HCl in a polypropylene centrifuge tube. One-half ml of pH 3 water was added and the sample was vortexed for 15 seconds before being transferred to the cartridge reservoir. The sample was allowed to flow through the cartridge slowly under slight vacuum pressure (<5 inches of mercury). After the sample was evacuated, 6 ml of pH 3 water was added under vacuum followed by 6 ml of hexane. The eicosanoids were eluted from the cartridge with 6 ml of ethyl acetate using gentle pressure applied with the syringe plunger. The ethyl acetate was then evaporated under a stream of nitrogen gas.

ii. ELISA

Quantitation of plasma and synovial fluid eicosanoids was achieved by commercially available PGE_2 and LTB_4 ELISA kits (Advanced Magnetics, Cambridge, MA). These assays were based on the principle of a competitive ELISA where PGE_2 or LTB_4 in the sample competed with fixed amounts of alkaline phosphatase labelled PGE_2 or LTB_4 for binding to a limited number of sites on the specific rabbit antibody (anti-PGE_2 or LTB_4) bound to the microtiter well. Absorbance (optical density) was read at 410 nm by a microtiter plate reader (Dynatech MR5000, Dynatech Laboratories, Alexandria, VA). Samples with the greatest color development or optical density values (ODs) contained the least PGE_2 or LTB_4. Absorbance was correlated with concentration by means of a standard curve ranging from 10 to 5000 pg/ml. All sample concentrations were then corrected for extraction efficiency. For a complete description of the ELISA procedure, refer to Chapter 3.

5. Hematology

All blood samples were kept at 4° C then warmed to room temperature before analysis. Total leukocyte (WBC) and red blood cell (RBC) counts were determined by the hemocytometer method [383,384] using the Unopette™ micro-collection system (WBC- model 5853; RBC- model 5850, Becton, Dickinson and Company, Rutherford, NJ). Determination of whole blood packed cell volume (PCV%) was accomplished by centrifugation of glass capillary tubes at 11,700 to 13,700 rpm for 5 minutes (Autocrit™ II, model 0574, Clay Adams; Becton, Dickinson and Company,

Parsippany, NJ) [384]. Plasma obtained from the centrifuged capillary tubes was analyzed for protein (g/dl) by a hand held refractometer method (TS Meter, American Optical, Keene, NH). Plasma fibrinogen was determined by heating these centrifuged capillary tubes for 4 minutes at 56° C and then centrifuging again for 5 minutes [384]. Protein in the heated plasma was determined by the refractometer. Fibrinogen (g/dl) was calculated by subtracting the heated plasma protein concentration from the original plasma protein value and multiplying by 1,000. Blood smears were prepared on glass slides for differential leukocyte counts, air dried and stained with modified Wright's stain (Hema-Tek™, Miles Laboratory, Inc., Elkhart, IN) using an automatic stainer (Hema-Tek™ slide stainer, model 4480, Ames Co.; Miles Laboratory, Inc., Elkhart, IN). Cells (100) were differentiated into neutrophils, lymphocytes, monocytes, eosinophils and basophils. Platelets were also enumerated from the stained blood smears. A standard light microscope with 10X, 40X and 100X oil immersion objective lenses was used for all hematological counts (Ernst Leitz, Wetzlar, Germany).

6. Study design and statistical analysis

Twenty-four horses received ketoprofen (2.2 mg/kg and 3.63 mg/kg), phenylbutazone (4.4 mg/kg) and saline intravenously according to a Latin Square design [385]. Each treatment was administered to 6 horses and each horse was used only once. Treatments assigned to horses were coded before the initiation of the study. All drug administrations, tests and analyses were performed by individuals

unaware of the treatment code. The treatment code was broken at the end of the study.

All data except for eicosanoid concentrations were analyzed using one-way analysis of variance (ANOVA) for repeated measures [386,387]. When indicated by the ANOVA, multiple comparisons were performed using Tukey's *w* procedure [388]. Differences between treatments at each time were considered significant when $P < 0.05$.

PGE_2 and LTB_4 synovial concentration data were analyzed independently using an analysis of variance for a split plot design with repeated measures where treatment constituted the main plot and the time by treatment interaction was the subplot [386,389]. The paucity of synovial fluid available for eicosanoid assay at some time points resulted in missing data. This precluded the use of the ANOVA as this procedure excludes all the data from a particular horse if only one time point is missing. Instead, data were analyzed initially by a univariate approach for overall treatment, time, and time by treatment interaction effects. Where indicated by the univariate analysis, treatment means (averaged over time) were compared using Tukey's *w* procedure. Eicosanoid concentrations (averaged over treatments) were compared at times 0, 1, 3, 6, 9, 12, 24 and 48 hours by Tukey's *w* procedure. Post treatment times that were found to have significantly ($P < 0.05$) different eicosanoid concentrations than baseline were analyzed for differences between individual treatments by pairwise Student's *t* tests, using the least squares means procedure to adjust for missing data [388,386]. Differences between treatments at each time were

considered significant when $P < 0.05$. Multiple comparisons of treatments were thus preplanned, *i.e.* performed only at times indicated by the Tukey's *w* procedure, so as to not inflate the comparisonwise error rate [388].

C. Results

Before initiation of this project, a pilot study was performed on 2 horses in order to refine sampling techniques and assay procedures and to ensure that the synovitis was transient with no lasting articular damage. The experimental protocol was similar except that these 2 horses were not administered systemic NSAIDs at the time of carrageenan induction. The first horse was humanely euthanized for an unrelated hindlimb musculoskeletal disorder 72 hours after carrageenan induction. The articular cartilage of the intercarpal joint (third and intermediate carpal bones) of this horse was grossly and histologically normal. Upon gross examination, the synovium was slightly hyperemic and thickened. Histologically, the synovial membrane showed a minimal non-suppurative inflammatory response with hypertrophy and hyperplasia of the synovial cells resulting in increased folding of the synovial villi. Lameness subsided in both of these horses by 24 hours. Synovial fluid collected from the second horse 5 months after induction was determined to be normal upon clinical pathology analysis. Based on the pilot study results, it was determined that the synovitis was transient and many of the parameters returned to near baseline by 48 hours.

Overall treatment effects for all parameters tested in the 24 carrageenan treated horses are listed in Table 12. For the clinical parameters, there was a significant difference among treatments for synovial fluid volume, lameness grade, carpal effusion grade and carpal thermography. For the synovial fluid clinical pathology parameters, significant treatment effects were observed for protein and PGE_2 concentrations. For the hematological parameters, only the number of eosinophils and basophils showed a significant treatment effect.

1. Synovial fluid volume and appearance

The volume (mean $\pm$ standard error (SEM)) of synovial fluid in 24 horses obtained from the left intercarpal joint at baseline was 4.54 ml $\pm$ 0.24. The volume of synovial fluid increased over time in all horses after induction. However, phenylbutazone significantly reduced the volume of synovial fluid as compared to saline at 1, 9 and 12 hours after administration (Figure 11A). Further, phenylbutazone significantly reduced the synovial fluid volume as compared both to doses of ketoprofen at 9 and 12 hours. The remaining treatments failed to affect synovial fluid volume significantly.

No significant treatment effects for subjective grade of hemorrhage were obtained. However the mean hemorrhage grade for all horses increased over time with a peak at 12 hours. When data were averaged over all horses, the hemorrhage grade ranged from 0.83 $\pm$ 0.19 at baseline to 3.29 $\pm$ 0.19 at 12 hours post-induction (Table 13). Synovial fluid samples often contained visible amounts of yellowish fibrin. This was removed from the sample before analysis. A few samples with

Table 12: Statistical analysis of overall treatment effects.

CLINICAL PARAMETERS	P	SYNOVIAL FLUID CLINICAL PATHOLOGY	P	HEMATOLOGY	P
SYNOVIAL FLUID VOLUME (ml)	0.0092*	NUCLEATED CELLS/μl	0.3611	WBC/μl	0.0908
SYNOVIAL FLUID HEMORRHAGE GRADE	0.7151	RBC/μl	0.4862	RBC/μl	0.7829
LAMENESS GRADE	0.0015*	MPQ grade	0.1296	PCV (%)	0.7261
MAX. FLEXION ANGLE (°)	0.1601	PROTEIN (g/dl)	0.0064*	PROTEIN (g/dl)	0.8187
JOINT CIRCUMFER. (cm)	0.3469	NEUTROPHILS	0.8492	FIBRINOGEN (g/dl)	0.8644
CARPAL EFFUSION GRADE	0.0432*	MONO. CELLS/μl	0.3681	NEUTROPHILS/μl	0.4993
RECTAL TEMP. (°C)	0.1848	LYMPHOCYTES/μl	0.8936	MONOCYTES/μl	0.4000
HEART RATE (bpm)	0.3653	EOSINOPHILS/μl	0.2448	LYMPHOCYTES/μl	0.4235
RESPIRATORY RATE (bpm)	0.9279	BASOPHILS/μl	0.2670	EOSINOPHILS/μl	0.0047*
CARPAL THERMOGRAPHY (°C)	0.0072*	PGE_2 (pg/ml)	0.0056*	BASOPHILS/μl	0.0375*
ROOM TEMP. (°C)	0.7151	LTB_4 (pg/ml)	0.0603	PLATELETS/μl	0.8886

P = Probability estimate obtained from analysis of variance, treatments include phenylbutazone (4.4 mg/kg), ketoprofen (3.63 mg/kg), ketoprofen (2.2 mg/kg) and saline.
*Statistically significant at $P < 0.05$

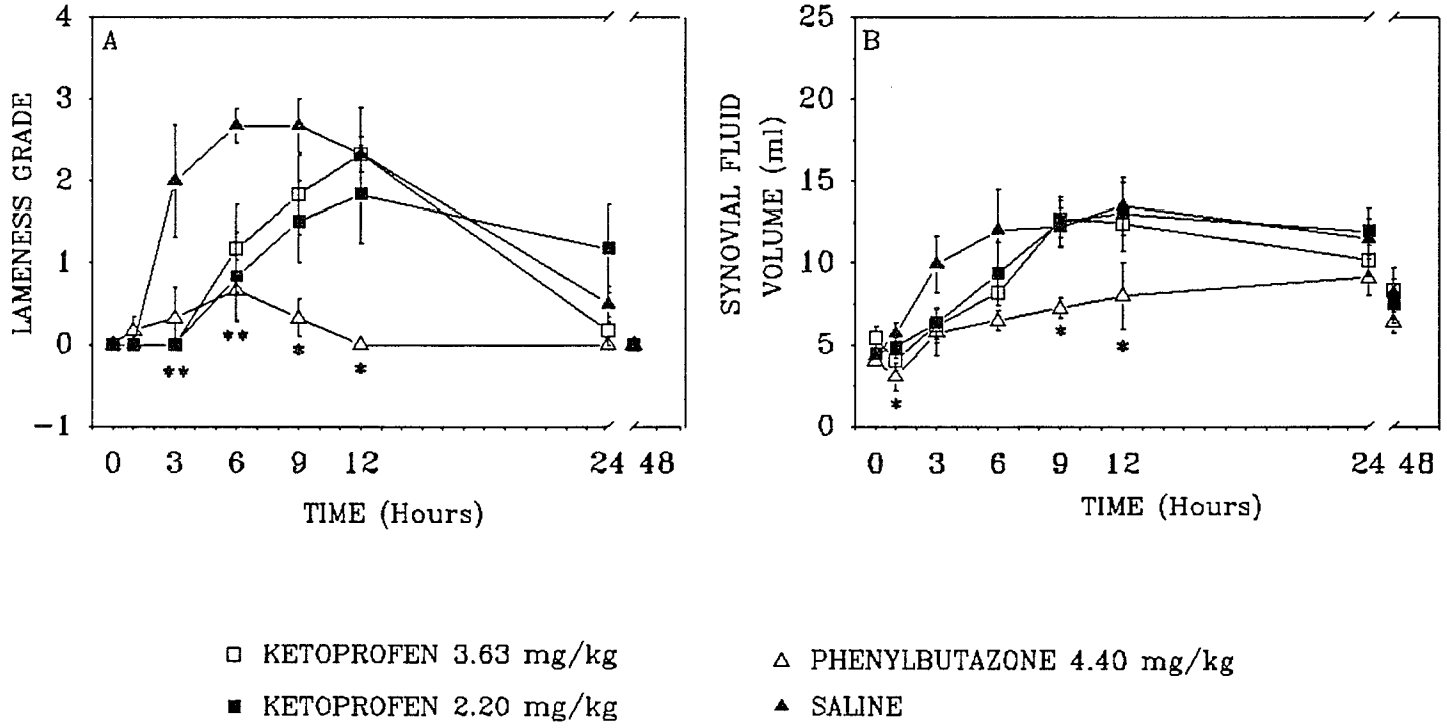


Figure 11: Effect of NSAIDs and saline on (A) lameness grade and (B) volume of synovial fluid obtained at each arthrocentesis. Statistically significant treatment means as compared to saline are indicated by (*). Carrageenan and NSAIDs were administered at time 0 (n=6).

Table 13: Means* (± SEM) of non-significant clinical parameters.

	TIME							
	0	**1**	**3**	**6**	**9**	**12**	**24**	**48**
HEMORRHAGE GRADE	0.83 ±0.19	2.17 ±0.25	2.54 ±0.26	2.63 ±0.27	2.75 ±0.26	3.29 ±0.19	3.09 ±0.23	2.57 ±0.25
MAX. FLEXION ANGLE (°)	21.08 ±1.01	22.94 ±0.92	26.13 ±1.34	28.63 ±1.55	27.58 ±1.41	30.21 ±2.26	27.22 ±1.80	24.00 ±1.01
JOINT CIRCUMFERENCE (cm)	28.72 ±0.25	28.81 ±0.27	28.85 ±0.26	29.24 ±0.27	29.41 ±0.27	29.55 ±0.27	29.47 ±0.32	29.37 ±0.31
RECTAL TEMPERATURE (°C)	37.85 ±0.06	37.81 ±0.07	37.70 ±0.07	37.90 ±0.09	38.18 ±0.07	38.30 ±0.08	37.98 ±0.08	37.80 ±0.07
HEART RATE (bpm)	29.29 ±0.70	29.67 ±0.76	28.75 ±0.71	30.42 ±0.75	30.63 ±0.61	31.71 ±0.71	29.83 ±0.74	28.87 ±0.75
RESPIRATORY RATE (bpm)	10.79 ±0.57	8.29 ±0.40	8.50 ±0.40	10.96 ±1.05	11.17 ±0.85	13.54 ±1.56	11.83 ±1.35	9.48 ±0.70

*Since there were no significant treatment effects for the above parameters, values reported above represent averages over 24 horses.

hemorrhage grades of 4 contained evidence of coagulation. These clots would often dissolve upon gentle inversion of the EDTA tube.

2. Lameness grade

All horses but five (3 with phenylbutazone, 4.4 mg/kg; 1 with ketoprofen, 2.2 mg/kg; and 1 with ketoprofen, 3.63 mg/kg) became lame after carrageenan administration. The remaining horses returned to soundness by 48 hours after induction (Figure 11B). Ketoprofen (3.63 mg/kg) significantly reduced the lameness score as compared to saline at 3 hours post dose. Ketoprofen (2.2 mg/kg) similarly reduced lameness at 3 hours and at 6 hours. Phenylbutazone (4.4 mg/kg) reduced the lameness score as compared to saline at 6, 9 and 12 hours after administration. Further, phenylbutazone significantly reduced lameness as compared to both doses of ketoprofen at 12 hours post dose.

3. Maximum flexion angle

There were no significant treatment effects for the maximum flexion angle as determined by radiography of the carpus held in rigid flexion by the examiner. However, the flexion angle increased over time with a peak at 12 hours. When data were averaged over all treatments, the maximum flexion angle ranged from 21.08° $\pm$ 1.10 at baseline to 30.21° $\pm$ 2.26 at 12 hours after induction (Table 13).

4. Joint circumference and effusion grade

There were no significant treatment effects for the circumference of the intercarpal joint (Figure 12A). The mean circumference ranged from 28.72 cm $\pm$ 0.25 at baseline to 29.55 cm $\pm$ 0.27 at 12 hours post-induction (Table 13).

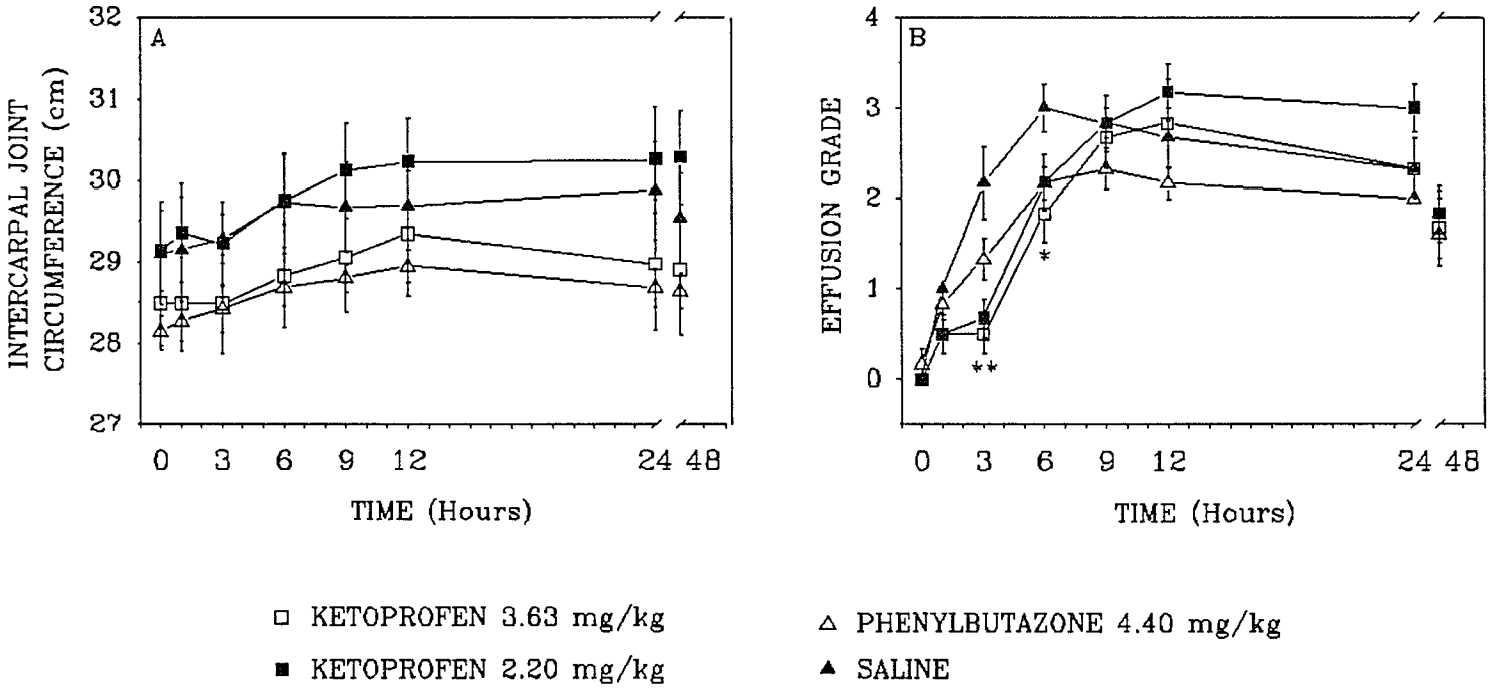


Figure 12: Effect of NSAIDs and saline on (A) intercarpal joint circumference and (B) carpal effusion grade. Statistically significant treatment means as compared to saline are indicated by (*). There were no significant treatment effects on joint circumference. Carrageenan and NSAIDs were administered at time 0 (n=6).

Both doses of ketoprofen reduced the subjective grade of joint effusion at 3 hours as compared to saline. The high dose of ketoprofen also reduced joint effusion at 6 hours as compared to saline (Figure 12B). There were no significant effects associated with phenylbutazone administration.

5. Temperature, heart and respiratory rates

There were no significant treatment effects for rectal temperature, heart rate or respiratory rate (Table 13). Rectal temperature ranged from 37.85° C ± 0.06 at baseline to 38.30° C ± 0.08 at 12 hours. Heart rate ranged from 29.29 bpm ± 0.70 to 31.71 bpm ± 0.71 at 12 hours. Respiratory rate ranged from 10.79 bpm ± 0.57 at baseline to 13.54 bpm ± 1.56 at 12 hours.

6. Carpal thermography

Joint temperature increased over time in the saline treated animals with a peak at 9 to 12 hours. Phenylbutazone significantly reduced the overall joint temperature as compared to saline from 3 to 48 hours after administration (Figure 13A). Phenylbutazone also significantly reduced joint temperatures as compared to ketoprofen (3.63 mg/kg) at 3 hours and ketoprofen (2.2 mg/kg) from 6 to 24 hours. A graph of room temperature is included for comparison in Figure 13B. Room temperature was constant over time and there were no significant treatment effects.

7. Synovial fluid clinical pathology

There were no significant treatment effects for total number of nucleated cells per μl, red blood cells per μl, MPQ grade and the number of neutrophils, large mononuclear cells, lymphocyte, eosinophils and basophils per μl (Table 14). Further,

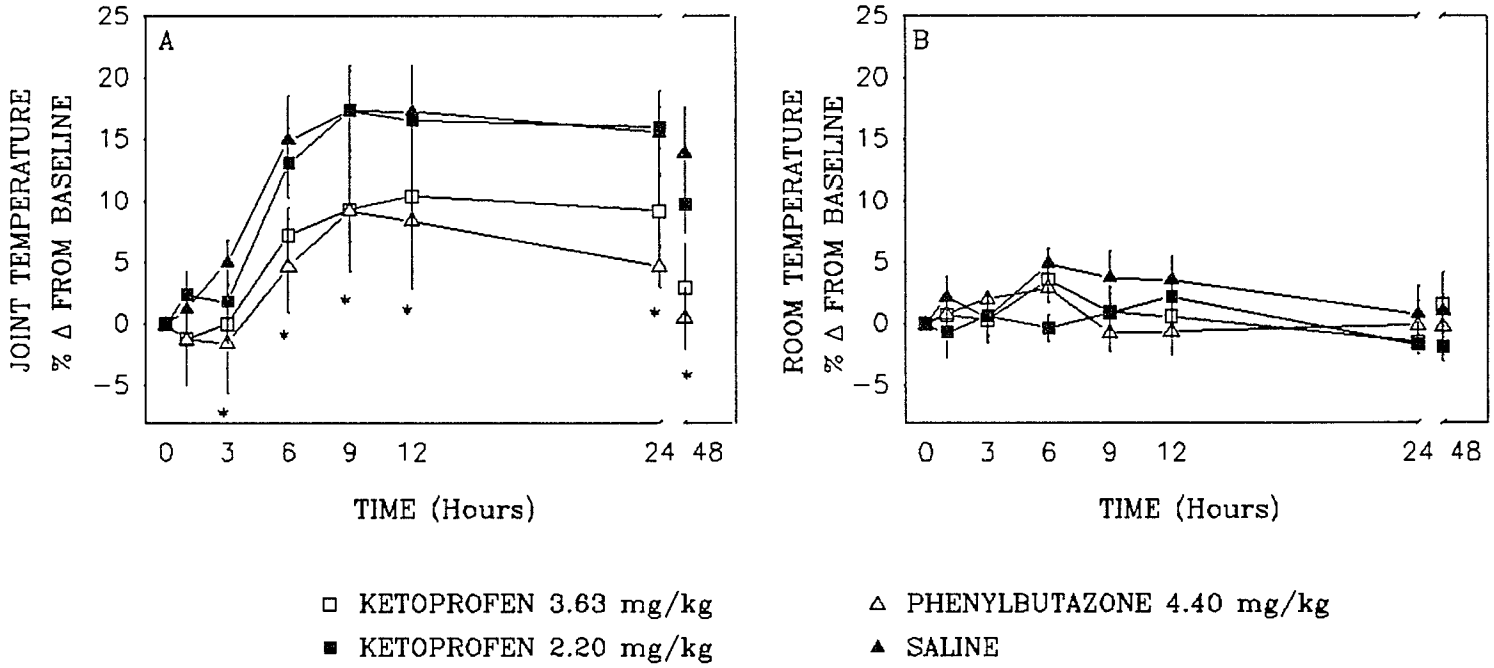


Figure 13: Effect of NSAIDs and saline on (A) joint temperature as expressed as % change from baseline. The graph of room temperature (B) indicates corresponding ambient temperature changes over time. Statistically significant treatment means as compared to saline are indicated by (*). There were no significant treatments effects of room temperature. Carrageenan and NSAIDs were administered at time 0 (n=6).

Table 14: Means* (± SEM) of non-significant synovial fluid clinical pathology parameters.

SYNOVIAL FLUID CLINICAL PATHOLOGY	TIME 0	1	3	6	9	12	24	48
NUCLEATED CELLS x $10^3/\mu l$	0.40 ±0.08	3.43 ±0.30	9.75 ±1.88	155.41 ±13.2	155.59 ±8.64	101.89 ±8.13	52.51 ±3.42	15.02 ±1.72
RED BLOOD CELLS x $10^3/\mu l$	121.64 ±54.87	417.08 ±116.40	575.63 ±172.29	562.08 ±118.13	517.12 ±76.80	542.67 ±89.08	413.70 ±90.02	318.04 ±81.27
MPQ GRADE	1.17 ±0.08	1.38 ±0.12	2.58 ±0.12	2.63 ±0.13	2.56 ±0.14	2.63 ±0.14	1.87 ±0.14	1.87 ±0.16
NEUTROPHILS x $10^3/\mu l$	0.16 ±0.05	1.90 ±0.22	9.45 ±1.87	144.98 ±12.07	145.26 ±8.24	91.70 ±8.07	38.20 ±3.06	8.75 ±1.35
MONONUCLEAR CELLS x 10^3 /μl	0.13 ±0.02	0.89 ±0.21	0.14 ±0.03	8.45 ±1.79	10.53 ±1.83	8.58 ±0.83	11.58 ±1.20	5.36 ±0.48
LYMPHOCYTES x $10^2/\mu l$	0.79 ±0.17	5.73 ±0.75	1.47 ±0.43	12.45 ±6.08	8.87 ±1.99	6.12 ±1.35	25.89 ±11.62	6.46 ±1.16
EOSINOPHILS/μl	18.01 ±10.18	110.67 ±30.86	10.48 ±5.63	594.04 ±378.45	116.92 ±84.24	0.00 ±0.00	34.96 ±34.96	0.00 ±0.00
BASOPHILS/μl	0.42 ±0.42	5.17 ±3.67	0.00 ±0.00	41.83 ±41.83	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00

*Since there were no significant treatment effects for the above parameters, values reported above represent averages over 24 horses.

there were no significant treatment effects when the differential cell counts were expressed as a percent of the total nucleated cell count. There was a significant treatment effect on protein concentrations (g/dl). The phenylbutazone-treated horses had significantly increased synovial fluid protein concentrations at 12, 24 and 48 hours as compared to saline. At 24 hours, phenylbutazone increased the protein concentration as compared to both doses of ketoprofen (Figure 14A). The mean synovial fluid protein concentrations at baseline were 2.51 g/dl $\pm$ 0.01. The peak levels occurred at 9 hours for all treatments except phenylbutazone which peaked at 24 hours. Synovial protein in normal horses as determined by the refractometer method is generally reported to be low (less than 2.5 g/dl) [75] as synovial fluid is considered a dialysate of blood plasma with hyaluronic acid [74].

The mean number of nucleated cells per μl of synovial fluid ranged from 0.40 $\pm$ 0.08 x 10^3 at baseline to 155.59 $\pm$ 8.64 x 10^3 at 9 hours (Table 14). Horses administered saline had peak levels occurring at 6 hours 202.13 $\pm$ 15.22 x 10^3 while the NSAID treated horses tended to have peak levels at 9 hours (Figure 14B). Normal levels for the horse are generally reported to be less than 500 cells/μl [382]. The cells were predominantly neutrophils from 1 hour to 48 hours with an increasing percentage of large mononuclear cells at 24 and 48 hours. There was also an increase in the number of red blood cells in the synovial fluid with peak in cell numbers at 3 hours (Table 14). There was considerable variation in the number of red cells in synovial fluid at all time points. The was a moderate decrease in the MPQ grade after carrageenan administration with values returning to near baseline at 48 hours. The

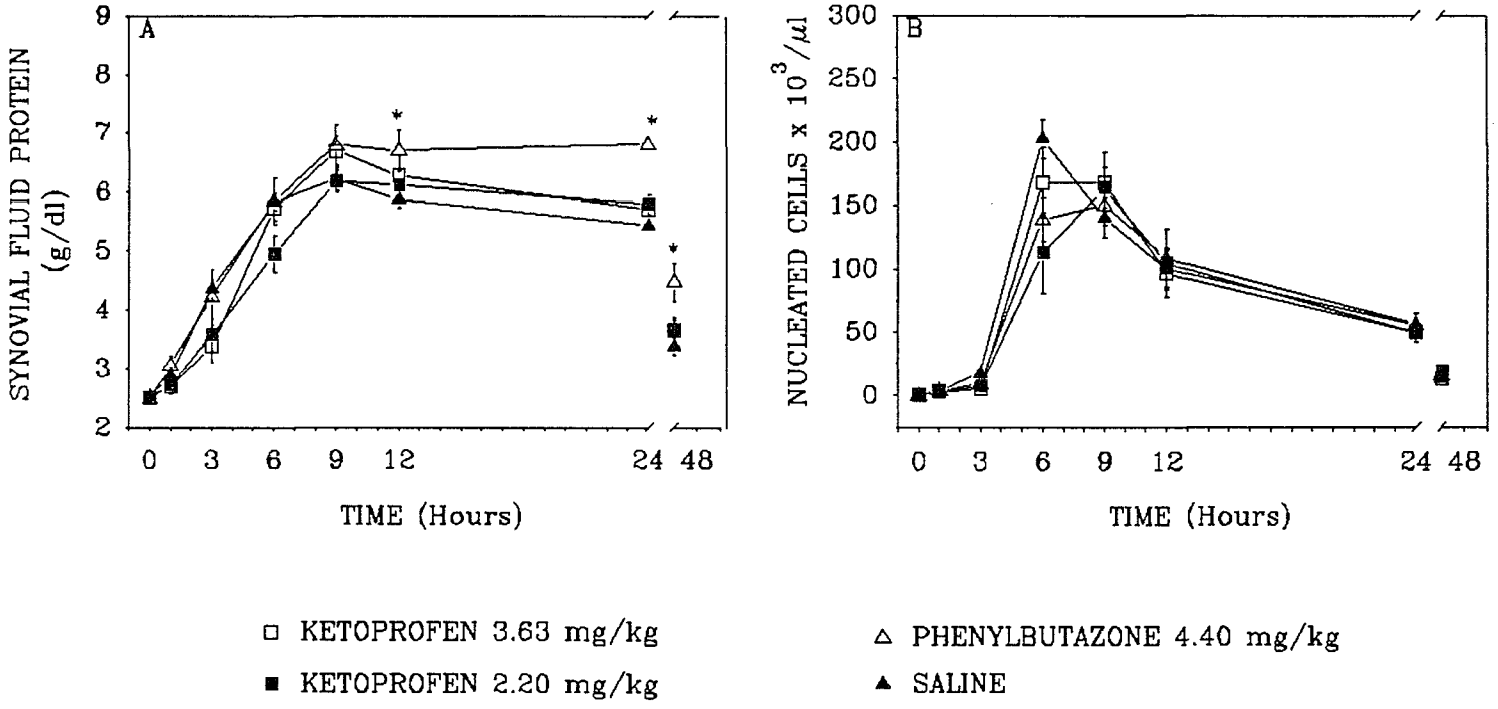


Figure 14: Effect of NSAIDs and saline on (A) synovial fluid protein concentration and (B) nucleated cells. Statistically significant treatment means as compared to saline are indicated by (*). There were no significant treatment effects on the number of nucleated cells. Carrageenan and NSAIDs were administered at time 0 (n=6).

mean MPQ grade ranged from 1.17 ± 0.08 at baseline to 2.63 ± 0.13 and 2.63 ± 0.14 at 6 and 12 hours, respectively (Table 14).

Microscopic examination of synovial fluid smears often revealed protein folding and degenerating or dead cells in varying stages of cellular breakdown from 3 through 24 hours after induction. Vacuolation of large mononuclear cells and neutrophils was often seen from 6 to 12 hours. Occasionally, clumps of platelets and synovial lining cells were noted.

8. Bacterial cultures

Examination of blood agar plates and thioglycollate broth tubes revealed no bacterial growth in any of the carrageenan cultures. Broth tubes and agar plates of a few synovial fluid samples contained evidence of bacterial or fungal growth. However, the growth was not consistent between duplicates or subsequent samples. Based on these inconsistencies and the clinical signs of the horses, these samples were most likely contaminated with non-pathogenic flora from the air or person performing the microbial culture.

9. Synovial fluid eicosanoid concentrations

The mean baseline concentration of PGE_2 was 0.42 ng/ml ± 0.07 (Figure 15A). The concentrations of PGE_2 increased dramatically over time with peak levels occurring at 9 hours (79.19 ng/ml ± 33.83) in horses administered saline. For all horses, concentrations returned to near baseline levels by 48 hours. There was a significant overall treatment effect for PGE_2 when data was analyzed over all sampling times according to the univariate procedure. Results from the Tukey's multiple

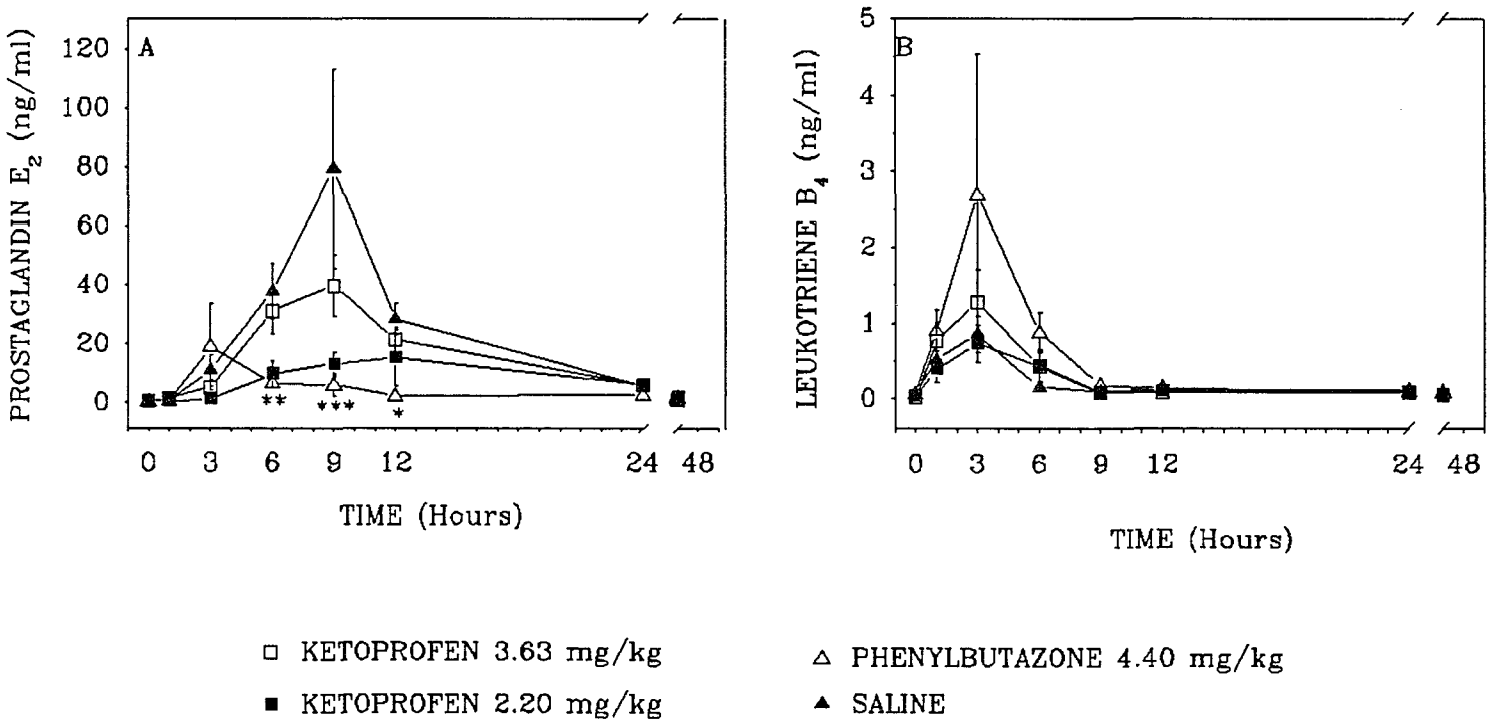


Figure 15: Effect of NSAIDs and saline on synovial fluid (A) PGE_2 and (B) LTB_4 concentrations. Statistically significant treatment means as compared to saline are indicated by (*). There were no significant treatment effects (P = 0.0603) on LTB_4 concentration. Carrageenan and NSAIDs were administered at time 0 (n=6).

comparison procedure indicated that ketoprofen (2.2 mg/kg) and phenylbutazone reduced the PGE_2 concentrations as compared to saline. Tukey's procedure was then used to compare eicosanoid concentrations (averaged over treatments) at each post-dose time to baseline, in order to determine when the concentrations were different from baseline. The results indicated that means for time 6, 9 and 12 hours were significantly different from baseline means. Based on the significant time effect, individual treatment means at 6, 9 and 12 hours were compared using the Student's *t* tests. At 6 hours, phenylbutazone and ketoprofen (2.2 mg/kg) significantly reduced the PGE_2 concentrations as compared to saline. At 9 hours post administration, both doses of ketoprofen and phenylbutazone reduced PGE_2 as compared to saline. At 12 hours after administration, only phenylbutazone reduced levels when compared to saline. Ketoprofen (3.63 mg/kg) produced PGE_2 concentrations significantly greater than phenylbutazone and ketoprofen (2.2 mg/kg) at 6 and 9 hours.

LTB_4 concentrations were associated with no significant ($P = 0.0603$) overall treatment effects (Figure 15B). The mean baseline concentration was 0.04 pg/ml $\pm$ 0.01. Levels peaked at 3 hours post-dose (1.26 pg/ml $\pm$ 0.37) and returned to near baseline values by 9 hours.

10. Hematology

No treatment effects were obtained for WBC count, RBC count, plasma protein (g/dl), fibrinogen (g/dl), PCV (%) or platelet count (Table 15). However there was a significant treatment effect for the number of eosinophils and basophils per μl (Figure 16A and B). At 1 hour post-dose, eosinophils in phenylbutazone treated

Table 15: Means* (± SEM) of non-significant hematological parameters.

HEMATOLOGY	TIME 0	1	3	6	9	12	24	48
WHITE BLOOD CELLS x $10^3/\mu L$	9.64 ±0.51	9.08 ±0.44	9.83 ± 0.38	10.28 ±0.43	11.99 ±0.65	12.58 ±0.56	10.06 ±0.36	9.64 ±0.38
RED BLOOD CELLS x $10^6/\mu L$	8.40 ±0.20	7.48 ±0.27	8.20 ±0.26	7.95 ±0.22	7.94 ±0.19	8.02 ±0.20	7.85 ±0.29	7.94 ±0.14
PROTEIN (g/dl)	7.36 ±0.09	7.12 ±0.08	7.32 ±0.11	7.20 ±0.11	7.18 ±0.11	7.20 ±0.10	7.26 ±0.08	7.42 ±0.09
FIBRINOGEN (g/dl)	287.50 ±32.03	250.00 ±20.85	233.33 ±18.71	237.50 ±13.20	279.17 ±28.22	250.00 ±21.70	308.70 ±22.59	334.78 ±30.55
PCV (%)	38.08 ±0.97	34.58 ±0.77	37.58 ±0.98	37.29 ±0.71	36.63 ±0.85	37.79 ±0.76	36.35 ±0.79	36.04 ±0.55
NEUTROPHILS x $10^3/\mu l$	5.70 ±0.36	5.83 ±0.40	6.30 ±0.36	6.83 ±0.40	8.58 ±0.59	8.44 ±0.43	6.28 ±0.28	5.90 ±0.25
MONOCYTES x $10^3/\mu l$	0.36 ±0.05	0.33 ±0.05	0.32 ±0.05	0.40 ±0.07	0.37 ±0.05	0.51 ±0.08	0.37 ±0.05	0.23 ±0.03
LYMPHOCYTES x $10^3/\mu l$	3.16 ±0.26	2.60 ±0.16	2.96 ±0.18	3.11 ±0.36	2.74 ±0.22	3.36 ±0.26	3.18 ±0.24	3.13 ±0.22
PLATELETS x $10^3/\mu l$	198.25 ±14.85	196.75 ±13.52	198.31 ±16.54	192.83 ±16.98	188.33 ±15.54	194.79 ±13.49	192.48 ±9.92	203.17 ±12.78

*Since there were no significant treatment effects for the above parameters, values reported above represent averages over 24 horses.

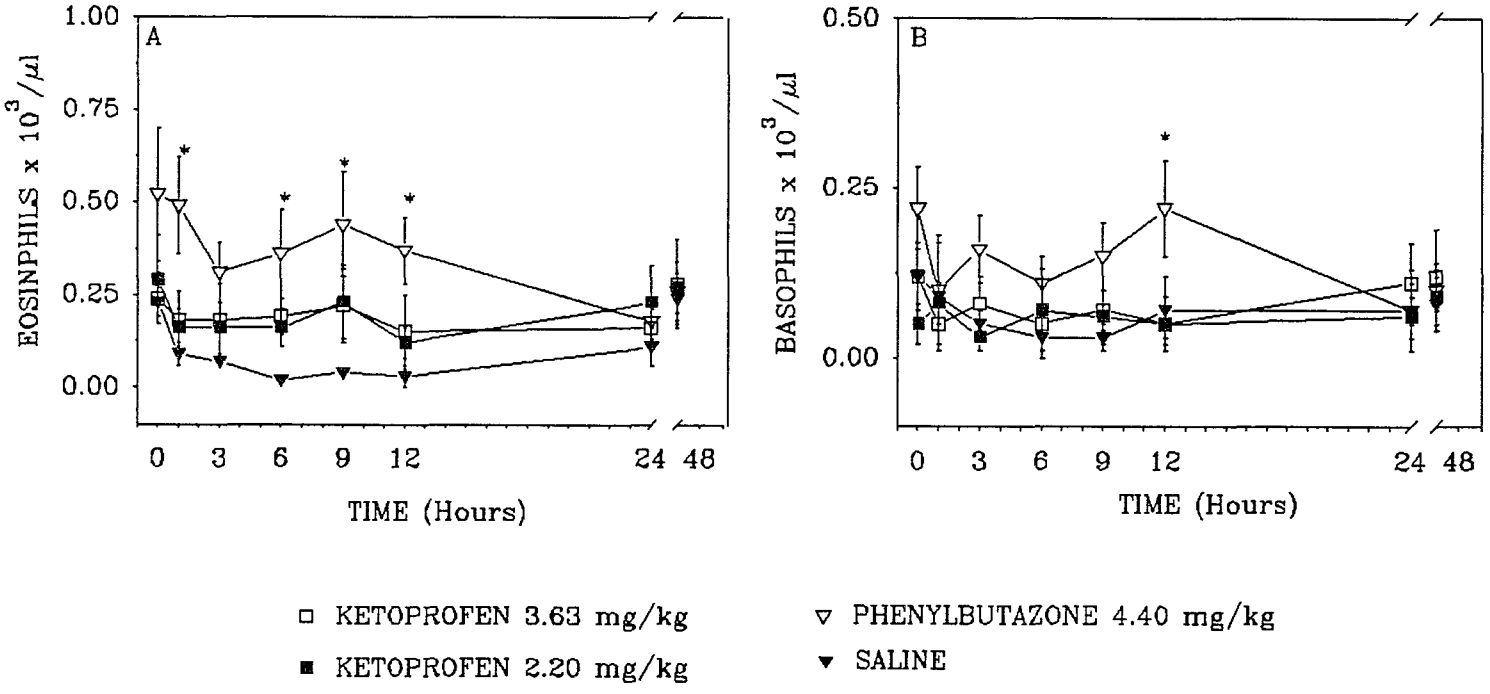


Figure 16: Effect of NSAIDs and saline on the number of blood (A) eosinophils and (B) basophils. Statistically significant treatment means as compared to saline are indicated by (*). Carrageenan and NSAIDs were administered at time 0 (n=6).

horses were greater than saline and both doses of ketoprofen. At 6 hours, phenylbutazone treated horses had higher eosinophil counts than ketoprofen (2.2 mg/kg) and saline. At 9 and 12 hours, phenylbutazone also was associated with higher numbers of eosinophils than saline. At 12 hours, basophils in horses administered phenylbutazone were higher than both doses of ketoprofen and saline. However, the eosinophil and basophil numbers were not outside of the normal ranges (eosinophils- 0.0-0.8 x 10^3 cells/μl; basophils- 0.0-0.3 x 10^3 cells/μl [390]. Mean peripheral blood WBC counts were within normal ranges except at 12 hours when the mean count rose to 12.58 x 10^3 cells/μl $\pm$ 0.56 (normal range for the horse is 6-12 x 10^3 cells/μl [390]). This leukocytosis was due to a mature neutrophilia in most horses. The mean neutrophil count at this time was 8.44 x 10^3 $\pm$ 0.43 cells/μl (normal range for the horse is 3-6 x 10^3 cells/μl [390]). Further, although the total WBC counts were not increased, many horses were neutrophilic at 3, 6, 9 and 24 hours after induction. No other hematologic abnormalities were noted.

D. Discussion

Carrageenan-induced synovitis has been used previously in the horse to isolate leukocytes from an inflammatory exudate, but the clinical effects and eicosanoid concentrations in synovial fluid were not described [52]. A pilot study was performed initially to determine the clinical effects of intra-articular carrageenan and to refine methodology. The pilot study showed that the carrageenan-induced synovitis produced no articular damage 72 hours after administration, although there was still evidence

of synovial inflammation. No synovial fluid abnormalities were noted 5 months after induction. Horses with carrageenan synovitis (n=24) appeared clinically normal within 2 weeks following induction with no lameness, heat, effusion or palpable thickening of the joint capsule or synovial membrane. These results indicate that the horses experienced a transient synovitis that resulted in no obvious lasting damage to the joint. Thus our initial objective of inducing self-limiting inflammation was achieved.

The volume of synovial fluid obtained from the left intercarpal joint and the size of these joints (effusion grade and joint circumference) rose dramatically after induction of synovitis. Phenylbutazone reduced the synovial fluid volume at the time of peak inflammation (9-12 hours) to a greater extent than either dose of ketoprofen. However, phenylbutazone did not reduce the subjective grade of effusion while both doses of ketoprofen produced significant reduction at 3 hours and there was no significant treatment effect on joint circumference. These parameters all measure joint distension, but they have subtle qualitative differences which may account for the discrepancies in treatment effects. An increase in effusion grade accounted for increases in subcutaneous and periarticular edema as well as synovial fluid distension, whereas the volume of synovial fluid reflected the amount of synovial fluid obtained from the intercarpal joint only. The circumference of the intercarpal joint appeared to be a less sensitive estimate of joint distension than the effusion grade and synovial fluid volume, due to presence of bony protuberances on the equine carpus. The

swelling and effusion associated with synovitis in the early stages of development often occurred between these prominences and thus were not directly measurable.

Carrageenan-induced synovitis produced a variable degree of lameness: 5 horses out of 24 did not show lameness at any time post-induction and while a few horses became markedly lame, others were only slightly effected. Despite this variability, there were significant treatment effects. Phenylbutazone more effectively relieved lameness than either dose of ketoprofen. There appeared to be no clear ketoprofen dose related effect, as the high dose (phenylbutazone molar equivalent dose) of ketoprofen had a short lived effect at 3 hours and the therapeutic dose reduced lameness from 3 to 6 hours.

Phenylbutazone reduced the overall temperature of the joint in the early stages of the inflammatory process with effects lasting to 48 hours after administration. This effect was greater than the therapeutic dose ketoprofen for most of the times tested. As the surface temperature of the joint is related to blood flow to the region, these results may indirectly reflect that phenylbutazone inhibits carrageenan-induced vasodilation over an extended period. A similar time course has been observed previously for phenylbutazone in a carrageenan-induced subcutaneous inflammation model [325].

The number of nucleated cells in synovial fluid rose dramatically with peak levels occurring at 6 to 9 hours. This primarily neutrophilic exudate was likely attributable to the increase in the synovial fluid concentration of the potent chemotactic agent, LTB_4 [27,28]. In addition, PGE_2 has been shown to be chemotactic for equine

neutrophils and both of these eicosanoids play a significant role in the recruitment of neutrophils to the site of inflammation [20]. These results are similar to leukocyte numbers from tissue cage studies with carrageenan. However, the number of cells obtained from joint fluid were an order of magnitude higher than the tissue cage [67]. Cellular migration was likely impeded in the tissue cage model as compared to the synovitis model by the presence of fibrous tissue around and within the polypropylene device. Synovitis accompanying clinical cases of degenerative joint disease in the horse results in nucleated cell counts of up to 10,000 cells/μl [75]. Nucleated cell counts in excess of 50,000 cells/μl with a predominance of neutrophils in clinical equine cases usually indicates septic arthritis [75,94]. Based on the cell count, protein concentration and the MPQ grade, this model of experimentally-induced synovitis appeared to be quite similar to clinical cases of equine joint infection [114].

There were no significant treatment effects associated with the number of nucleated cells in the inflamed synovial fluid or any of the other clinical pathology parameters except for protein concentration. The effects of NSAIDs on leukocyte numbers in inflammatory exudate are contradictory and have not been correlated with cyclooxygenase inhibition [15]. Ketoprofen when administered within the therapeutic dose range has been shown to reduce leukocyte counts in other carrageenan-induced inflammation models [15,299]. Phenylbutazone (4.4 mg/kg) did not affect leukocyte numbers in an equine model of subcutaneous inflammation as compared to control [323], while low doses of phenylbutazone and other NSAIDs stimulated leukocyte migration [50]. Phenylbutazone increased the synovial fluid protein concentration in

the late stages of the inflammatory process. This unexpected effect is likely due to the significant reduction in synovial fluid volume associated with phenylbutazone treatment.

Erythrocytes are not usually found in normal synovial fluid unless contamination occurred during arthrocentesis (iatrogenic hemorrhage) [376]. However, hyperemia of the synovial membrane during synovitis greatly increases the potential for hemorrhage into the joint [75]. Further, joints in this study were aspirated 8 times over a 48 hour period which increased the likelihood of hemorrhage into the joint cavity. The iatrogenic hemorrhage in these joints most likely contributed to the total nucleated cell counts [382]. The eosinophils and basophils noted in the synovial fluid are likely the result of intra-articular hemorrhage as eosinophils are rare constituents of synovial fluid and basophils have not been reported to be present in non-hemorrhagic synovial fluid [376]. In addition, blood contamination of synovial fluid could have contributed to eicosanoid production (refer to data described in Chapter 6). However, this probably had little impact on the treatment effects of these parameters as the grade of hemorrhage was not significantly different among treatments.

There was no bacterial growth in the carrageenan cultures and synovial fluid cultures. Joint infection in these horses cannot be ruled out definitively as it is not uncommon for synovial fluid from infected joints to culture negatively [75]. However, the rapid reversal of lameness and the decrease in the number of synovial fluid nucleated cells over time in all horses would indicate non-septic synovitis.

There was a dramatic increase in PGE_2 concentrations in the synovial fluid after carrageenan administration. The peak concentration in horses administered saline was approximately 200 times the baseline level at 9 hours. The LTB_4 concentrations also increased markedly in all horses. Peak levels were 14 times over baseline concentrations at 3 hours in horses administered saline. The peak levels of PGE_2 and LTB_4 in control animals were similar to those found in subcutaneous models of carrageenan-induced inflammation [69-72]. The LTB_4 levels were in the range of reported concentrations from carrageenan-induced arthritis in dogs [123] and clinical osteoarthritis in man [136]. However, the PGE_2 levels were generally higher in this study than those reported in polycation-induced arthritis [118] and clinical joint diseases in man [143] and the horse [134]. PGE_2 levels from carrageenan models are apparently higher than those in other forms of inflammation. *In vitro* research on cells from humans with rheumatoid arthritis indicates that activated synovial lining cells produce PGE_2 whereas LTB_4 originates mainly from synovial fluid neutrophils [142], although monocytes from patients with rheumatoid arthritis have also been shown to produce large amounts of LTB_4 [139]. In the horse, chondrocytes and synovial cells secrete PGE_2 with very little contribution from PGE_1 [391]. Studies in other inflammatory exudates have also shown that the primary arachidonic acid products of neutrophils are leukotrienes [7,392].

There was considerable variability in the magnitude in the peak eicosanoid levels after carrageenan administration in these horses. Despite the variability among and within horses, there were significant treatment effects at 6, 9 and 12 hours for

PGE_2, but not for LTB_4. Phenylbutazone apparently inhibited cyclooxygenase activity longer than ketoprofen in this model of acute inflammation. Studies have indicated that phenylbutazone is more potent in producing cyclooxygenase inhibition and reducing carrageenan-induced edema than ketoprofen [8,64]. In contrast, others have shown that ketoprofen is more potent in producing cyclooxygenase inhibition [290], and in carrageenan-induced inflammatory models [44,63] and pain models [44]. In this study, the therapeutic dose of ketoprofen reduced PGE_2 concentrations longer than the high dose. This lack of a dose-response effect with ketoprofen may in part be due to the small difference between the two doses chosen coupled with the large degree of variability associated with PGE_2 concentrations. Unexpectedly, at 6 and 9 hours the 3.63 mg/kg dose of ketoprofen resulted in higher PGE_2 concentrations than the therapeutic dose. Perhaps the high dose of ketoprofen decreases prostaglandin degradation thereby resulting in apparent potentiation of prostaglandin formation. Several NSAIDs inhibit the 15-hydroxy prostanoate dehydrogenase enzyme that oxidizes and inactivates PGE_2 and $PGF_{2\alpha}$ at concentrations that are generally higher than for cyclooxygenase inhibition [393,394]. Perhaps the high dose, but not the therapeutic dose of ketoprofen has activity against this degradative enzyme in the joint thereby leading to a net increase in the concentration of PGE_2 in the synovial fluid.

There were no significant treatment effects on synovial fluid LTB_4 concentrations for ketoprofen or phenylbutazone. Previous studies have indicated that ketoprofen inhibits rabbit neutrophil [250,293] and human lung [292] lipoxygenase activity, while others have found no effect [290,293]. Lipoxygenase inhibition data

varies considerably depending on the source of the enzyme and the species tested [293,295]. Although not significant, most NSAIDs appeared to increase the LTB_4 concentrations as compared to saline at 3 hours. Phenylbutazone appeared to have the greatest potentiation followed by the high dose of ketoprofen. This effect has been reported previously for diclofenac, ketoprofen and aspirin in the guinea pig [293] and for indomethacin, ibuprofen and aspirin in man [392] and has been attributed to a diversion of arachidonic acid substrate after cyclooxygenase inhibition to the lipoxygenase pathway. The apparent K_m of 5-lipoxygenase for arachidonic acid is comparable to that of cyclooxygenase. Since most cells with 5-lipoxygenase activity also contain cyclooxygenase, it is thought that the activated enzymes compete for arachidonic acid substrate in the same cell types [10].

Many horses were neutrophilic from 3 to 24 hours with a slight leukocytosis occurring at 12 hours. This effect most likely represented a mobilization of mature neutrophils from the marginal blood pool and the bone marrow maturation pool in response to the synovitis [395]. The reason for the effect of phenylbutazone on blood eosinophil and basophil numbers is unclear. However, the mean eosinophil and basophil numbers per μl for horses treated with phenylbutazone were within normal clinical limits. This effect is most likely artifactual as there are no reports of phenylbutazone increasing these white cells. On the contrary, toxic doses of phenylbutazone produce leukopenia in the horse [396,397].

Phenylbutazone was more effective than ketoprofen in alleviating many of the signs of pain and inflammation in this model of synovitis. These results are consistent

to those obtained in models of carrageenan-induced subcutaneous inflammation where peak reduction in PGE_2 exudate levels occurred at 6 hours [323]. High doses of phenylbutazone in the dog have been effective in both preventing and suppressing the inflammatory response from urate-induced joint inflammation [101]. Ketoprofen was effective in alleviating inflammation in this model, but did not result in inhibition of the leukotriene pathway as determined by the measurement of LTB_4 in the synovial fluid.

CHAPTER 5

PHARMACOKINETICS AND SYNOVIAL FLUID LEVELS OF KETOPROFEN IN NORMAL HORSES AND HORSES WITH ACUTE SYNOVITIS

A. Introduction

Ketoprofen or (±)-2(3-benzoylphenyl)propionic acid is a nonsteroidal anti-inflammatory drug (NSAID) of the propionic acid family that includes ibuprofen, naproxen, benoxaprofen, fenoprofen and carprofen. This agent has been used in human medicine in the treatment of arthritis and mild-to-moderate pain since its introduction in France in 1973. Ketoprofen, at an intravenous dose of 2.2 mg/kg, was approved by the United States Food and Drug Administration for alleviation of musculoskeletal pain and inflammation in the horse in 1990.

The plasma or serum pharmacokinetics of ketoprofen has been studied in humans [282,398], rabbits [287], rats [289], dogs [399], and horses [283,286]. Synovial fluid levels of ketoprofen have been measured in humans with various forms of arthritis [280,297,400,401]. Peak synovial fluid concentrations of total (plasma protein bound and free) ketoprofen in humans with arthritis are lower and occur later than do peak plasma concentrations [280]. However, the peak synovial fluid concentrations often exceed corresponding plasma levels after an equilibrium has been established.

Water and small solutes, including most NSAIDs, cross the synovial membrane from plasma principally by diffusion through the intercellular, interstitial matrix of the synovium. This diffusion by small molecules is considered bi-directional [402]. Compounds that are lipid soluble may also cross the membrane through transcellular diffusion [402]. The extravascular pharmacokinetics of a particular drug depend on the agent's molecular weight, lipid solubility, protein binding, plasma half-life and pK_a. For example, ketoprofen ($C_{16}H_{14}O_3$, molecular weight 254.29) has an octanol-buffer partition coefficient at pH 7.4 of approximately 1 with a pK_a of 5.02 and a high percentage of plasma protein binding [399]. However, this pK_a results in ionization of ketoprofen at a physiological pH. In contrast to ketoprofen, phenylbutazone ($C_{19}H_{20}N_2O_2$, molecular weight 308.37) has a similar pK_a (4.8) and degree of protein binding, but is much more lipid soluble with an octanol-buffer partition coefficient of 5.01 at a neutral pH and would be expected to cross biological membrane to a greater extent than ketoprofen [162].

Evidence indicates that synovial NSAID concentrations are higher in patients with joint inflammation than those in normal joints [403]. This is due to the increase in vascular permeability and blood flow, loss of endothelial integrity and hemorrhage that allows for the exudation of plasma proteins with the bound drug. Normally, only the free drug is able to diffuse freely between plasma and synovial fluid, but with inflammation, bound drug may enter into the synovial fluid [402]. In contrast to small molecules, plasma protein diffusion into the synovial fluid is a one-way transport mechanism [258]. Plasma protein concentrations in synovial fluid are

normally low, but the permeability increases with joint inflammation [402,404]. The increased protein concentration and drug receptor binding at the site of action may serve to increase the joint drug concentration of highly bound drugs [258,402].

The purpose of this study was to compare the pharmacokinetics of ketoprofen in healthy horses and those with experimentally-induced acute synovitis. More importantly, this study was designed to evaluate the concentration of ketoprofen at its site of action in animals with joint disease. Synovial fluid levels of ketoprofen in inflamed joints were compared to levels in normal joints in order to determine the effect of acute joint inflammation on the magnitude and duration of ketoprofen synovial fluid concentrations.

B. Materials and methods

1. Experimental animals

a. Normal horses

Three healthy geldings (2 American Quarter Horses and one Thoroughbred) and one Thoroughbred mare weighing from 406 to 554 kg and ranging from 5 to 12 years of age were used. Horses were judged to be healthy and sound based on physical examination and lameness evaluation.

b. Horses with experimentally-induced synovitis

Four geldings (one American Quarter Horse and 3 Thoroughbreds) weighing from 437 to 509 kg and ranging from 8 to 12 years of age with carrageenan-induced synovitis of the left intercarpal joint were used. Prior to induction, horses were judged

to be healthy and sound based on physical examination, lameness evaluation, synovial fluid analysis and radiographic examination of the left carpus.

All horses were vaccinated at least three weeks prior against eastern and western equine encephalomyelitis, influenza, tetanus (Equi-Flu EWT™, Coopers, Mundelein, IL) and rhinopneumonitis (Rhinomune™, SmithKline Beecham Animal Health, Exton, PA). The horses were dewormed with oxibendazole (Equipar™, Coopers, Mundelein, IL). Horses were housed in 10.9 x 12.5 feet stalls and were maintained on 5 pounds of a pelleted ration (Purina Horse Chow 100™, Purina Mills Inc., St. Louis, MO) containing: a minimum of 10% protein; 2% fat; a maximum of 25% fiber; and a vitamin/trace mineral supplement twice daily with mixed grass hay (11% protein, 1.4% fat, 24% fiber) and water provided *ad libitum.*

2. Drugs and reagents

a. Drug solution for administration

Ketoprofen (Ketofen™) was obtained commercially from Aveco Company, Inc., Fort Dodge, IA. Each ml of the sterile solution contained: 100 mg ketoprofen; L-arginine, 70 mg; citric acid to adjust pH to approximately 7; and benzyl alcohol, 0.25 ml as a preservative.

b. Standard solutions

A stock solution of ketoprofen was prepared by dissolving 10 mg of the compound in 10 ml of methanol. The stock solution was serially diluted with methanol to produce working standard solutions of 100 μg/ml, 10 μg/ml, and 1 μg/ml. The working solution of fenoprofen was prepared by dissolving 10 mg of the

compound in 10 ml of methanol. Ketoprofen and fenoprofen were obtained from Sigma Chemical Co., St. Louis, MO.

c. Solvents

Liquid chromatography grade solvents were obtained from commercial sources. Phosphate buffer (0.001M) for high performance liquid chromatography (HPLC) mobile phase was prepared by placing 68.027 μl of *o*-phosphoric acid (44.6N) in triple distilled water sufficient to make 1 L total volume. The pH of this solution was adjusted to 7.4 with sodium hydroxide (2.157 ml of 1 N).

d. Water

Triple distilled, filtered water was prepared by passing through a Modulab™ Polisher I water purification system (Continental Water Systems Corp., San Antonio, TX).

3. Experimental protocol

a. Horse preparation

Each horse was shaved over the entire carpal region 24 hours prior to drug administration. Indwelling catheters (14 gauge, 5.7 cm) (Quick-Cath™, Baxter Healthcare Corp., Deerfield, IL) were aseptically placed bilaterally in the jugular veins of each horse before each session.

b. Blood collection

Ketoprofen (2.2 mg/kg) as Ketofen™ and detomidine (Dormosedan™, Norden Laboratories, Lincoln, NE) were administered via the left jugular catheter. The right jugular catheter was used for blood collection before drug administration and at 3, 5,

10, 15, 20, 30, and 45 minutes and at 1, 1.5, 2, 3, 4, 5, 6, 9, 12, and 24 hours after drug injection for determination of plasma drug concentrations. The patency of each catheter was maintained after each blood collection with 3 ml of 100 IU/ml of heparinized saline. Before blood was collected, 2-3 ml was discarded to remove any residual heparin from the catheter. Blood (14 ml) was collected with a 18 gauge, 1.5 inch needle and a 20 ml syringe and then placed immediately into 7 ml evacuated glass tubes containing 10.5 mg of ethylenediamine tetraacetate K_3 (EDTA) as an anticoagulant (Vacutainer, Becton Dickinson, Rutherford, NJ). Tubes were immediately centrifuged at 2,000 rpm for 15 minutes (Dynac, Becton Dickenson and Company, Parsippany, NJ). Plasma was transferred into 15 ml polypropylene centrifuge tubes (Sarstedt, Newton, NC) and stored at -20° C until analyzed. Previous studies have shown that freezing does not affect ketoprofen concentrations in biological matrices [280,405].

c. Synovial fluid collection from normal horses

Horses received detomidine HCl intravenously (10 μg/kg for the first dose and 5.0 μg/kg for subsequent doses) before each arthrocentesis as a sedative to reduce the risk of iatrogenic joint hemorrhage and trauma. In addition, all horses were nose twitched during arthrocentesis. Before each arthrocentesis the entire carpal area was scrubbed with Hibiclens™ (Stuart Pharmaceuticals, Wilmington, DE) and alcohol at least three times with a final scrub directly over the site for arthrocentesis. Sterile needles (20 gauge, 1 inch), syringes (12 ml or 20 ml) and gloves were used during each arthrocentesis. Synovial fluid was collected from the intercarpal joint before and

at 1, 3, 6, 9, 12, and 24 hours after drug administration for determination of ketoprofen. Synovial fluid (approximately 3.5 ml) was obtained alternatingly from the left and right intercarpal joints due to the paucity of synovial fluid in these normal joints. Synovial fluid was placed into 2 ml evacuated glass tubes containing 3 mg of EDTA as an anticoagulant (Vacutainer, Becton Dickinson, Rutherford, NJ). The fluid was immediately centrifuged at 4° C and 2,500 rpm for 15 minutes (Eppendorf 5415C, Brinkman Instruments, Westbury, NJ). The supernatant was stored in 1.5 ml polypropylene micro-centrifuge tubes (Dot Scientific, Inc., Flint, MI) at 20° C until analyzed.

d. Synovial fluid collection from horses with experimental synovitis

Arthrocentesis was accomplished as above except that acute inflammation was induced in the left intercarpal joints of these horses by the injection of 0.3 ml of 1% solution of sterile carrageenan at the same time as ketoprofen administration (time 0). In these horses synovial fluid was obtained only from the inflamed left intercarpal joint at the times designated above. At each time point, the maximum volume of synovial fluid was withdrawn in order to reduce the degree of discomfort in the horse caused by joint effusion. The amount of synovial fluid withdrawn ranged from 2.5 to 15 ml. Synovial fluid was processed as above.

4. Ketoprofen determination

The concentrations of ketoprofen in plasma and synovial fluid were determined by high performance liquid chromatograph (HPLC) analysis of ethyl acetate extracts

of acidified samples. Fenoprofen was added to the samples before extraction as an internal standard.

Plasma and synovial fluid calibrators were prepared by adding ketoprofen and fenoprofen standard solutions to drug-free equine plasma and synovial fluid. For plasma, concentrations of 0.1, 0.5, 1, 10, and 40 μg of ketoprofen/ml of plasma were prepared. Ketoprofen was added to synovial fluid to make concentrations of 1, 0.5, 0.1, 0.05, and 0.025 μg/ml. These calibrators were prepared and analyzed daily in duplicate with each set of unknown samples.

a. Extraction

For plasma samples, 25 μl of 1000 μg/ml fenoprofen was added to 500 μl of sample to achieve a final concentration of 50 μg/ml. For synovial fluid samples, 5 μl of 1000 μg/ml of fenoprofen was added to 500 μl of sample to achieve a final concentration of 10 μg/ml. Plasma or synovial fluid samples were acidified with 0.5 ml 1 N HCl and 2.0 ml of ethyl acetate was added. Samples were vortexed for 30 seconds, then centrifuged at 2,000 rpm for 15 min (Dynac, Becton Dickenson and Company, Parsippany, NJ). The ethyl acetate extraction was repeated. The upper organic layers were transferred to a separate tube and evaporated to dryness without heat under a flow of nitrogen. Samples were reconstituted in 250 μl of mobile phase solvent mixture, vortexed and filtered through a 0.45 μm teflon syringe filter (Poretics Co., Livermore, CA) into to a 2.0 ml HPLC autosampler vial. The injection volume was 10 μl.

b. Chromatography

All analyses were performed using a Hewlett Packard 1090 high performance liquid chromatograph equipped with a variable volume auto-injector and a photodiode array detector set at 256 nm (10 nm bandwidth) with a reference spectrum of 550 nm (100 nm bandwidth). Instrument control, data acquisition, and peak integration were accomplished with Hewlett-Packard HPLC ChemStation software (Waldbronn, Germany). A 100 x 4.6 mm reversed phase column packed with 3 μm octadecylsilyl derivatized silica particles (Spherosorb™ ODS-2, 80 Å pore) equipped with 10 x 4 mm guard column (5 μm, 300 Å pore) from Keystone Scientific (Bellefonte, PA) maintained at ambient temperature was used for all analyses.

The mobile phase consisted of 0.001 M phosphate buffer (pH 7.4) and acetonitrile delivered at a ratio of 85:15 or 82.45:17.55. The mobile phase was filtered through a 0.45 μm membrane filter (FP Vericel™, Gelman Sciences, Inc., Ann Arbor, MI) and degassed with helium immediately before use. A 10 μl aliquot of synovial fluid or plasma extract was injected at a rate of 833.3 μl/minute for analysis. The analyte and internal standard were eluted at a flow rate of 1 ml/minute The total run time was 8.5 minutes.

c. Linearity of response

Plasma and synovial fluid concentrations were calculated by determining the peak height ratio of ketoprofen to the internal standard, fenoprofen for each incurred sample and calibrator. Five point calibration curves for ketoprofen in plasma and synovial fluid were prepared by plotting the peak height ratios against concentration

using data derived from the average of duplicate injections. The slope, intercept and correlation coefficients for each of the calibration curves were determined before each analytical session.

d. Recovery

Relative recovery of ketoprofen from synovial fluid and plasma was evaluated by comparison of analyte peak heights of 5 injections of non-extracted standard solutions to peak heights of 5 extracted fortified samples. Percent recovery was determined for the lowest and highest concentrations of ketoprofen tested in synovial fluid and plasma.

e. Intra-assay variability

The within run precision was determined by replicate injections of the same sample run under identical conditions during the same analytical session. This was determined for fortified samples of the lowest and highest concentrations of ketoprofen tested in synovial fluid and plasma. The intra-assay variation of each concentration in each matrix was defined as the coefficient of variation of triplicate injections of the same sample run in duplicate so that $n=6$ per concentration. The coefficient of variation of each concentration was calculated from the mean and standard deviation of the peak height ratios.

f. Inter-assay variability

The between day precision was determined from samples of the same concentration extracted and analyzed under identical conditions during different analytical sessions. This was determined for fortified samples of the lowest and

highest concentrations of ketoprofen tested in synovial fluid and plasma. The inter-assay variability at each concentration in each matrix was defined as the coefficient of variation of 5 duplicate injections analyzed during 5 different analytical sessions. The overall inter-assay variation for each matrix was obtained from averaging the coefficient of variations from each concentration examined.

g. Plasma pharmacokinetic analysis

The plasma ketoprofen concentration versus time data for each horse were analyzed by an automated curve-stripping procedure using the RSTRIP™ computer program (MicroMath Scientific Software, Salt Lake City, UT). This provided initial estimates of pharmacokinetic parameters and the best exponential disposition function to fit the data. These initial estimates were then used to generate a best fit by nonlinear least-squares regression analysis with equal weighting of the data using the MINSQ™ computer program (MicroMath Scientific Software, Salt Lake City, UT). Visual inspection of the fitted curves, examination of the plot of the residuals versus calculated concentration [406], and application of a modified Akaike's information criterion [407] were used to determine the number of exponential terms required to describe the data. For the 2-compartment open model, the equation was:

$$C_p = A \cdot e^{-\alpha \cdot Time} + B \cdot e^{-\beta \cdot Time}$$

where C_p is the plasma concentration, A = distribution phase intercept, α = distribution phase rate constant, B = elimination phase intercept, ß = elimination phase rate constant. The MINSQ™ computer program calculated the following dependent variables: area under the plasma concentration versus time curve (calculated

to infinity), AUC; the elimination rate from the central compartment, K_{el}; the transfer rate from the first compartment to the second compartment, K_{12}; the transfer rate from the second compartment to the first compartment, K_{21}; the half-life of α, $t_{½\alpha}$; and the half-life of β, $t_{½ß}$. The following parameters were derived from standard kinetic formulas [408].

The total plasma clearance was calculated from:

$$CL_T = \text{Dose/AUC}$$

where dose = dose of ketoprofen in μg/kg and AUC = $A/\alpha + B/ß$.

The volume of the central compartment was calculated from the dose and the area under the curve:

$$V_c = \text{Dose/AUC}$$

The volume of distribution based on the area method was determined by the total clearance and elimination rate constant:

$$Vd_{(area)} = CL_T/ß$$

The volume of distribution at steady state or the whole body estimate of the volume of distribution was calculated as follows:

$$Vd_{ss} = \text{Dose}\cdot\text{AUMC/AUC}^2$$

The elimination half-life ($t_{½ß}$) was determined by the elimination rate constant:

$$t_{½ß} = \ln(2)/ß$$

A noncompartmental method was used to determine the statistical moments [409]. The area under the first moment curve ($\int C_p \, t \cdot dt$) was calculated from the intercepts and rate constants:

$$AUMC = A/\alpha^2 = B/\beta^2$$

The first statistical moment of the drug concentration time curve is the mean residence time (MRT) and is analogous to the half-life of compartmental analysis. This value represents the time for 63.2% of the dose to be eliminated and was calculated from the area under the curve and the area under the first moment curve [409]:

$$MRT = AUMC/AUC$$

h. Synovial fluid pharmacokinetic analysi

The limited number of time-concentration data for synovial fluid in normal horses (3 points) and synovitis horses (4 points) did not allow for a calculation of a complete pharmacokinetic profile. However, the area under the curve and time-concentration data for each horse were calculated. The area under the time versus concentration curve (AUC) was determined for each horse by the Pharmacologic Calculation System computer program (MicroComputer Specialists, Philadelphia, PA) using the trapezoidal rule [410].

i. Plasma Statistical analysis

The elimination half-life ($t_{½\beta}$) was expressed as an arithmetic mean of four horses. In addition the half-life was expressed as the harmonic mean ($Ht_{½}$) based on the mean elimination rate constant. The harmonic is the more appropriate estimate

of the half-life and always produces a lesser value than the arithmetic mean [411]. The harmonic mean is calculated as follows:

$$H_{t½} = n /(1/t_{½ß,1} + 1/t_{½ß,2} + 1/t_{½ß,n})$$

or

$$H_{t½} = \ln(2)/ß^{a}$$

where $ß^{a}$ = the mean elimination rate constant of four horses. The pseudo standard deviation (pSD) of the harmonic half-life was based on the jackknife variance and was estimated by the following:

$$pSD = \sqrt{[(n-1)\ \Sigma(H_i - H_t½)^2]}$$

where H_i = the harmonic mean of n-1 values which is calculated n times deleting a different half-life value each time. Estimates of half-life, volume of distribution, AUC and Cp° (time 0 plasma concentration) are not considered to be normally distributed [412]. Therefore, all pharmacokinetic parameters were compared for normal horses and those with acute synovitis by the Mann-Whitney rank sum test [413] using SigmaStat (Jandel, Corp., San Rafael, CA). All results were considered significant when $P < 0.05$.

k. Synovial fluid statistical analysis

Values for AUC from both groups of horses were compared by the Mann-Whitney rank sum test. Synovial fluid concentrations from both groups of horses were compared at each time using the Mann-Whitney rank sum test. Within each group, synovial fluid concentrations were compared to corresponding plasma

concentrations with paired Student's t tests [414] using SigmaStat (Jandel, Corp., San Rafael, CA). All results were considered significant when $P < 0.05$.

C. Results

1. Chromatography

The mobile phase composition reported by Satterwhite and Boudinot [405] of 0.01 M phosphate buffer and acetonitrile (82.5:17.5) did not achieve adequate resolution of the analyte and the reported internal standard, naproxen when used with the 3 μm octadecylsilyl column. Several other internal standards were also tested using this system without success including, oxyphenbutazone, phenylbutazone and ibuprofen. Fenoprofen was determined to have adequate chromatography and a unique retention time as compared to the analyte when a mobile phase of 0.001 phosphate buffer and acetonitrile was used with a ratio of 85:15 or 82.45:17.55. The latter mobile phase ratio was found to be optimal for some sessions using a newly purchased column. The limits of quantitation of ketoprofen in this system were 0.025 μg/ml in synovial fluid and 0.1 μg/ml in plasma.

Chromatograms of blank plasma and synovial fluid are shown in Figures 17 and 18. Chromatograms of plasma and synovial fluid from horses administered ketoprofen are shown in Figures 19 and 20. All chromatograms are expressed as milliabsorbance units (mAU) versus time. The retention time of ketoprofen in both fluids was 1.75 minutes. The retention time of fenoprofen varied slightly from 1.90 to 2.20 minutes. As can be seen from the blank chromatograms in Figures 17 and 18,

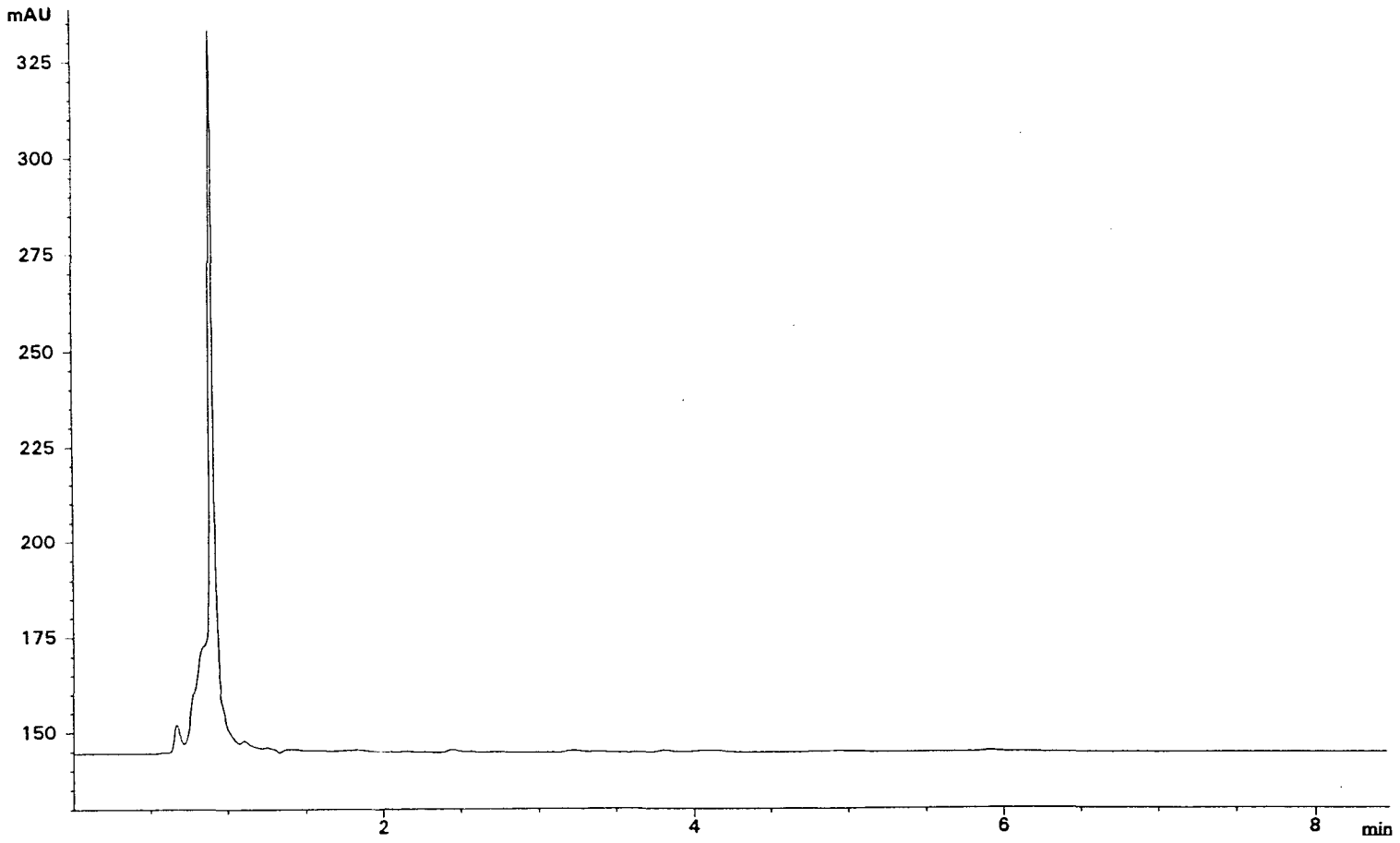


Figure 17: Liquid chromatogram of extracted plasma containing no drug.

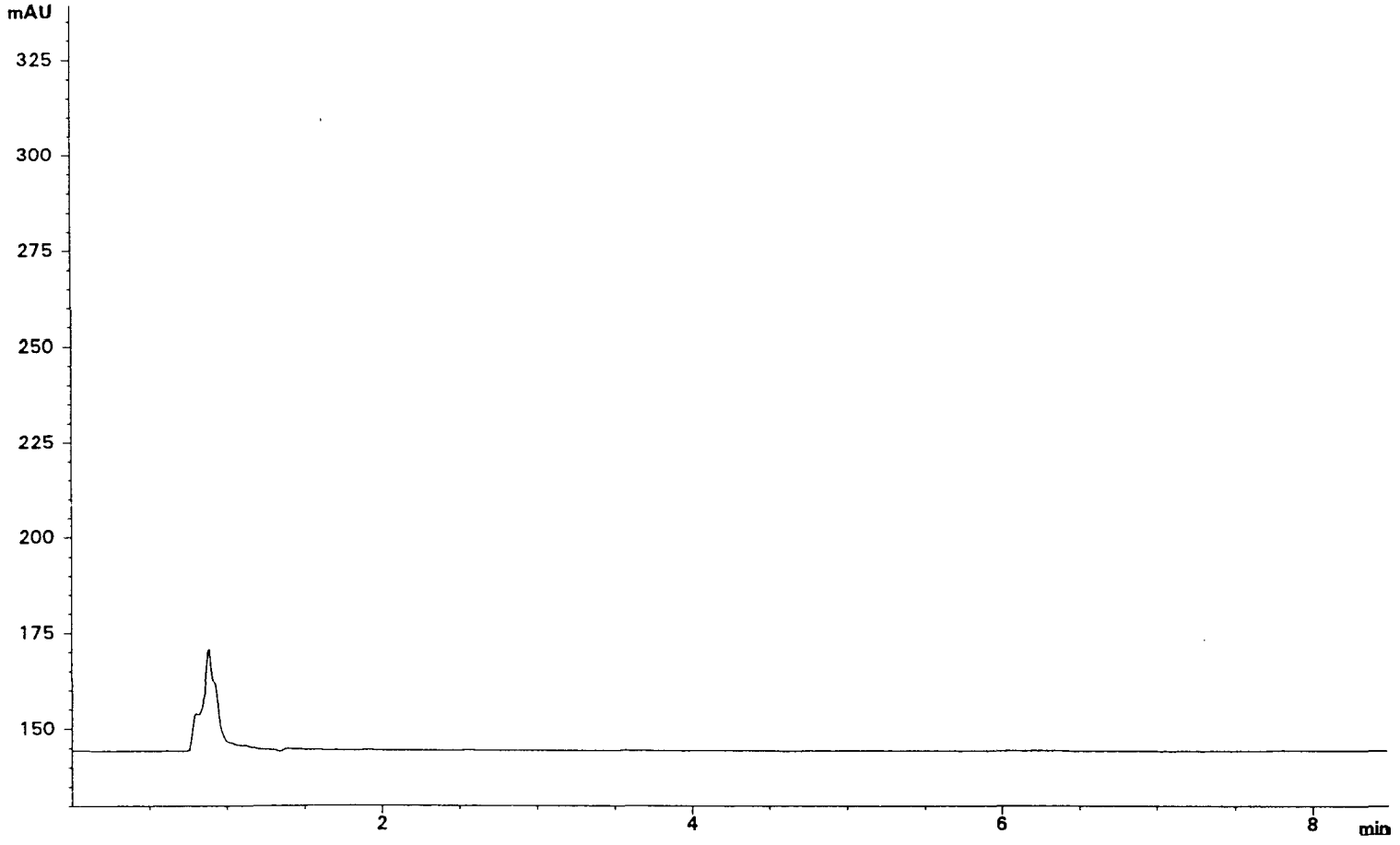


Figure 18: Liquid chromatogram of extracted synovial fluid containing no drug.

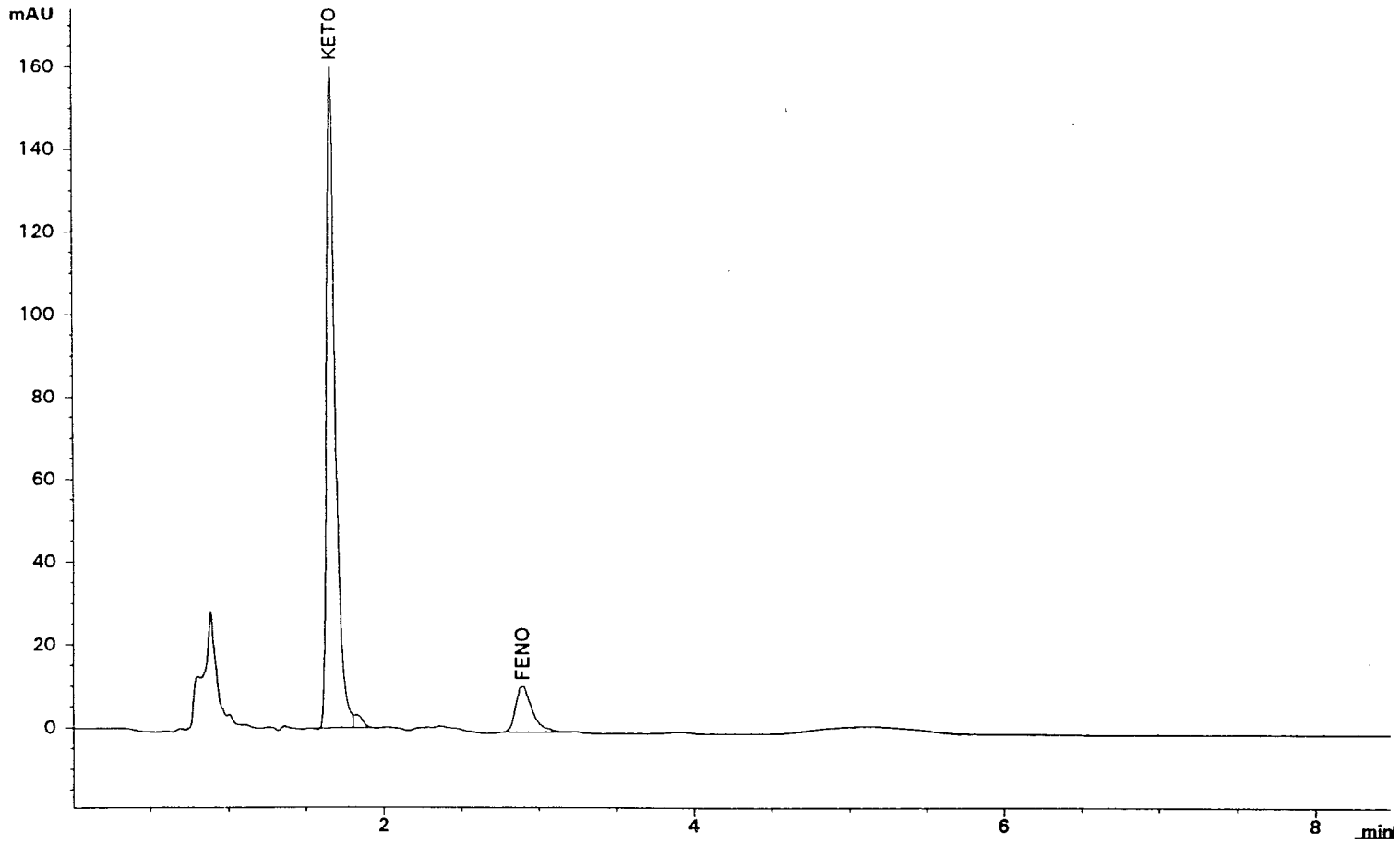


Figure 19: Liquid chromatogram of extracted plasma from a horse administered ketoprofen. The calculated ketoprofen (KETO) concentration was 11.89 μg/ml. The sample was fortified with fenoprofen (FENO) at 50 μg/ml as an internal standard.

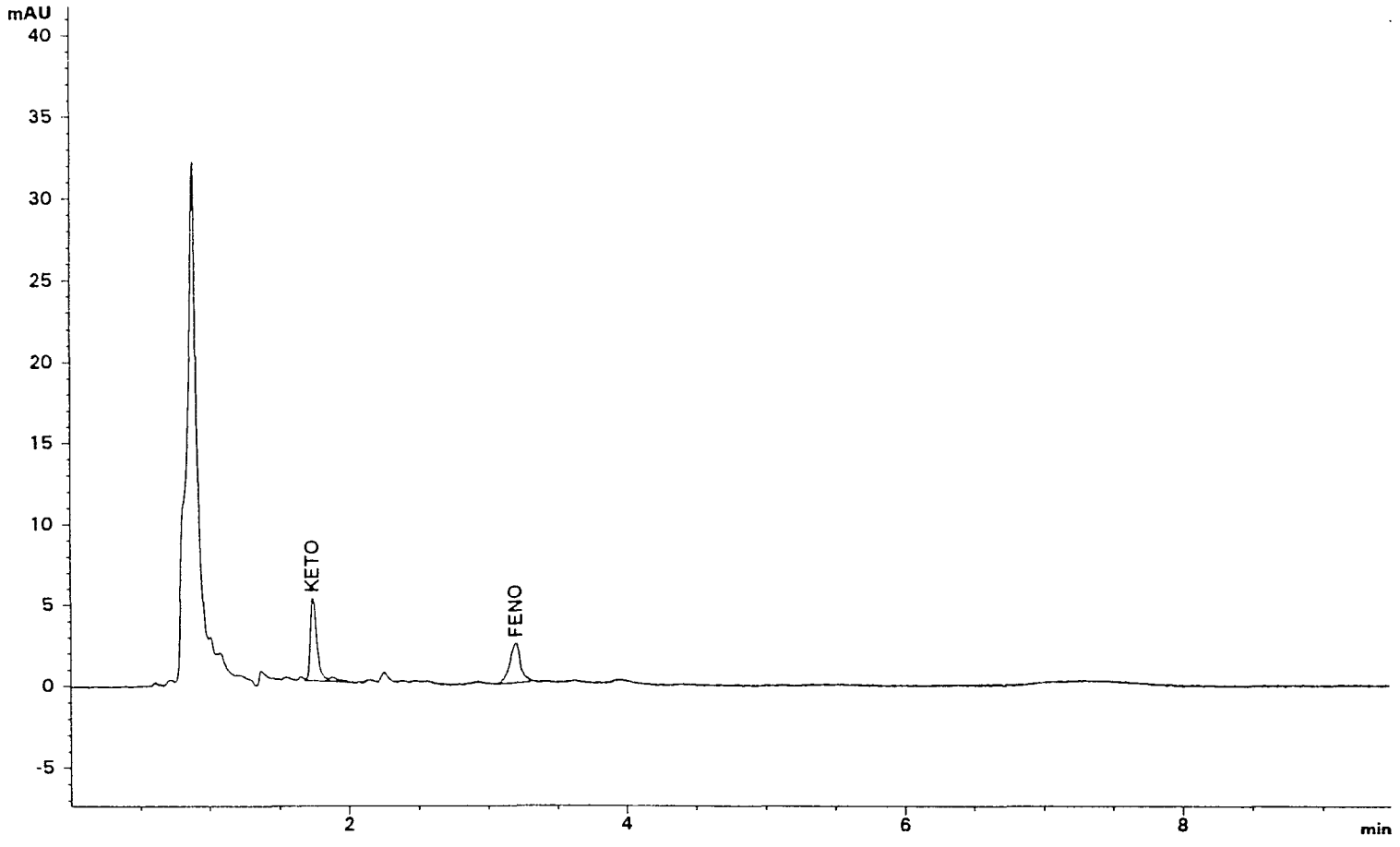


Figure 20: Liquid chromatogram of extracted synovial fluid from a horse administered ketoprofen. The calculated ketoprofen (KETO) concentration was 0.53 μg/ml. The sample was fortified with fenoprofen (FENO) (10 μg/ml) as an internal standard.

there were no interfering substances eluting near the retention times for ketoprofen or fenoprofen in plasma or synovial fluid. The ultraviolet (UV) absorbance spectra of ketoprofen from non-extracted standards and incurred samples are shown in Figures 21 and 22. Comparison of the UV spectra of ketoprofen from standards and incurred samples indicates excellent peak purity. The UV absorbance spectrum of fenoprofen is shown in Figure 23. Thus, ketoprofen and fenoprofen were differentiated based on both the unique retention time and UV absorbance spectrum of each compound.

a. Linearity of response

Calibration curves of ketoprofen fortified plasma and synovial fluid were obtained using the mean peak height ratio values of duplicate injections. These curves were linear over the range of concentrations used (0.1 to 40 μg/ml for plasma, and 0.025 to 1.0 μg/ml for synovial fluid) for each analytical session with correlation coefficients (r) ranging from 0.9964 to 0.9989.

b. Recovery

The mean relative recoveries of ketoprofen as determined by comparison of peak heights of five injections of standard solutions to five injections of extracted fortified samples were 100.2% $\pm$ 10.0 and 92.1% $\pm$ 6.5% for synovial fluid samples containing 0.025 μg/ml and 1.0 μg/ml, respectively, with an overall mean recovery of 96.2%. Mean relative recoveries of ketoprofen from fortified plasma samples were 109.4% $\pm$ 13.4% and 89.8% $\pm$ 12.5% for samples containing 0.1 μg/ml and 40.0 μg/ml, respectively, with an overall mean of 99.6% (Table 16). The extraction

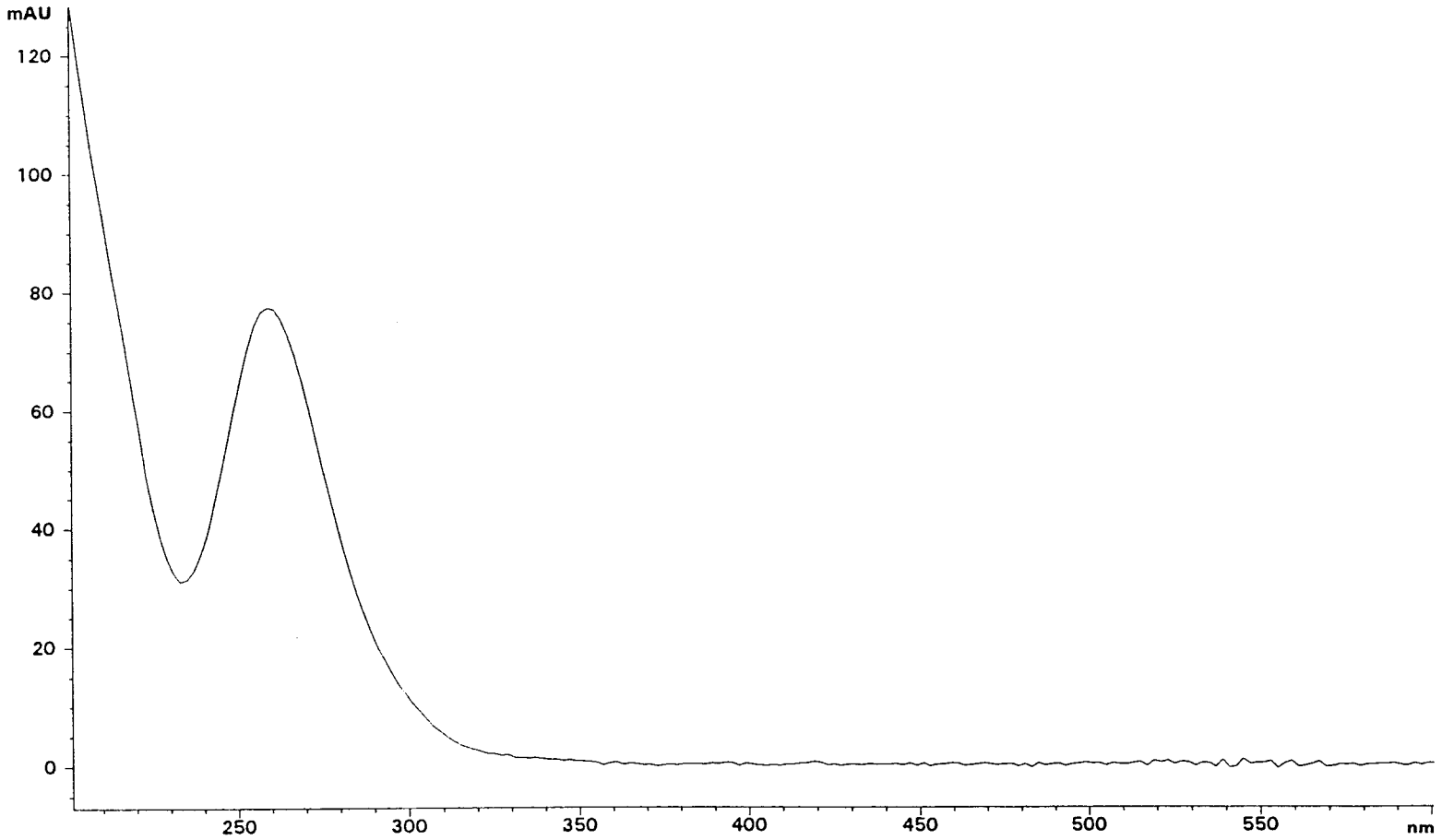


Figure 21: UV absorbance spectrum of ketoprofen from a liquid chromatogram of a non-extracted standard solution.

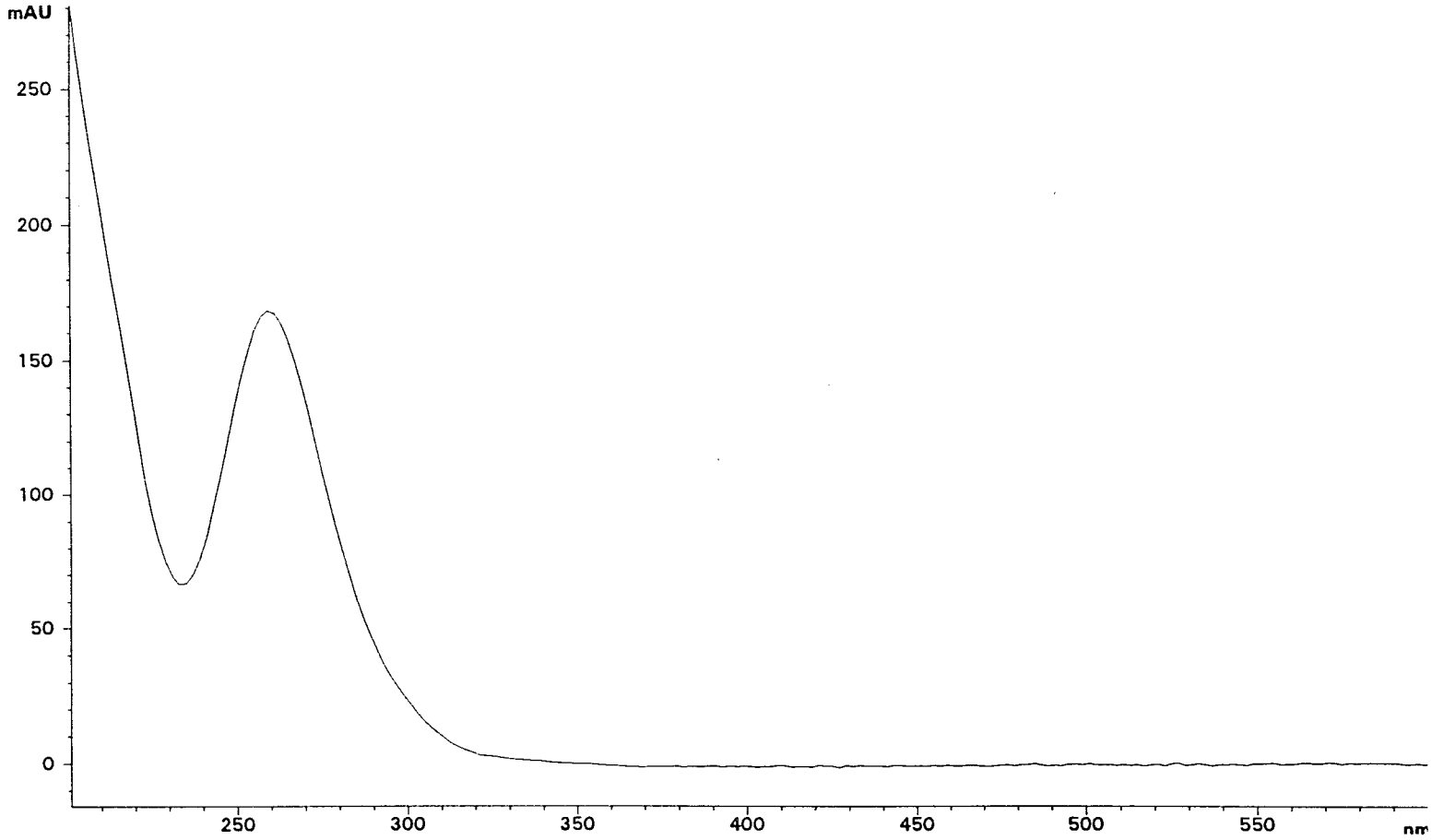


Figure 22: UV absorbance spectrum of ketoprofen from a liquid chromatogram of an extracted incurred plasma sample.

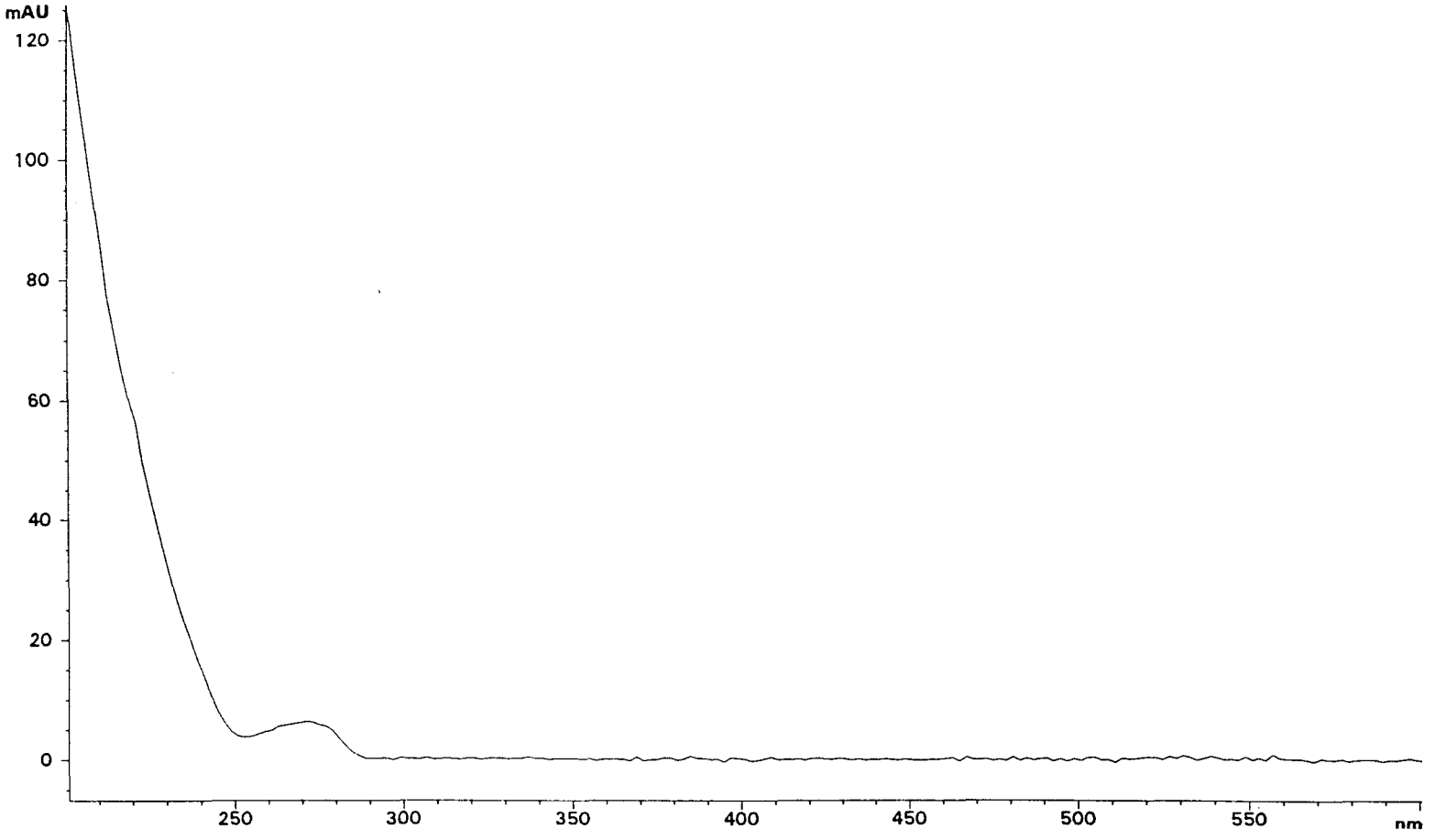


Figure 23: UV absorbance spectrum of fenoprofen from a liquid chromatogram of an extracted plasma sample.

Table 16: Recovery of ketoprofen as determined by the comparison of peak heights from 5 standard solutions and extracted fortified samples. Percent recovery is reported ± SD.

CONCENTRATION (μg/ml)	RECOVERY (%)	CONCENTRATION (μg/ml)	RECOVERY (%)
0.025	100.2 (± 10.0)	**0.10**	109.4 (± 13.4)
1.000	92.1 (± 6.5)	**40.0**	89.8 (± 12.5)
OVERALL	96.1	**OVERALL**	99.6

technique used showed excellent recoveries for synovial fluid and plasma and were similar to other methods using diethyl ether [405] and dichloromethane [283].

c. Intra-assay variability

The intra-assay variances for synovial fluid as expressed as the coefficient of variation ranged from 5.2% for the 1.0 μg/ml concentration to 11.0% for the 0.025 μg/ml level. The plasma values were 3.5% for the 0.1 μg/ml concentration and 4.6% for samples containing 40.0 μg/ml of ketoprofen (Table 17).

d. Inter-assay variability

The between session variability based on the coefficient of variation of five samples run during subsequent analytical sessions ranged from 8.0% for fortified plasma samples containing 0.1 μg/ml of ketoprofen to 10.3% for fortified synovial fluid containing 0.025 μg/ml of ketoprofen (Table 17).

2. Plasma pharmacokinetics

Ketoprofen disposition in plasma for normal horses and those with acute synovitis was best described by a bi-exponential equation, except in one normal horse and one horse with acute synovitis which were better fitted to a mono-exponential or one-compartment model. These unusual animals were force-fitted to a two-compartment model which permitted comparison between horse groups. The bi-exponential equation, $C_p = A \cdot e^{-\alpha \cdot Time} + B \cdot e^{-\beta \cdot Time}$, was used to determine the pharmacokinetic parameters of all horses where C_p = plasma concentration, A = distribution phase intercept, α = distribution phase rate constant, B = elimination phase intercept, and ß = elimination phase rate constant. The individual coefficients

Table 17: Intra-assay variability as represented by the CV of 6 injections. Inter-assay variability as represented by the CV of 5 injections.

SYNOVIAL FLUID		
CONCENTRATION (μg/ml)	**INTRA-ASSAY (CV)**	**INTER-ASSAY (CV)**
0.025	11.0	14.2
1.000	5.1	9.2
OVERALL	8.1	11.7
PLASMA		
0.1	3.5	8.0
40.0	4.6	9.5
OVERALL	4.1	8.8

of determination (correlation coefficient^2 or r^2) ranged from 0.96217 to 0.99753. This value is a measure of the fraction of the total variance accounted for by the model.

The mean total plasma ketoprofen concentration versus time curve for normal horses and horses with acute synovitis are shown in Figures 24 and 25 and the derived pharmacologic parameter values are outlined in Tables 18 and 19. The mean ± standard deviation (SD) plasma concentration of normal horses 3 minutes after dosing was 33.72 ± 0.43. The corresponding concentration in horses with acute synovitis was 23.61 ± 1.96. The distribution phase intercept (A) of normal horses was 41.31 ± 17.08. This value was significantly higher (P = 0.029) than the corresponding value in horses with acute synovitis, 24.65 ± 4.90. Both groups had a similar rapid distribution phase followed by slower elimination phase. At 4 hours post-dose plasma levels were 0.36 μg/ml ± 0.02 in normal horses and 0.16 μg/ml ± 0.06 in horses with acute synovitis. Plasma ketoprofen concentrations were below the limit of quantitation by 5 hours. The harmonic plasma half-life ($t½_\beta$) ± the pseudo standard deviation of the horses with acute synovitis was 0.55 hour ± 0.21. This harmonic half-life was shorter (P = 0.029) than that of the normal horses, 0.88 hour ± 0.35. All other plasma pharmacokinetic parameters were not statistically different between horse groups.

3. Synovial fluid pharmacokinetics

Ketoprofen disposition in synovial fluid in normal horses was minimal with mean peak levels ± SD of 0.39 μg/ml ± 0.03 occurring at one hour with levels falling to 0.08 μg/ml ± 0.04 by 6 hours (Figure 26). Concentrations were below the

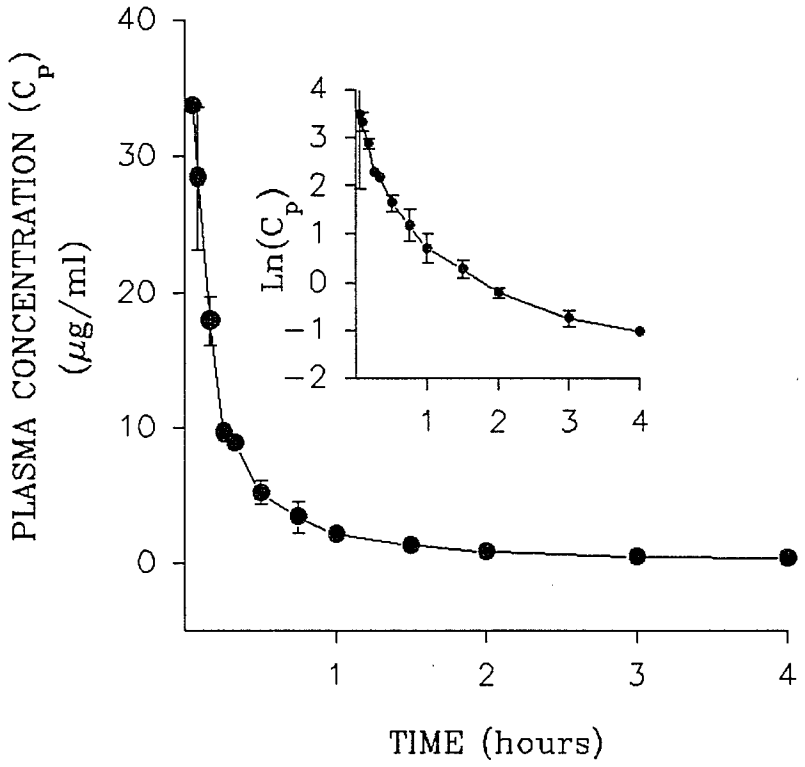


Figure 24: Plasma disposition curves of intravenous ketoprofen (2.2 mg/kg) in 4 normal horses. Mean ketoprofen concentrations in μg/ml are presented as $\pm$ standard deviations (SD). Inset graph represents the natural log of plasma concentrations versus time.

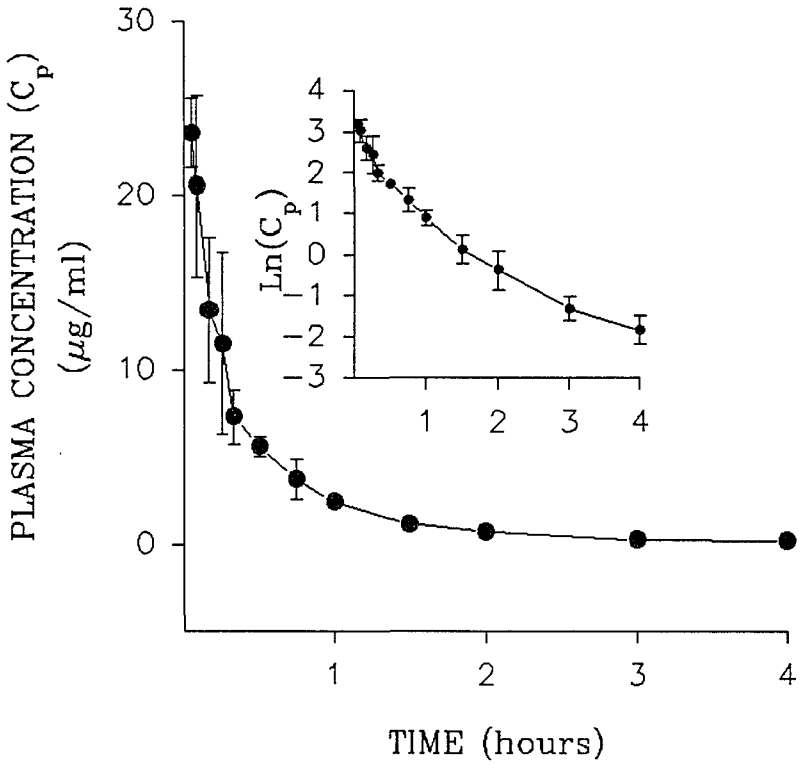


Figure 25: Plasma disposition curves of intravenous ketoprofen (2.2 mg/kg) in 4 horses with acute synovitis. Mean ketoprofen concentrations in μg/ml are presented as $\pm$ SD. Inset graph represents the natural log of plasma concentrations versus time.

Table 18: Plasma pharmacokinetic parameters of intravenous ketoprofen (2.2 mg/kg) in normal horses and horses with acute synovitis.

	NORMAL HORSES			SYNOVITIS HORSES			
PARAMETER	**MEAN**	**ST.DEV.**	**RANGE**	**MEAN**	**ST.DEV.**	**RANGE**	**P**
A (μg/ml)	41.31	17.08	31.06-66.82	24.65	4.90	18.36-30.24	0.029*
α (1/hr)	6.86	2.89	4.57-10.92	9.47	3.74	3.95-11.88	>0.10
B (μg/ml)	5.10	3.40	2.02-9.18	9.29	5.26	2.99-15.80	>0.10
β (1/hr)	0.79	0.31	0.42-1.10	1.27	0.49	0.67-1.83	>0.10
Cp° (μg/ml)	46.16	20.07	33.08-76.04	33.78	7.26	26.44-40.11	>0.10
K_{el} (1/hr)	3.73	1.05	2.84-5.25	3.37	0.56	2.62-3.83	>0.10
K_{12} (1/hr)	2.44	1.45	1.33-4.47	3.67	1.93	0.99-5.58	>0.10
K_{21} (1/hr)	1.47	0.78	0.67-2.28	3.69	2.03	1.01-4.51	>0.10

P = Probability estimate
*Statistically significant at $P < 0.05$

Table 19: Plasma pharmacokinetic parameters of intravenous ketoprofen (2.2 mg/kg) in normal horses and horses with acute synovitis.

	NORMAL HORSES			SYNOVITIS HORSES			
PARAMETER	**MEAN**	**ST.DEV.**	**RANGE**	**MEAN**	**ST.DEV.**	**RANGE**	**P**
$t_{½\alpha}$ (hr)	0.11	0.04	0.06-0.15	0.09	0.06	0.05-0.17	>0.10
$t_{½\beta}$ (hr)	1.02 0.88[a]	0.47 0.35[b]	0.62-1.65	0.63 0.55[a]	0.29 0.21[b]	0.37-1.03	>0.10 0.029[a*]
$t_{½Kel}$ (hr)	0.20	0.05	0.13-0.24	0.23	0.04	0.18-0.26	>0.10
MRT (hr)	0.76	0.25	0.56-1.11	0.62	0.13	0.57-0.75	>0.10
AUC_{∞} (μg•hr/ml)	12.06	1.64	10.75-14.46	10.10	1.00	8.70-10.94	0.057
$AUMC_{\infty}$ (μg•hr^2/ml)	9.07	2.64	7.49-12.99	6.25	1.63	4.87-8.26	>0.10
CL_T (ml/hr•kg)	184.72	22.70	152.08-204.48	219.55	23.25	201.02-252.68	0.057
V_c (ml/kg)	52.67	16.50	28.93-66.48	66.55	12.40	54.83-77.80	>0.10
$Vd_{(area)}$ (ml/kg)	276.30	139.16	138.20-319.16	195.27	77.46	112.51-299.04	>0.10
Vd_{ss} (ml/kg)	142.30	52.53	85.63-211.74	134.79	27.21	94.24-151.74	>0.10

P = Probability estimate
[a]Harmonic mean
[b]Psuedo Standard Deviation
*Statistically significant at $P < 0.05$

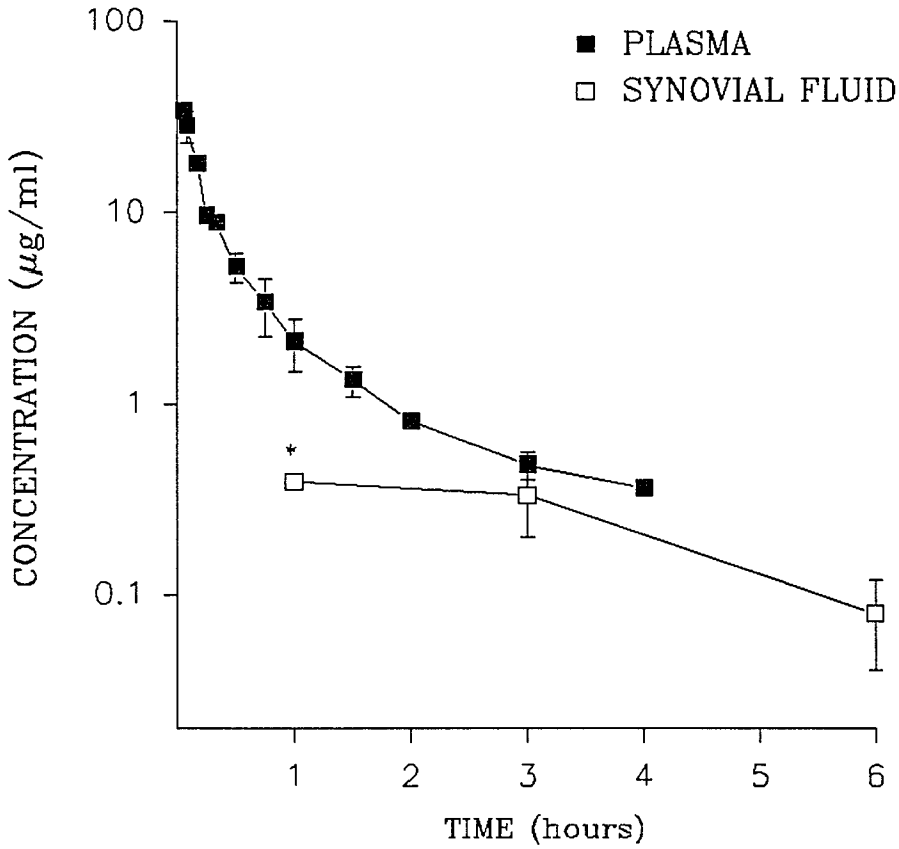


Figure 26: Mean plasma and synovial fluid ketoprofen concentrations in μg/ml (± SD) following intravenous ketoprofen (2.2 mg/kg) in 4 normal horses. Significantly different synovial fluid and plasma concentrations are indicated by (*).

limit of quantitation in all normal horses by 9 hours. Synovial fluid ketoprofen concentrations at one hour were below ($P = 0.015$) corresponding plasma levels. Synovial fluid levels at 3 hours were not different from the corresponding plasma concentrations.

In contrast, synovial fluid disposition of ketoprofen in horses with acute synovitis was extensive with peak concentrations (2.53 μg/ml $\pm$ 0.39) occurring at one hour with levels falling to 0.06 μg/ml $\pm$ 0.02 by 9 hours. Concentrations were below the limit of quantitation by 12 hours. Although not significant, synovial fluid levels at 3 hours appeared greater than the corresponding plasma concentrations ($P = 0.09$). In contrast to the normal horses, synovial fluid concentrations at one hour were not statistically different from one hour plasma levels (Figure 27). The average AUC for normal horses (1.37 μg·hr/ml $\pm$ 0.38) was less ($P = 0.029$) than that of horses with acute synovitis (6.24 μg·hr/ml $\pm$ 2.41). At one hour post-dose, synovial fluid levels of ketoprofen in horses with acute synovitis were 6.5 times greater ($P = 0.017$) than normal horses. There was no difference in synovial fluid concentrations between the two groups at 3 or 6 hours.

D. Discussion

The harmonic means of plasma elimination half-lives of ketoprofen in both groups of horses were between 0.5 and 1 hour. This was expected based on the polarity of ketoprofen, as most polar compounds are rapidly excreted. The plasma half-life is proportional to the volume of distribution and inversely proportion to total

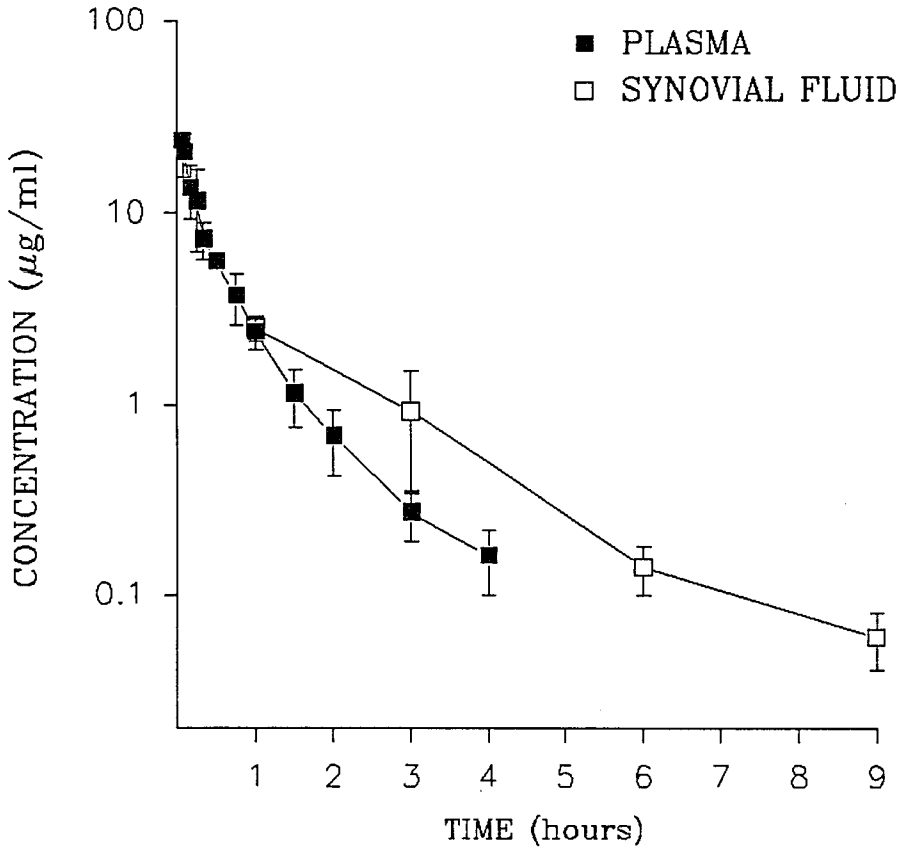


Figure 27: Mean plasma and synovial fluid ketoprofen concentrations in μg/ml (± SD) following intravenous ketoprofen in 4 horses with acute synovitis.

clearance. The three volumes of distribution for both groups were less than or approximately equal to the extracellular volume (200 ml/kg), which would also be expected for a polar drug. The steady state volume of distribution is largely dependent on the intravascular albumin binding which for most NSAIDs results in limited distribution volumes [415].

The mean harmonic plasma half-life of normal horses (0.88 hours $\pm$ 0.35) was significantly higher than that of horses with acute synovitis (0.55 hours $\pm$ 0.21). This may be due to the increased penetration of ketoprofen into the inflamed joint, particularly from 1 to 3 hours post-dose as compared to the normal joints. In addition, the inflamed joints of these horses may have acted as a sequestered site of elimination, as the synovial fluid withdrawn from horses with acute synovitis ranged from 2.5 to 15 ml as compared to approximately 3.5 ml in normal horses. However, this cannot be confirmed as elimination data from concurrent urinary and fecal ketoprofen levels were not obtained in this study. To date, there are two published studies on the plasma kinetics of ketoprofen in the horse. In the study by Sams *et al.*,1993, the reported harmonic mean of the half-life after one dose of 2.2 mg/kg of intravenous ketoprofen was 1.6 hours with a range of 1.18 to 2.65 hours. After five daily intravenous doses the harmonic mean of the half-life was 1.3 hours with a range of 0.76 to 1.86 hours [283]. The half-life in normal horses in the present study ranged from 0.62 to 1.65 hours. Several differences in study design and pharmacokinetic analysis are evident in these two studies. There may be a breed difference in the pharmacokinetics of ketoprofen as most of the horses in the previous

study were standardbreds weighing from 465 to 660 kg whereas most horses in this study were Thoroughbreds weighing from 406 to 554 kg. The ketoprofen disposition was described as a tri-exponential equation in the previous study whereas the data in this study were best described by a bi-exponential equation. In addition, blood was sampled in this study more often than in the Sams study, particularly during the early stages (17 times versus 13). Further, all three volumes of distribution and the total clearance values were lower in the present study as compared to data collected by Sams. Again, these differences may be accounted for in part by the larger horses used in the Sams study as these parameters are known to change in proportion to body weight [416].

The distribution phase intercept (A) of normal horses (41.31 μg/ml $\pm$ 17.08) was significantly greater than that of horses with acute synovitis (24.65 μg/ml $\pm$ 4.90). The significance of this effect is unknown. However, the other pharmacokinetic values were not different between groups with the exception of the harmonic half-life. This may be due to the effect of an outlier in the normal horse group with a particularly high distribution phase intercept value (66.82 μg/ml).

Ketoprofen is a chiral compound with the center of asymmetry at C2. The commercial preparation is a racemic mixture of the *R* and *S* enantiomers. Upon administration many species show conversion of the biologically inactive *R* enantiomer to the active *S* enantiomer [417,418]. In humans, the there is an approximately 10% inversion of the *R* enantiomer to the *S* after oral administration with little stereoselectivity in the pharmacokinetics of these enantiomers. Therefore,

nonstereospecific assays may be used to explain the pharmacokinetics of the individual enantiomers in man [284]. In a recent study by Jaussaud *et al.*, the plasma pharmacokinetics of ketoprofen enantiomers in the horse were evaluated after administration of the racemate by means of a stereoselective HPLC technique [286]. The plasma disposition curves were fitted to a bi-exponential equation. In contrast to humans, the ratio of the *S* to *R* enantiomer increased over time and attained a 70:30 ratio at 50 minutes in the horse. The elimination half-lives of the enantiomers were not significantly different statistically but the clearance rate and initial concentration of the *S* enantiomer were lower than that of the *R* enantiomer. The total ketoprofen disposition curve closely paralleled the *S* enantiomer curve. Further, these authors noted a short elimination half-life (0.29 to 0.47 hours) as compared to the present study, which may be accounted for by their relatively short sampling interval of 90 minutes.

Synovial fluid ketoprofen levels in normal horses were lower than plasma levels at one hour, but the drug reached an equilibrium between the joint and systemic circulation by 3 hours as synovial fluid levels approximated plasma concentrations at this time. In contrast, ketoprofen synovial fluid and ketoprofen levels in horses with acute synovitis reached equilibrium at one hour post-administration. In these horses with acute synovitis, synovial fluid concentrations at 3 hours tended to be higher than the corresponding plasma levels. In humans with arthritis this effect is common as synovial levels often exceed plasma levels after the equilibrium time [280]. This results from the relative sequestration of the drug in the synovial fluid with the lack

of significant elimination occurring from the joint [402]. In horses with acute synovitis the amount and persistence of ketoprofen in synovial fluid was greater than in normal horses. This was based on the significantly larger AUC, higher concentration at one hour and longer duration of synovial fluid ketoprofen quantitation for the horses with joint disease.

The effect of increased penetration of an NSAID into inflamed joints versus normal ones has been reported in several species. In humans, NSAID joint levels were higher in patients with synovial inflammation than normal non-inflamed joints. NSAIDs reached levels in inflamed chicken joints that were three times higher than in control joints. The inflamed joint levels approximated the corresponding plasma levels at 2 hours post-dose [265].

Distribution of a drug to the peripheral tissues depends on the physicochemical properties of the drug, the concentration of the drug in the plasma and tissue, the blood flow to the tissue, percentage of plasma protein binding and the affinity of the drug for the particular tissue [399,416]. Only free (unbound to plasma proteins) drug is available to penetrate the endothelium and enter the synovial fluid. However, in cases of synovitis, the damaged endothelial barrier allows transudation of plasma proteins along with frank hemorrhage into the joint space leading to the accumulation of albumin bound drug in the synovial fluid. NSAIDs bound to albumin are thought to be released by the degradation of albumin by lysosomal enzymes in sites of inflammation [257]. This one way flow of albumin serves to increase the tissue concentration of the drug [258]. Also the lowered pH of the inflamed environment

serves to keep these planar, anionic molecules unionized and thus increase membrane permeability [247]. Ion trapping is postulated to occur intracellularly as the environment inside the cell is relatively alkaline compared to the acidic inflamed tissue [260,261,419].

The effects of protein binding on pharmacokinetics are greater for drugs that are highly bound to plasma proteins and are bound in a concentration dependent manner. For these drugs, small changes in binding often result in marked alterations in drug disposition [420]. In the horse, ketoprofen is greater than 90.0% bound to plasma proteins and this binding is constant over the range of therapeutic drug levels [283]. Plasma and synovial fluid protein binding was not determined in the present study. However, due to the reported linear protein binding in the horse, these data obtained from total drug analytical methods were used as an estimate of free drug kinetics. In addition, in humans there does not appear to be any difference in the degree of protein binding between plasma and synovial fluid for ketoprofen [280].

Certain drugs have a high affinity for tissues and undergo sequestration or depot formation. These drugs may reversibly or covalently bind to subcellular organelles, nucleic acids or cellular proteins and lipids. In cases of covalent binding the drug release is dependent on the cell life span [416]. The R-enantiomer of profens acts as a substitute for endogenous fatty acids and forms hybrid triacylglycerols. The drug may then be bound to lipid membranes for extended periods of time [236,417]. Data from rat studies based on unbound drug disposition suggest that ketoprofen has dose-dependent binding to tissue components [415]. This

tissue binding may account for the low reported urine recovery of ketoprofen (57.2%) in horses [283].

CHAPTER 6

EFFECT OF KETOPROFEN AND PHENYLBUTAZONE ON CHRONIC PAIN AND DIGITAL VEIN EICOSANOID LEVELS IN LAMINITIC HORSES

A. Introduction

Laminitis has been cited as one of the major causes of chronic hoof pain in the horse [198]. Laminitis is classified as chronic after 48 hours of continual pain from a laminitic episode or when ventral deviation or rotation of the distal phalanx occurs [200]. It is associated with debilitation, hypertension, lameness and hoof pain which may last for the life of the animal. The complex syndrome of equine laminitis has multiple etiologies with a complex and poorly understood pathophysiology [199]. The pathology within the foot is a manifestation of a systemic metabolic disorder that affects the cardiovascular, endocrine and renal systems.

This disease is no longer defined as inflammation of the laminae of the foot, but is more precisely defined as a peripheral vascular disease [200] characterized by ischemic necrosis of the epidermal laminae, microvascular thrombosis, epithelial hyperplasia, and hemorrhage [220,224-226]. It has been hypothesized that the laminar ischemia develops acutely due to decreased capillary perfusion from opening of arteriovenous shunts and vasoconstriction [220].

Bacterial endotoxin has been implicated as an inciting cause in laminitis. Many horses with endotoxemia develop laminitis [220] and horses with carbohydrate

overload-induced laminitis have increased plasma endotoxin levels and lameness [221]. Endotoxemia is associated with increased plasma levels of leukotriene B_4 (LTB_4) [222], prostaglandin E_2 (PGE_2) as well as other eicosanoids. The administration of nonsteroidal anti-inflammatory drugs (NSAIDs) prevents many of the effects of experimentally induced endotoxemia [223]. Eicosanoids have been implicated as mediators in laminitis due to the powerful vaso- and venoconstriction produced by $PGF_{2\alpha}$ and thromboxane [217] along with the well known hyperalgesic effects of PGE_2 [157,158] and LTB_4 [166]. However, the association between endotoxin-induced eicosanoid production and the development of laminitis remains a theory due to the inability to produce laminitis following endotoxin administration. Further, concentrations of eicosanoids have not been measured in horses with acute or chronic laminitis [220].

NSAIDs are commonly administered to reduce the pain and suffering associated with laminitis and allow the animal to more comfortably stand and ambulate, thereby promoting blood flow to the foot and reducing the adverse effects of long term recumbency. According to Hood [199], horses that are treated with analgesics early in the course of the disease have a lower incidence of rotation of the distal phalanx than those treated later. NSAIDs are thought to be particular useful in treating the coagulopathy within the foot because of their inhibitory effects on eicosanoid formation and platelet function [199,234]. Previously, phenylbutazone (4.4 mg/kg) has been regarded as the single most important therapeutic agent in treating

laminitis [231,233]. The effects of the recently approved NSAID, ketoprofen have not been evaluated in horses with laminitis.

The purpose of this study was to determine if horses with chronic laminitis have increased digital vein levels of PGE_2 and LTB_4 as compared to normal horses. An increase in the concentration of eicosanoids in the venous drainage of the foot would lend further evidence that these metabolites play a role in mediating the pain and pathology within the foot. The digital vein was chosen as a sampling site due to the difficulty in obtaining laminar tissue for eicosanoid level determination in live horses. Horses were evaluated both at rest and after a brief exercise period in order to determine if eicosanoids are released into the circulation after mild concussion to the laminitic foot.

Provided that there were higher eicosanoid concentrations in laminitic versus normal horses, the severity of hoof pain in these laminitic animals was to be correlated with the eicosanoid concentrations. These horses were then to receive ketoprofen and phenylbutazone in order to determine the magnitude and time course of the eicosanoid inhibitory effects of these NSAIDs in animals with chronic laminitis. Further, this study objectively quantitated and compared the analgesic effects of phenylbutazone and the newly approved NSAID, ketoprofen in horses with chronic hoof pain by means of an electronic hoof tester. The approved therapeutic doses of ketoprofen (2.2 mg/kg) and phenylbutazone (4.4 mg/kg) were used. In addition, the phenylbutazone molar equivalent dose of ketoprofen (3.63 mg/kg) was tested in the laminitic animals in order to compare the potency of ketoprofen and phenylbutazone.

B. Materials and methods

1. Experimental animals

a. Normal horses

Six healthy Thoroughbred mares ranging from 7 to 18 years of age were used to determine digital vein PGE_2 and LTB_4 concentrations. Horses were judged to be healthy and sound based on physical examination and lameness evaluation.

b. Horses with chronic laminitis

Six horses (4 geldings, 2 mares; 4 American Quarter Horses, 1 Thoroughbred, and 1 Morgan) diagnosed with chronic laminitis ranging from 3 to 22 years of age were used for the collection of digital venous blood for determination of PGE_2 and LTB_4 concentrations. Seven horses with chronic laminitis (4 mares, 3 geldings; 5 Quarter Horses, 1 Thoroughbred and 1 Morgan) ranging from 3 to 18 years of age were used in determining the analgesic effects of ketoprofen and phenylbutazone. The diagnosis was based on: **1) degree of lameness** as evaluated at a walk, trot and while turning on a concrete surface; **2) hoof abnormalities** associated with chronic laminitis such as diverging hoof growth patterns with wider growth rings at the heel, subsolar or mural abscesses, a convex shaped sole, and the presence of a 'seedy toe' or an abnormally large white line [200]; **3) radiographic evidence** of laminitis such as ventral deviation or rotation of the third phalanx as determined by Stick's method [228] (mean rotation $\pm$ standard error, $13.75° \pm 2.25$), and osteolysis with demineralization of the distodorsal aspect of the third phalanx [234]; **4) response to**

the hoof tester, particularly over the sole midway between the apex of the frog and the toe; and **5) clinical history** of a laminitic episode [231].

All horses were vaccinated at least three weeks prior against eastern and western equine encephalomyelitis, influenza, tetanus (Equi-Flu EWT™, Coopers, Mundelein, IL) and rhinopneumonitis (Rhinomune™, SmithKline Beecham Animal Health, Exton, PA). The horses were dewormed with oxibendazole (Equipar™, Coopers, Mundelein, IL). Horses were housed in 10.9 x 12.5 feet stalls and were maintained on 5 pounds of a pelleted ration (Purina Horse Chow 100™, Purina Mills Inc., St. Louis, MO) containing: a minimum of 10% protein; 2% fat; a maximum of 25% fiber; and a vitamin/trace mineral supplement twice daily with mixed grass hay (11% protein, 1.4% fat, 24% fiber) and water provided *ad libitum.*

2. Drugs and reagents

a. Drug solutions for administration

Ketoprofen (Ketofen™) was obtained commercially from Aveco Company, Inc., Fort Dodge, IA. Each ml of the sterile solution contained: 100 mg ketoprofen; L-arginine, 70 mg; citric acid to adjust pH to approximately 7; and benzyl alcohol, 0.25 ml as a preservative. Phenylbutazone (Butazolidin™) was obtained commercially from Coopers Animal Health, Inc., Kansas City, KS. Each ml of the sterile solution contained: phenylbutazone, 200 mg; sodium hydroxide to adjust pH to between 9.5 and 10.0; and benzyl alcohol, 10.45 mg as a preservative.

3. Experimental protocol

a. Collection of blood from digital veins

Blood was collected from 6 normal horses and 6 horses with chronic laminitis before and after trotting for 5 minutes on a concrete surface. The lateral region of the metacarpophalangeal joint of each normal and laminitic horse was shaved and scrubbed with a povidone-iodine preparation and alcohol before each venipuncture. Horses were restrained in a stanchion and nose twitched during venipuncture. Blood was collected from the digital vein at the level of the metacarpophalangeal joint with a 20 gauge, 1.0 inch needle. One to 2 ml of blood were discarded before collection of 10 ml into chilled evacuated siliconized glass tubes (Vacutainer, Becton Dickinson, Rutherford, NJ) containing 3.3 μg/ml of BW755C (Wellcome Research Laboratories, Beckenham, Kent, England) as an inhibitor of *ex vivo* eicosanoid synthesis and 10 mg/ml of the anticoagulant, disodium ethylenediamine tetraacetate (EDTA) purchased from Sigma Chemical Company, St. Louis, MO. Tubes were immediately centrifuged at 2,000 rpm for 15 minutes at 4°C (Beckman J21-B, Palo Alto, CA). The platelet poor plasma was transferred into 15 ml polypropylene centrifuge tubes (Sarstedt, Newton, NC) and stored at -20°C until analyzed.

b. Quantitation of nociceptive thresholds in horses with chronic laminitis

Ketoprofen (2.2 mg/kg and 3.63 mg/kg), phenylbutazone (4.4 mg/kg) and saline as a control were administered via the left jugular vein to 7 horses with chronic laminitis. The following methods were used to evaluate analgesia before

administration of NSAIDs (baseline) and at 3, 6 and 24 hours post-treatment. Nociceptive or pain thresholds were determined in the test animals using a calibrated electronic hoof tester according to the method of Kamerling, *et al.*, [421-423]. This device is a standard hoof tester equipped with a load cell welded to the tip of one jaw. The tension/compression load cell (Sensotec Model 31, Columbus, OH) had 10 V excitation, 2 mV/V output and a load capacity of 250 pounds. The calculated full scale accuracy was 0.15% with 0.15% linearity and hysteresis.

The hoof tester was manually applied to the solar surface of each forefoot in the traditional fashion [377]. Gradually increasing force over 2 seconds was applied at 16 separate loci on the solar and posterior surfaces of each forefoot (Figure 28). The force in Newtons required to produce a ***hoof withdrawal response*** by the laminitic horse was designated the ***hoof compression threshold*** (***HCT***). A 'response' to hoof compression was considered painful when the hoof was withdrawn from the examiner's hand or a contraction of the antebrachial musculature was noted by the examiner and an independent observer. The examiner terminated hoof compression at the initiation of the withdrawal response to avoid tissue damage. Loci that did not result in hoof withdrawal or muscle contraction by the end of the 2 second compression were deemed 'non-responsive'. The peak analog output (mV) of the hoof tester transducer was converted to Newtons and recorded for each locus by a computer (Commodore 64, England) equipped with customized software designed to test nociceptive thresholds.

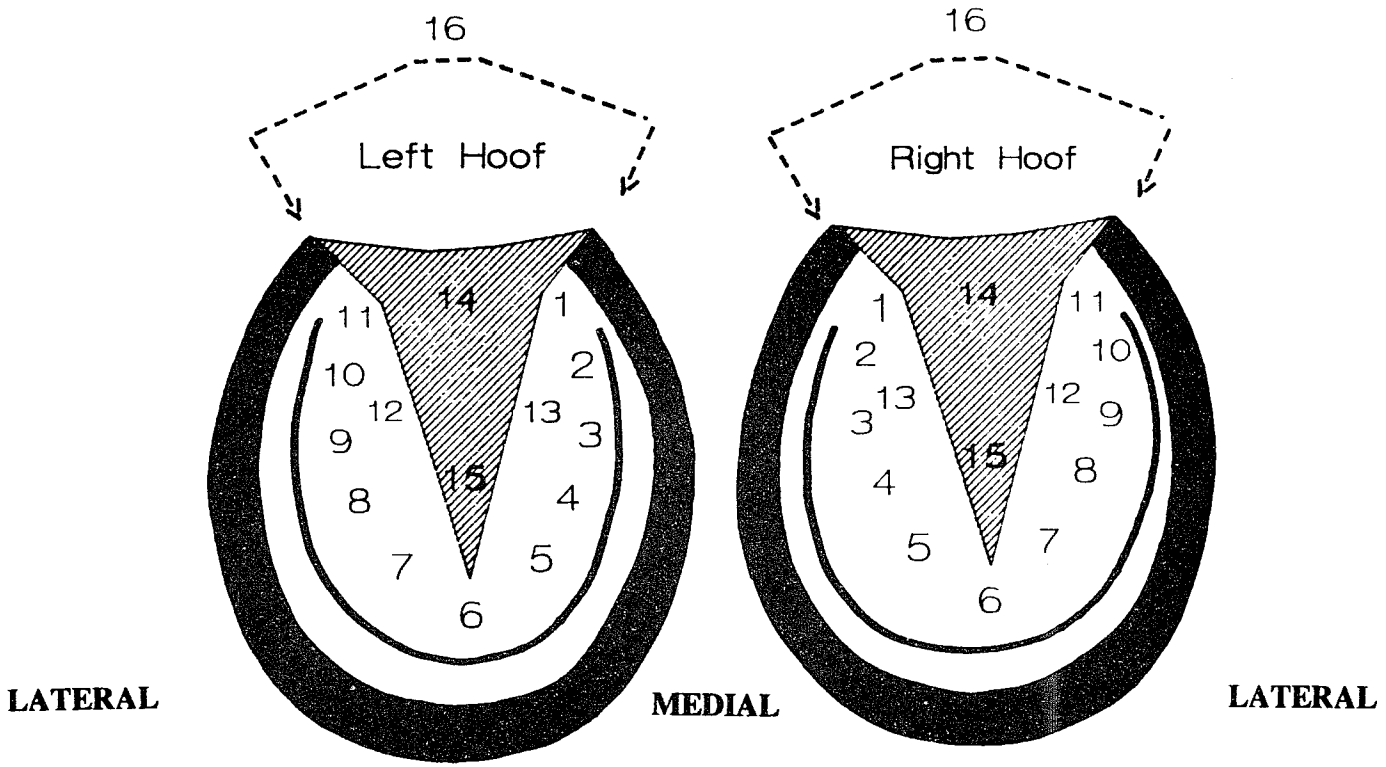


Figure 28: Diagram showing the position of the 16 loci tested on the solar surface of the left and right forefeet of the laminitic horses. Locus 16 is tested by applying the hoof tester to the medial and lateral heel bulbs.

During baseline hoof testing sessions, the computer recorded the peak force applied to each locus and an independent observer entered (into the computer) whether the locus was 'responsive' or 'non-responsive'. All loci were tested again at 3, 6 and 24 hours after drug (or saline) administration. However, in the responsive loci, HCTs were elicited using a compression cutoff of 50% above baseline. This was calculated by the computer for each baseline locus which was 'responsive' to hoof compression. The computer was equipped with an audible bell that signaled when 50% above baseline HCT had been reached so that the examiner could terminate compression. Therefore, post-treatment compression in the 'responsive' loci was terminated either when a withdrawal response was initiated by the horse or the cutoff value was reached. 'Non-responsive' loci received approximately the same pressure on post-treatment sessions as baseline, unless the loci became responsive in a post-treatment session. In that case compression was terminated when a withdrawal response was initiated.

The number of ***responsive (pain sensitive) loci*** on each foot was recorded during each hoof test session and expressed as a percentage of the total number of loci tested per horse. A subjective assessment of the ***hoof withdrawal response*** following hoof compression was made by the examiner. A subjective grade of 0 to 4 was used to indicate the weakest to strongest response as follows:

Grade 0: No response to hoof compression.

Grade 1: The horse contracted the antebrachial musculature with mild extension of the shoulder, but the foot was not moved appreciably.

Grade 2: The horse extended the shoulder and briefly attempted to withdraw the foot from the examiners grasp.

Grade 3: Moderate excursion of the limb and foot occurred, but the foot was not removed from the examiners grasp.

Grade 4: The horse quickly and forcefully withdrew the limb and attempted to place the foot on the ground.

c. Evaluation of lameness in laminitic horses

Lameness was assessed (***lameness score***) immediately before each hoof test session according to a modified Obel scale [200] as follows:

Grade 1: The horse exhibited a normal gait at a walk. The trot showed a shortened stride with an audible cadence abnormality, but showed even head and neck lifting for each foot.

Grade 2: The walk was stilted, but showed no abnormal head or neck lifting. The trot showed obvious lameness with uneven head and neck lifting. A forefoot could be lifted off the ground easily.

Grade 3: The lameness was obvious at a walk and trot. The horse resisted attempts to have a forefoot lifted and was reluctant to move.

Grade 4: The horse experienced difficulty bearing weight at rest or was very reluctant to move.

Preliminary studies indicated that this scale was not sensitive to subtle changes in lameness that occur over time in laminitic horses *e.g.*, head and neck lifting becoming more/less pronounced. Further, numerical rating scales consisting of only 4 lameness

grades are known to be less sensitive compared to other grading schemes [424]. Consequently, the above enumerated scale was expanded to include 3 divisions per grade by adding a + or - to each lameness grade to indicate the degree of severity associated with the score. Therefore, this modified lameness scale ranged from 0 (sound) to 12 (non-weight bearing).

4. Eicosanoid determination

a. Extraction

Plasma obtained from digital venous blood was extracted using a liquid/liquid extraction technique prior to determination of PGE_2 and LTB_4 by enzyme-linked immunosorbent assay (ELISA) [333]. Four ml were extracted for both the LTB_4 and PGE_2 assays. All samples were processed in duplicate. Plasma was acidified and then three volumes of ethyl acetate were added to extract the eicosanoids. After vortexing, centrifugation and separation of aqueous and organic layers, the ethyl acetate was evaporated under nitrogen. Samples were then dissolved in phosphate buffer just prior to the ELISA procedure. For a more complete description of the extraction procedure and recovery information refer to Chapter 3.

b. ELISA

Quantitation of plasma eicosanoids was achieved using commercially available PGE_2 and LTB_4 ELISA kits (Advanced Magnetics, Cambridge, MA) [425]. These assays were based on the principle of a competitive ELISA where PGE_2 or LTB_4 in the sample competed with fixed amounts of alkaline phosphate labelled PGE_2 or LTB_4 for binding to the specific rabbit antibody bound to the microtiter well. Results were

determined by means of a Dynatech MR5000 ELISA micro-titer plate reader (Dynatech Laboratories, Alexandria, VA). Absorbance (read at 410 nm) was correlated with concentration by means of a standard curve ranging from 10 to 5000 pg/ml. Quantitation of unknown samples was achieved by averaging the absorbance of sample duplicates and calculation of the concentration from the standard curve. All sample concentrations were then corrected for extraction efficiency. For a more complete description of the ELISA procedure see Chapter 3.

5. Statistical design and data analysis

a. Eicosanoid concentrations

PGE_2 and LTB_4 plasma concentration data were analyzed using a univariate analysis of variance for a split-plot design [389] where group (normal or laminitic) constituted the main plot and status (resting digital vein or post-exercise digital vein) was the subplot [386,387]. When indicated, multiple comparisons between groups or status categories were performed using a Tukey's *w* Procedure [388]. Differences between groups or status categories were considered significant when $P < 0.05$.

b. Nociceptive thresholds and lameness grade

Each laminitic test subject received ketoprofen (2.2 mg/kg and 3.63 mg/kg), phenylbutazone (4.4 mg/kg) and saline intravenously according to a Latin square design [385]. Horses received one treatment per week for four weeks. The study was performed in a double-blind fashion as neither the person performing the analgesic tests nor the injector were aware of the treatment.

To minimize variance in pain thresholds over experimental days, all data were expressed as percent change from baseline for each horse at each post-treatment time within a given session. In addition, HCT (for responsive loci) and subjective grade of hoof withdrawal reaction data were expressed as an average over all baseline responsive loci for each horse at the post-treatment times. Data were analyzed using one-way analysis of variance (ANOVA) for repeated measures [386,387]. When indicated by ANOVA, multiple comparisons were performed using a Tukey's *w* Procedure [388]. Differences between treatments at each time were considered significant when $P < 0.05$.

C. Results

1. Digital vein eicosanoid concentrations

Mean digital vein PGE_2 and LTB_4 concentrations are shown in Figure 29. Within each group (normal and laminitic), there were no differences in resting and post-exercise PGE_2 digital vein concentrations. Between groups, there were no differences in digital vein concentrations of PGE_2. The mean PGE_2 digital vein concentration for the two groups was 187.18 pg/ml. For the LTB_4 digital vein concentrations, there were no differences in resting and post-exercise levels within groups (normal and laminitic). As with PGE_2, there were no differences in digital vein concentrations of LTB_4 between groups. The mean LTB_4 digital vein concentration for the two groups was 74.71 pg/ml.

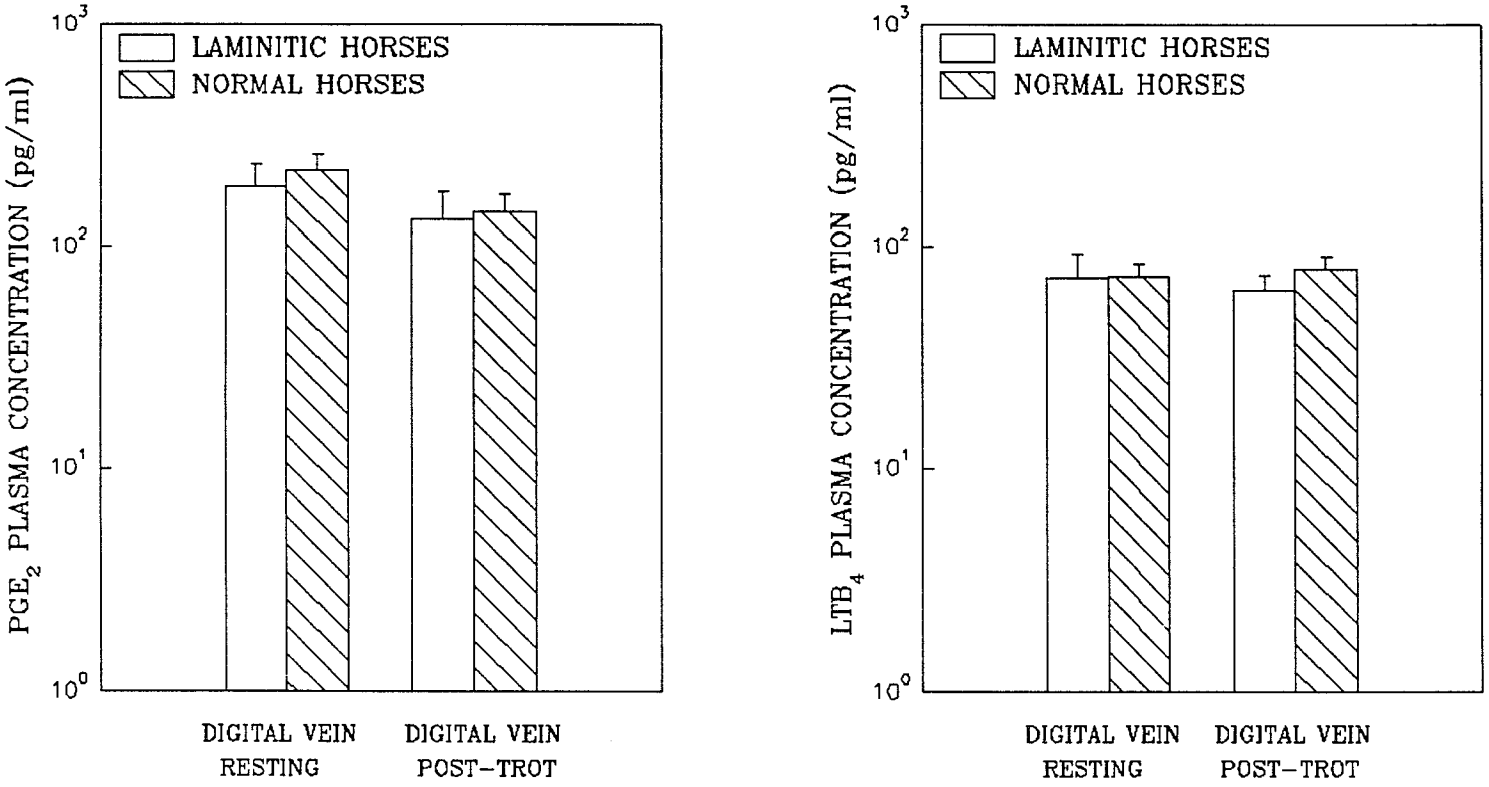


Figure 29: Mean plasma PGE_2 and LTB_4 concentrations in pg/ml ($\pm$ SEM) obtained from digital vein of 6 normal and 6 laminitic horses at rest and after exercise.

2. Nociceptive thresholds and lameness grade

The effect of NSAIDs and saline on HCTs in horses with chronic laminitis is shown in Figure 30. Ketoprofen (3.63 mg/kg) significantly increased the HCT as compared to saline at 3 and 6 hours post-treatment. At 3 and 6 hours after treatment, the horses administered this dose of ketoprofen tolerated an average increase in compressive force (as compared to baseline) of 19.74% and 23.35%, respectively. Even though there were significant effects as compared to saline, there were no differences between NSAIDs at 3 or 6 hours.

In order to determine the degree of variation in compression applied to a non-responsive locus over the course of time, the coefficient of variation (CV) of non-responsive loci HCT's elicited during the experimental day was determined. This value was obtained for each of 4 non-responsive loci in 4 horses by obtaining the mean and standard deviation of HCTs elicited at hoof test sessions before treatment and at 3, 6 and 24 hours after treatment. An overall CV was calculated by averaging all individual CVs across horses. Non-responsive locus CVs for an experimental day ranged from 1.18% to 9.50%. The overall average CV for a total of 16 loci in 4 horses was 3.81% with a standard deviation of 2.30.

The percentage of responsive loci per horse was unaffected by any treatment at 3 hours after drug dosing (Figure 31). However, at 6 hours all NSAIDs produced a lower percentage of responsive loci as compared to the saline control (29.49%). By 24 hours, ketoprofen at 3.63 mg/kg (-3.87%), and phenylbutazone (-2.50%) continued to produce a significantly lower percentage of responsive loci as compared to saline

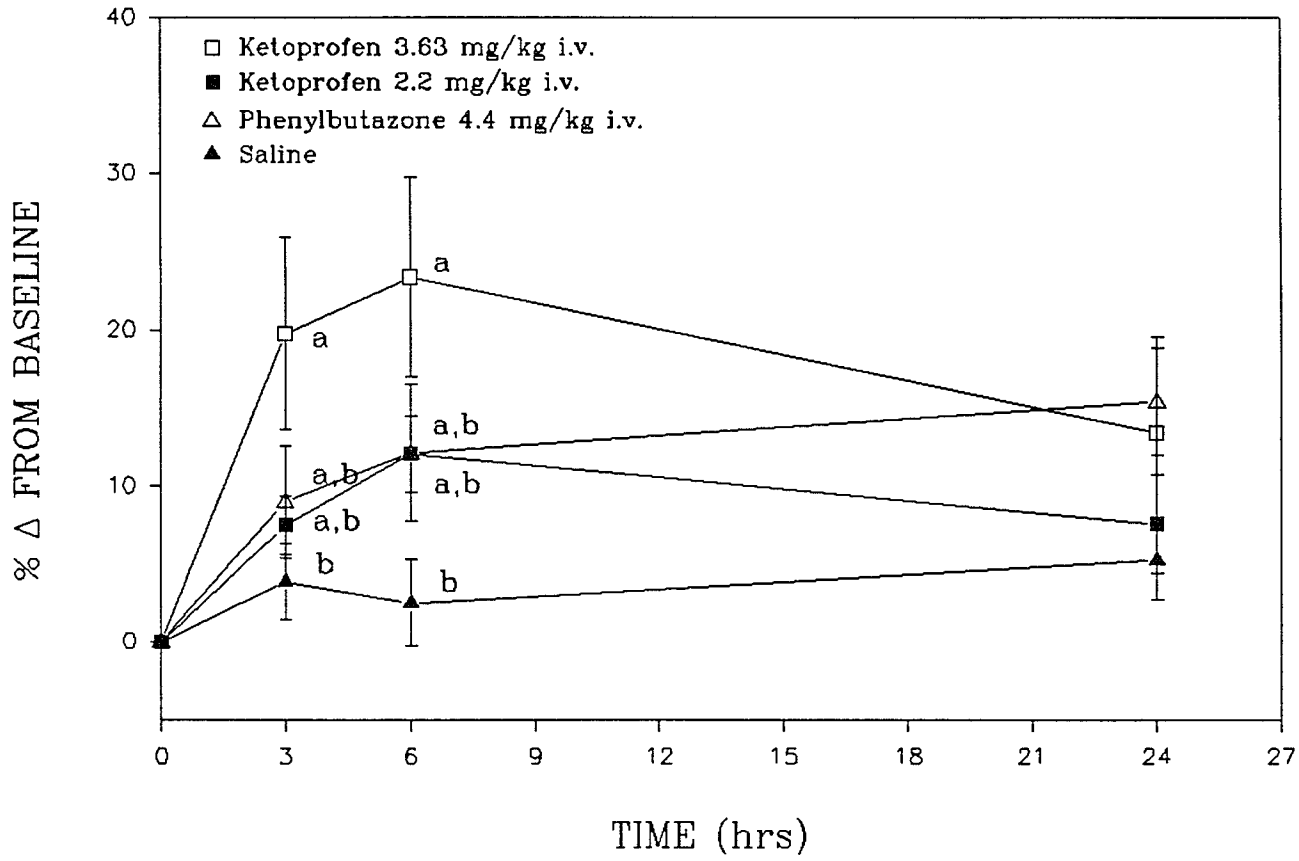


Figure 30: Effect of NSAIDs and saline on mean hoof compression thresholds (± SEM) in 7 laminitic horses. Dissimilar superscripts indicate statistically significant differences in treatments at $P < 0.05$.

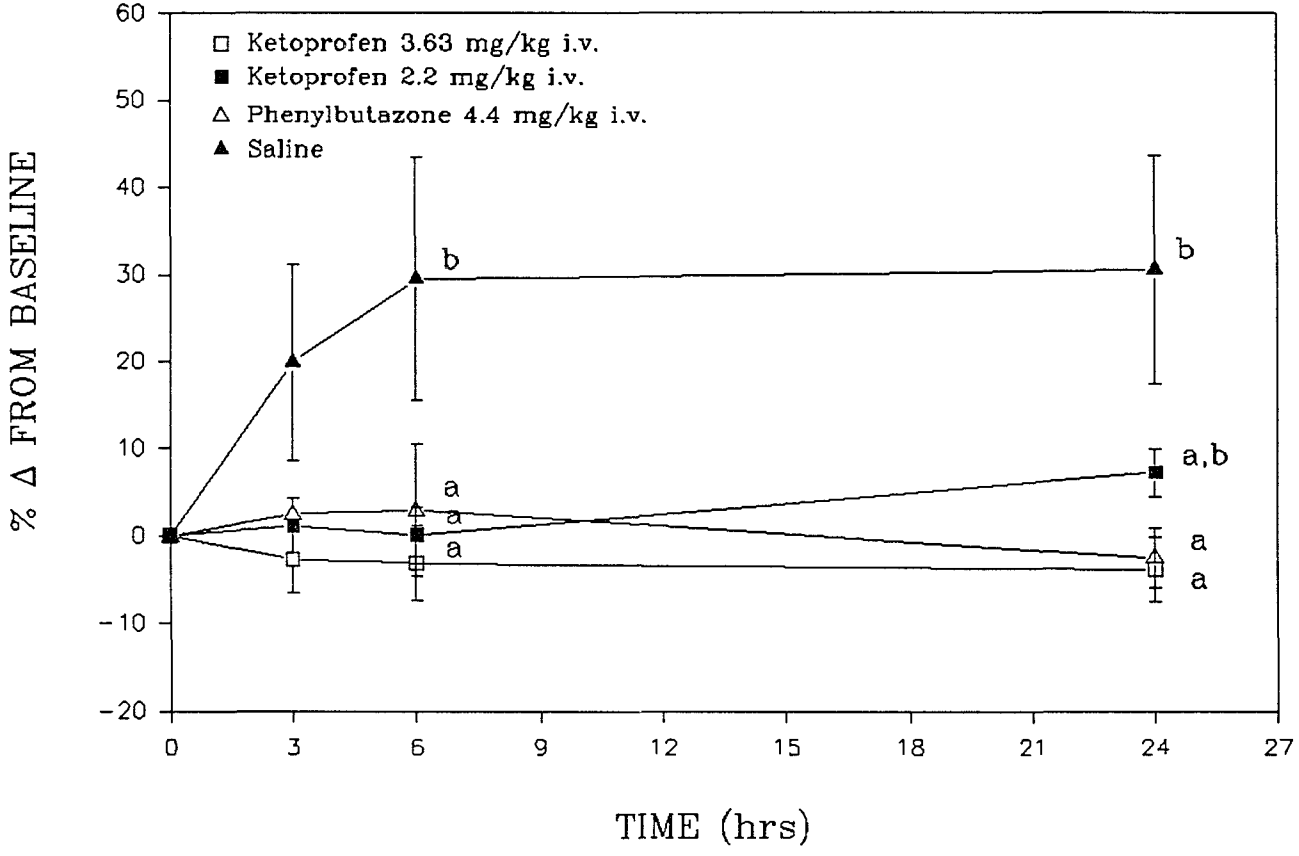


Figure 31: Effect of NSAIDs on mean percentage of responsive or pain sensitive loci (± SEM) in 7 laminitic horses. Dissimilar superscripts indicate statistically significant treatment differences at $P < 0.05$.

(30.48%), with apparent recovery in the horses treated with 2.2 mg/kg of ketoprofen (7.19%). There were no differences among NSAIDs at 6 or 24 hours. The number of responsive loci in horses administered saline increased over time while there was very little change in horses administered NSAIDs.

The 3.63 mg/kg dose of ketoprofen (-8.25%) reduced the subjective grade of hoof withdrawal response at 3 hours when compared to saline (9.89%). All NSAIDs reduced this grade at 6 hours (Figure 32). By 24 hours, only the 3.63 mg/kg dose of ketoprofen (-9.63%) had reduced the grade of withdrawal response as compared to saline (11.64%). There were no differences among NSAIDs at any of the times tested.

At 3 hours post-treatment, there were no significant effects on the lameness grade (Figure 33). By 6 hours, only the 3.63 mg/kg dose of ketoprofen (-21.43%) reduced the lameness grade when compared to saline (14.29%). There were no significant differences between individual NSAIDs. This effect continued through the 24 hour post-treatment period.

D. Discussion

In the 7 horses with chronic laminitis, the digital vein concentrations of PGE_2 and LTB_4 were not significantly different from corresponding concentrations in the 6 normal horses. Further, there was no difference in the digital vein concentrations of PGE_2 and LTB_4 after a brief exercise period in any of the horses. These results show that horses with hoof pain and lameness from chronic laminitis do not have an

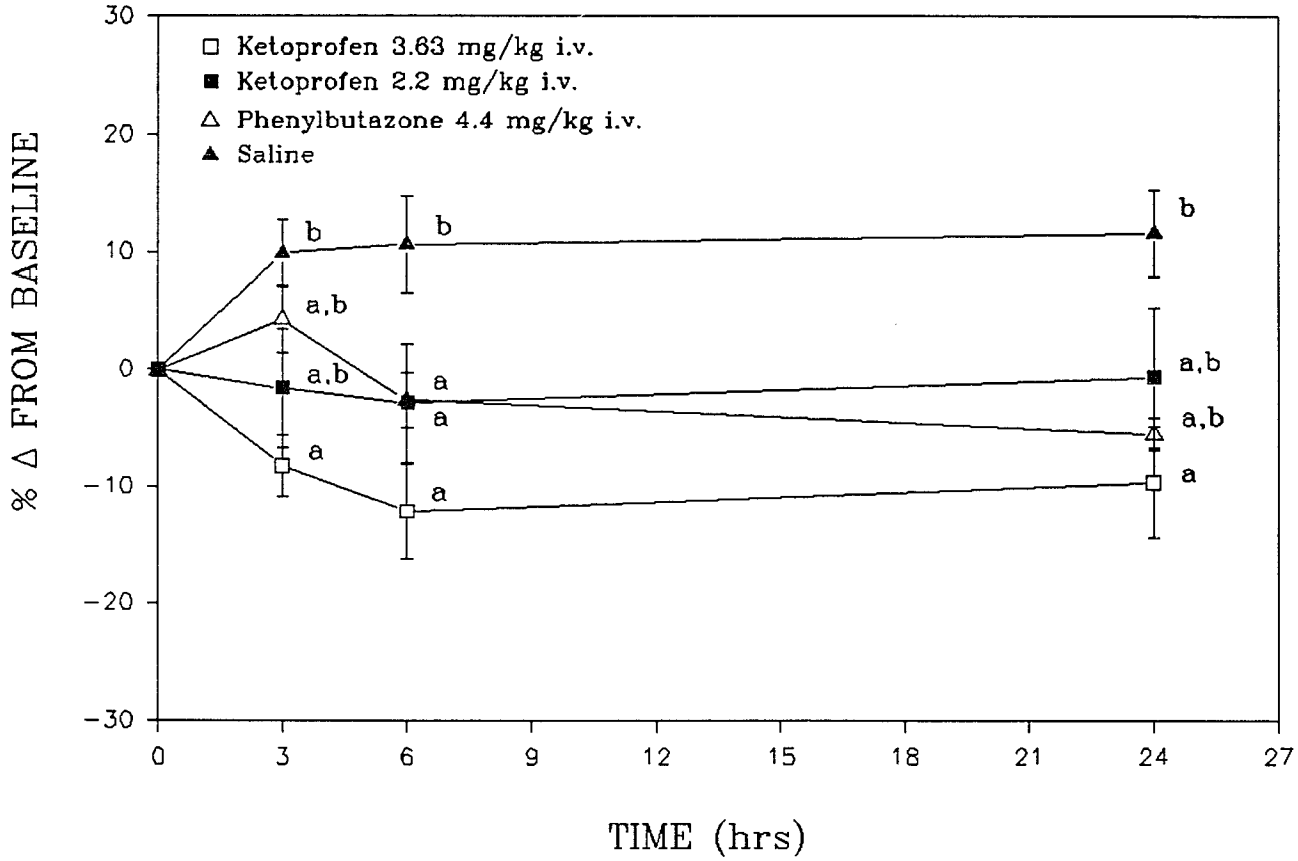


Figure 32: Effect of NSAIDs and saline on mean subjective grade of hoof withdrawal response (± SEM) in 7 laminitic horses. Dissimilar superscripts indicate statistically significant treatment differences at $P < 0.05$.

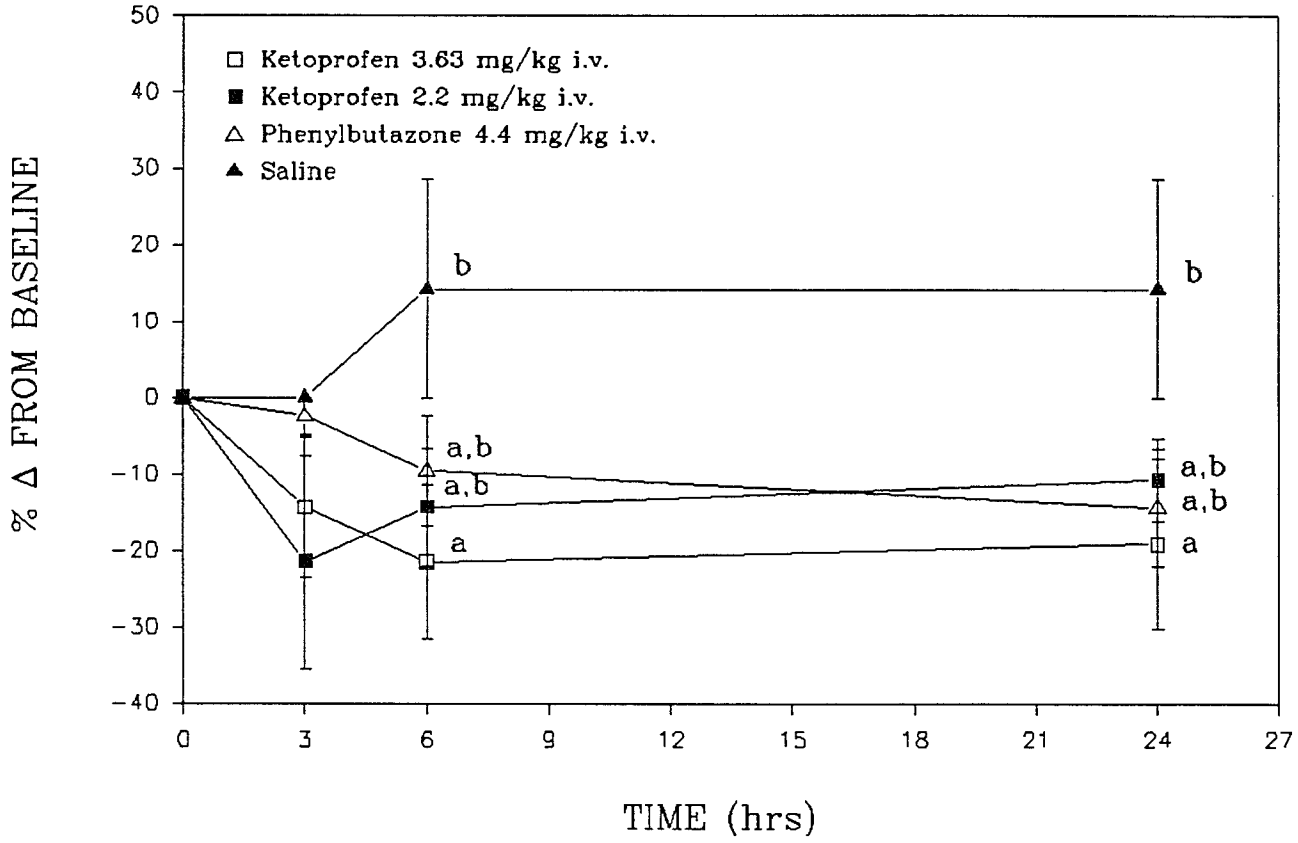


Figure 33: Effect of NSAIDs and saline on mean lameness grade (± SEM) in 7 laminitic horses. Dissimilar superscripts indicate statistically significant treatment differences at $P < 0.05$.

increase in the plasma eicosanoids in the venous drainage of the foot as compared to normal horses even after mild concussion to the foot. These data do not support the hypothesis that eicosanoids play a role in mediating the pain and pathology of chronic laminitis. However, it must be noted that eicosanoids were not measured in laminar tissue which is the site of pathology within the foot. It may be that tissue concentrations were not sufficiently elevated to increase levels in the venous blood. Other researchers have been able to demonstrate increased circulating levels of eicosanoids with a local disease state. An increased plasma leukotriene concentration has been reported in children with asthma as compared to healthy subjects [426]. Similarly, significantly higher than normal venous plasma prostaglandin and thromboxane levels have been described in calves [427] and humans with lung disease [428]. Further, plasma PGE_2 [140] and serum LTB_4 [141] levels of patients with rheumatoid arthritis are reportedly higher than normal controls and the time course of changes of PGE_2 in blood plasma levels reflects the disease dynamics [140]. It is possible that eicosanoids may play a role only in the acute phase of the laminitis where hoof pain and the circulatory changes are more pronounced [220].

The overall average PGE_2 level in the digital veins of normal and laminitic horses was 187.18 pg/ml. Plasma PGE_2 levels obtained from other species which range from 14.35 to 517 pg/ml [333,349,427,429]. The mean LTB_4 level in digital plasma as determined in normal and laminitic horses was 74.71 pg/ml. This is approximate to the plasma levels in humans which are reported to be less than 100 pg/ml [430]. It should be noted that eicosanoid concentrations vary considerably

depending on the sampling site, species, extraction and method of quantitation. It is theorized that actual circulating eicosanoid levels do not exceed 2 pg/ml [24]. Therefore, even minor trauma occurring during collection of blood plasma may result in *ex vivo* formation of eicosanoids which accounts for the wide range of concentrations reported [24]. These reported values then cannot be regarded as absolute and should only be used for comparison when obtained under identical circumstances.

One of the objectives of this study was to correlate the severity of hoof pain in these laminitic animals with the eicosanoid concentrations, provided that there were higher eicosanoid concentrations in laminitic versus normal horses. These horses were then to be administered ketoprofen and phenylbutazone in order to determine the magnitude and time course of the eicosanoid inhibitory effects of these NSAIDs. This objective could not be accomplished due to the lack of significant eicosanoid differences in laminitic versus normal horses

Although the pain and lameness in these laminitic horses could not be definitively attributed to eicosanoids, both effects were reduced by the systemic administration of NSAIDs. Statistically significant overall treatment effects were observed for all measures of nociceptive threshold and lameness. However, ketoprofen at a dose of 3.63 mg/kg (1.65 times the approved therapeutic dose) produced a more pronounced and longer lasting reduction in most of the objective and subjective indices of pain and lameness. For lameness grade and HCT, the high dose of ketoprofen produced the only significant treatment effects that were different from

saline upon multiple comparison. This dose of ketoprofen produced effects even at 24 hours for 3 of the 4 pain tests, including lameness grade. However, it should be noted that there were no significant differences between the NSAIDs at any of the times for all 4 tests. These treatment means were within $\pm$ 20% of one another. The hoof is somewhat limited in terms of sensitivity as a bioassay for analgesia. This device is better at demonstrating the pain relieving effects of potent central analgesics [425].

In this study, horses that were administered saline became progressively more lame and the number of responsive loci along with the grade of withdrawal response increased over the course of the hoof test session as compared to horses administered NSAIDs. This would indicate that these animals were becoming hyperalgesic (excessive sensitivity to mechanical stimuli) and hyperpathic (an abnormally exaggerated subjective response to painful stimuli) [155]. It is known that NSAIDs usually do not completely block pain, but primarily reverse the hyperalgesia associated with painful conditions [163]. These agents reduce the peripheral afferent discharge from hyperalgesic regions [197,266], and also have central anti-hyperalgesic effects [272,305] that may not be dependent on eicosanoid inhibition [258,276]. The results of this study indicate that NSAIDs reduced the hyperalgesia associated with chronic laminitis.

Ketoprofen is approved at a intravenous dosage rate of 2.2 mg/kg, once daily. This dose of ketoprofen did not produced any significant effects on hoof pain or lameness at 24 hours. This would not support the recommended once daily dosing

interval for ketoprofen at the 2.2 mg/kg dose. It appears that laminitic horses might benefit from a slightly increased dosage rate of ketoprofen. This dose was not associated with any apparent clinical toxicity in the animals in this study. Although more extensive toxicity studies are needed, one study has shown that up to five times the therapeutic dose (11.0 mg/kg,IV) produced no evidence of toxicity after 15 days [309].

This study also served to further evaluate the electronic hoof tester and the model of chronic pain in horses. There was close agreement among the data from all 4 pain tests which supports the accuracy and validity of the subjective measures of hoof pain and lameness (subjective grade of hoof withdrawal, lameness score) as compared to the objective measures (HCT, number of responsive loci). The degree of variation in compression applied to a non-responsive locus as determined by the CVs of non-responsive loci HCT's elicited during the experimental day was minimal. This indicates that the compression applied to a particular loci was consistent over time.

CHAPTER 7

GENERAL CONCLUSIONS

Carrageenan-induced synovitis of the equine carpus proved to be self-limiting and reproducible model of acute inflammation. The synovitis was associated with quantifiable increases in prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) concentrations in synovial fluid along with other measurable joint inflammatory responses. PGE_2 concentrations rose dramatically over time with peak levels occurring at 9 hours, with gradual decreases to near baseline at 48 hours. LTB_4 concentrations in synovial fluid peaked early in the inflammatory process at 3 hours then quickly returned to baseline. The primarily neutrophilic cellular influx into the synovial fluid was extensive, with peak levels occurring from 6 to 9 hours. These findings are consistent with other forms of carrageenan-induced inflammation. The inflammation was apparently non-septic and transient as horses returned to soundness by 48 hours with no subsequent clinical signs of degenerative joint disease.

Phenylbutazone decreased the concentrations of PGE_2 from 6 to 12 hours post-dose, whereas the therapeutic and equimolar doses of ketoprofen were associated with more short lived prostaglandin inhibitory effects. There was no ketoprofen dose-response relationship observed for prostaglandin concentrations or any of the other parameters. Further, the therapeutic dose of ketoprofen inhibited prostaglandin production to a greater extent than the higher dose at 6 and 9 hours post-dose. This may in part be due to the small difference in the two doses chosen and the variability

associated with PGE_2 measurement. Future studies using ketoprofen at doses of 4.4 mg/kg and higher, and perhaps *in vitro* experiments on the effect of ketoprofen on prostaglandin metabolizing enzymes, would possibly clarify this effect.

Although ketoprofen and phenylbutazone had no significant influence on synovial fluid LTB_4 concentrations, all drugs appeared to elevate the levels as compared to saline at the time of peak increases. Phenylbutazone appeared to have the greatest potentiation followed by the therapeutic dose of ketoprofen. This effect has been reported for many nonsteroidal anti-inflammatory drugs (NSAIDs) and has been attributed to a diversion of arachidonic acid substrate after cyclooxygenase inhibition to the lipoxygenase pathway. Based on these experiments in this study, ketoprofen was not an inhibitor of lipoxygenase. Other reports have both confirmed and refuted these results, as inhibition of cyclo- and lipoxygenase enzymes by NSAIDs varies depending on the dose, species, metabolite measured and the model of inflammation. These studies do support data from other species suggesting that leukotrienes are involved in joint inflammation, and development of drugs which inhibit their synthesis may be of therapeutic value.

Phenylbutazone was more effective than ketoprofen in alleviating many of the clinical signs of pain and inflammation in this model of acute synovitis. Phenylbutazone had activity lasting as long as 24 and 48 hours post-dose for some of these measures of joint inflammation.

The plasma half-life of the therapeutic dose of ketoprofen was less than one hour. Horses with acute synovitis had significantly shorter plasma half-lives than

normal horses. This can be attributed to the significantly higher synovial fluid concentration of ketoprofen in horses with acute synovitis. In addition, ketoprofen may have been sequestered in the inflamed joint as the synovial fluid levels were greater than plasma concentrations at 3 hours post-administration. Synovial fluid levels of ketoprofen in horses with acute synovitis were detectable from 1 to 9 hours post-dose while this dose produced significant anti-inflammatory activity from 3 to 9 hours. Data from other studies suggest that ketoprofen and other NSAIDs may bind to tissue components for substantial lengths of time in a dose-dependent manner. Such data may help to explain the relatively long duration of action in comparison to the short plasma half-life of these drugs.

Digital vein eicosanoid concentrations were not different between horses with chronic laminitis and normal horses. These data do not support the hypothesis that eicosanoids play a role in mediation of the pain and pathology of chronic laminitis. However, eicosanoids may play a role locally in the laminar tissue without producing measurable concentrations in the digital vein. Alternatively, these compounds may be released during the acute phase of the laminitis where hoof pain and circulatory changes are more pronounced. Although the pain and lameness in the laminitic horses could not be attributed to eicosanoids, both effects were reduced by the systemic administration of NSAIDs at doses which reduced PGE_2 in synovial fluid. The high dose of ketoprofen produced a more pronounced and longer lasting reduction in most of the measures of hoof pain and lameness than the other NSAIDs. This dose produced effects lasting for 24 hours for most of the pain tests, including lameness

grade. The therapeutic dose of ketoprofen was effective in 2 of the 4 pain tests at 6 hours. Horses administered saline became progressively more lame and had increasing hoof pain over the course of the experimental session as compared to horses administered NSAIDs. These results indicate that rather than producing analgesia, these NSAIDs reduced the hyperalgesia associated with chronic laminitis. This effect has been well documented for NSAIDs and may be independent of eicosanoid inhibition. The severity of hoof pain could not be correlated with eicosanoid concentrations in the horses with chronic laminitis due to the lack of significant differences in normal and laminitic horses.

Procedures for extraction and measurement of PGE_2 and LTB_4 equine plasma and synovial were evaluated. The solid phase extraction procedure that was developed resulted in adequate recovery for both compounds from equine biological samples. An enzyme-linked immunosorbent assay (ELISA) was used for quantitation of LTB_4 and PGE_2 that had not been previously documented in the horse. Available information suggested that considerable cross reactivity with PGE_1 occurred in the PGE_2 ELISA while the LTB_4 was very specific. The results from the validation procedures for the PGE_2 ELISA indicated that the assay was specific for this compound in equine synovial fluid.

In conclusion, the results of these studies indicate that NSAIDs differ in potency and efficacy depending on the type of pain and inflammation present. In acute experimentally-induced synovitis, where the duration of clinical signs lasted approximally 48 hours, phenylbutazone was more potent in relieving the joint

inflammation and pain than either the therapeutic or the equimolar dose of ketoprofen. In naturally occurring chronic laminitis, where horses experience pain and pathology within the foot for months or years, the high dose of ketoprofen was more potent than either the therapeutic dose of ketoprofen or the equimolar dose of phenylbutazone.

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VITA

Jane Granville Owens was born in Pikeville, Kentucky on December 26, 1963. She attended Pikeville High School from which she graduated in May, 1981. She enrolled in the University of Kentucky, Lexington and received a Bachelor of Science in Animal Science in May 1985. She then entered the School of Veterinary Medicine at Tuskegee University. She graduated with a Doctor of Veterinary Medicine in May, 1989. After practicing as a resident veterinarian on a horse farm in Kentucky, she was awarded the Risen Star Fellowship by the School of Veterinary Medicine, Louisiana State University. Her studies were guided by Dr. Steven Kamerling, Associate Professor, Department of Veterinary Physiology, Pharmacology and Toxicology. She completed the Ph.D. degree with a major in veterinary pharmacology in May 1994.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Jane Granville Owens

Major Field: Veterinary Medical Sciences

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Approved:

Steven G. Kamerling
Major Professor and Chairman

George L. Hajiadis
Dean of the Graduate School

EXAMINING COMMITTEE:

Charles V Short

J W McClure Jr

George M Strain

C Venugopalan

Date of Examination:

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