STUDIES WITH NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

by

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To Ian

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DECLARATION

The estimation of serum thromboxane inhibition after oral administration of flunixin meglumine to dogs was carried out by Professor Peter Lees of London University. Platelet numbers were estimated by the staff of the Department of Veterinary Pathology at he University of Glasgow Veterinary School. Otherwise the contents of this thesis are the work of the author.

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Galbraith, E.A. and McKellar, Q.A.
Pharmacokinetics and pharmacodynamics of piroxicam in dogs.
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McKellar, Q.A., Pearson, T., Galbraith, E.A., Boyle, J. and Bell, G.
Kinetics and clinical efficacy of cinchophen and prednisolone combination in the dog.
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SUMMARY

The pharmacokinetics and serum thromboxane inhibition of four non-steroidal anti-inflammatory drugs (NSAIDs) were determined after administration to dogs by the intravenous, subcutaneous and oral routes.

Flunixin meglumine was first administered at three oral dose rates, 0.55, 1.10 and 1.65 mg/kg, to determine a likely suitable dose rate by this route. At each dose rate the mean maximum plasma concentration of drug (C_{max}) occurred between 1 and 2 hours. After 0.55, 1.10 and 1.65 mg/kg the C_{max} increased approximately in relation to the dose rate and values were 2.77 ± 0.63, 5.03 ± 0.99 and 8.17 ± 2.02 µg/ml respectively. The mean area under the plasma concentration versus time curve (AUC) was 9.01, 14.62 and 25.98 µg/ml.h after 0.55, 1.10 and 1.65 mg/kg. Maximum mean inhibition of serum thromboxane (TxB_2) was 91.47, 98.84 and 97.31 % and occurred between 1 and 2 hours. The area under the TxB_2 inhibition versus time curve was not linearly related to dose rate and was determined to be 5238, 5952 and 6169 %.h respectively for 0.55, 1.10 and 1.65 mg/kg dose rates . On the basis of the findings from the oral experiments the 1.10 mg/kg dose rate was selected for study after intravenous and subcutaneous administration.

The decline in plasma concentration of flunixin meglumine after intravenous administration was best described by a bi-exponential equation. The mean half life of elimination ($t^1/_2ß$) in dogs was 2.97 hours and the mean volume of distribution at steady state (Vd_{ss}) was 189.23 ± 46.70 ml/kg. The AUC from observed values after administration at a dose rate of 1.10 mg/kg was 20.47 ± 2.60 µg/ml.h.

Determination of the AUC after intravenous administration allowed calculation of the bioavailability (F) after oral administration. After normalisation for dose rate the bioavailability after oral administration of flunixin to dogs was 74.8, 60.66 and 69.8 %.for the 0.55, 1.10 and 1.65 mg/kg dose rates respectively, thus indicating that absorption was approximately linearly related to dose rate administered.

The maximum mean inhibition of serum TxB_2 after intravenous administration was 99.87 % (0.5 hours), and the area under the TxB_2 inhibition versus time curve was 2148 %.h, approximately 36 % of the area under the TxB_2 inhibition versus time curve obtained after the equivalent dose rate administered by the oral route.

After subcutaneous administration at a dose rate of 1.10 mg/kg the C_{max} of flunixin in plasma was 6.36 µg/ml, and this occurred at 0.92 hours. Bioavailability was excellent after subcutaneous administration, the AUC being 26.30 ± 4.73 µg/ml.h (F=101.09 %).

The maximum mean inhibition of TxB_2 after subcutaneous administration was approximately equal to that after intravenous administration (99.73 % at 1 hour), as was the
area under the TxB_2 inhibition versus time curve. This AUC was also approximately half of the AUC produced by the oral administration of the same dose rate.
No adverse reactions were observed after administration of flunixin meglumine to dogs.

After administration at a dose rate of 0.3 mg/kg by the intravenous route to dogs, the decline in plasma concentration of piroxicam was best described by a single exponential equation. The fit of the mathematically modelled best fit curve was very poor for all animals. The $t^1/_2\beta$ was calculated to be 40.16 hours and the Vd_{ss} calculated from observed data was 178.37 ml/kg. The observed AUC of piroxicam after intravenous administration was 47.39 µg/ml.h. The maximum mean inhibition of serum TxB_2 was 96.85 % and this occurred at 0.25 hours after drug administration. The area under the mean TxB_2 inhibition versus time curve was 5309 %.h.
The C_{max} of piroxicam after oral administration at a dose rate of 0.3 mg/kg was 1.35 ± 0.11 µg/ml, and occurred at 3.33 (± 1.09 hours). The AUC was 46.77 µg/ml.h showing that the oral bioavailability of piroxicam was excellent (F = 102.69 %). The mean maximum inhibition of serum TxB_2 occurred at 1 hour and was lower than that measured after intravenous administration (72.45 ± 3.38 %). The AUC for mean TxB_2 inhibition versus time was approximately 77 % of that after intravenous administration (4067 %.h).
The intravenous administration of piroxicam was complicated by its poor aqueous solubility and the need to administer it in ethanol. Some symptoms typical of ethanol intoxication were noted after intravenous administration, however these did not persist beyond the first 30 minutes after drug administration.
Large decreases in the number of blood platelets were also detected in dogs after intravenous administration of piroxicam. Although some improvement was noted throughout the sampling times, numbers had not returned to normal by the final sampling times. It is unlikely that this effect was as a result of piroxicam administration as a similar effect was not observed after oral administration of the drug. It is possible that the decrease in platelet numbers was a result of ethanol administration. However, previously recorded ethanol induced thrombocytopenia has only been been associated with chronic alcohol consumption.
Piroxicam was otherwise well tolerated after administration to dogs by the intravenous and oral routes.

Cinchophen was administered to dogs in combined preparations which contained prednisolone. Both intravenous and oral administration were at a dose rate of 12.5 mg/kg for cinchophen. As proportions of cinchophen and prednisolone differed in the intravenous and oral preparations, this resulted in dose rate of 0.15 and 0.06 mg/kg for prednisolone.
The decline in plasma concentration of cinchophen after intravenous administration was best described by a bi-exponential equation for four of the six animals. The plasma concentration versus time curves for dogs 5 and 6 were best described by a single exponential equation. The profile of the curves however, suggested that a proportion of the drug may have been injected peri-venously although there was no indication of this at the time of drug administration. Pharmacokinetic values from these animals have been excluded from mean values. The $t^1/_2\beta$ of cinchophen after intravenous administration was 6.84 hours and the Vd_{ss} was 109.73 ± 6.88 ml/kg.The AUC calculated from observed values was 1067.76 (± 148.87) µg/ml.h.
Mean maximum inhibition of TxB_2 was 71.28 % and occurred at 10 minutes (0.17 hours). The area under the serum TxB_2 inhibition versus time curve was 865 %.h.
After oral administration the C_{max} of cinchophen was 80.54 (±5.96) µg/ml and occurred at 1.92 ± 0.49 hours. As a result of differences in the final sampling times after intravenous and oral administration it was necessary to calculate the bioavailability over a period of 48 hours. The oral bioavailability of cinchophen based on data from four dogs was 95.93 (± 14.65) %.
The maximum mean inhibition of serum TxB_2 was considerably lower after oral administration than after intravenous administration (50.43 ± 9.29 %) and occurred at 1 hour. Inhibition did not exceed 71 % in any sample. The mean area under the serum TxB_2 inhibition versus time curve was 636 %.h, approximately 73 % of that obtained after intravenous administration.
No adverse reactions were noted after administration of cinchophen by either route.

Tolfenamic acid was administered to dogs at a dose rate of 4.0 mg/kg by the intravenous and subcutaneous routes. After intravenous administration the decline in plasma drug concentration was best defined by a bi-exponential equation, however, in all animals this model was a poor fit. The $t^1/_2\beta$ was 5.91 hours and the observed AUC was 18.10 ± 4.12 µg/ml.h. A comparatively large Vdss was measured (1246.66 ± 293.34 ml/kg). The mean maximum TxB_2 inhibition was 91.30 %, and the area under the serum TxB_2 inhibition versus time curve after intravenous administration was 1857.02 %.h.

After subcutaneous administration a C_{max} of 4.39 (± 0.55) μg/ml was measured at 1.40 (± 0.24) hours and the observed AUC was 29.62 μg/ml.h. In all but one animal the bioavailability after subcutaneous administration was calculated to be in excess of 100 % and the mean value was 165.70 ± 3.10 %. The maximum mean TxB_2 inhibition was slightly lower than that measured after intravenous administration (80.37 %). However, the overall area under the TxB_2 inhibition versus time curve was approximately 1.5 times that after intravenous administration (2784.64 %.h).

The profiles of the plasma concentration versus time curves for flunixin, piroxicam, cinchophen and tolfenamic acid, were typical of those seen for drugs known to undergo enterohepatic circulation.

Serum protein binding of flunixin and meclofenamic acid was measured for dogs, goats and horses. A wide inter-species variation in protein binding was found. Inter-species variation was greatest for meclofenamic acid for which binding was 94, 84 and 100 % respectively for dogs, goats and horses. Protein binding for flunixin in these species was 92, 87 and 87 % respectively.

In vitro inhibition of serum TxB_2 production by flunixin and meclofenamic acid was determined using blood from dogs, goats and horses. Production of TxB_2 was less sensitive to inhibition by flunixin in the dog than in the goat or horse and IC_{50} values of 0.104, 0.018 and 0.036 μM, respectively, were measured. Overall, considerably higher concentrations were required to achieve the IC_{50} in these species using meclofenamic acid. The IC_{50} values calculated for this drug in vitro were 0.707, 0.757 and 0.299 μM respectively, for dog goat and horse.

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ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
ADH	Antidiuretic hormone
ADP	Adensoine diphosphate
AIC	Akaike's Information Criterion
AMP	Adenosine monophosphate
ASA	Acetylsalicylic acid
ATP	Adenosine triphosphate
AUC	Area under the curve
AUFS	Absorbance units full scale deflection
^{o}C	Degrees Celcius
ED_{50}	Effective dose in 50 % of sample
F	Bioavailability
GAGPs	Glycosaminoglycan polysulphated esters
h	Hours
HPLC	High pressure (performance) liquid chromatography
5-HT	5-Hydroxytryptamine
IC_{50}	Concentration which produces 50 % inhibition
kg	Kilogram
l	Litre
LTs	Leukotrienes
LTB_4	Leukotriene B_4
M	Molar
MAT	Mean absorbance time
MN	Mononuclear phagocyte

mg	Milligram
min.	Minute
ml	Millilitre
MRT	Mean residence time
nm	Nanometers
No	Number
NSAID	Non-steroidal anti-inflammatory drug
p	Probability
PG	Prostaglandin
PGI_2	Prostacyclin
pH	Negative logarithm of hydrogen ion concentration of an aqueous solution
pKa	The pH at which half of the functional groups in a solution are charged and half are neutral (for acidic and basic functional groups)
PMN	Polymorphonuclear leukocytes
RNA	Ribonucleic acid
mRNA	Messenger Ribonucleic acid
r	Pearson linear correlation coefficient
SD	Standard deviation of the mean
SEM	Standard error of the mean
$t^1/_2$ ß	Half life of elimination
Tx	Thromboxane
Vc	Volume of the central compartment
Vd	Volume of distribution
Vd_a	Volume of distribution (area)
Vd_{ss}	Volume of distribution at steady state

vs	Versus
μ	Mu
μg	Micrograms
μl	Microlitre

Chapter 1

GENERAL INTRODUCTION

1.1 SOME HISTORIC ASPECTS OF INFLAMMATION

It is thought that the process of inflammation may have first been recognised almost 2000 years ago. Early medical writings from Egypt dating from about 1650 B.C. include a word which can be translated as "inflammation" and which was used to describe wounds. These scrolls are thought to contain material first recorded in writings produced 1000 years earlier. It was not, however, until sometime during the first century A.D. that the major signs of inflammation were set out by Cornelius Celsus, thereby providing an early clinical definition of inflammation.

The four "cardinal signs" - "rubor et tumor cum calor et dolore" (redness and swelling with heat and pain) remain the classical definition to this day. The only change being the addition of a fifth sign "functio laesa" ("disturbed function") by Rudolph Virchow during the 19th century.

Advances in science during the 19th century allowed some progress to be made towards understanding the tissue changes responsible for the appearance of the cardinal signs. The study of the microcirculation of frog mesentry after trauma led Cohnheim (1867) to recognise and describe its role in the pathology of inflammation.

1.2. CHANGES SEEN IN INFLAMMATION

The macroscopic changes - erythema, oedema, tenderness and pain seen as a response to tissue damage are a product of many underlying processes both vascular and cellular (figure 1.1). The precise nature of the processes is complex and is dependant to some extent upon whether the inflammation is acute or chronic.

During acute inflammation characteristic changes at the site of injury include dilation and increased permeability of the small blood vessels resulting in leakage of fluid into the interstitial spaces. Dilation of vessels at the site of injury has been shown to result in an increased blood flow of up to ten times normal levels (Ascheim and Zweifach, 1962) and this response may last for several hours. Small vessels are normally fully permeable to water but only slightly permeable to plasma proteins. As a result of increased permeability, passage of plasma proteins into the extravascular tissue increases (Majno and Palade, 1961) and leucocytes also pass through the walls of small blood vessels in the damaged area (Cohnheim, 1882). Initially, polymorphonuclear leucocytes (PMNs, neutrophils) are most abundant at the site of inflammation. These cells migrate in response to chemotactic factors released from injured cells which exist in a concentration gradient, increasing towards the site of injury. It is thought that PMNs are able to detect

the concentration gradient of chemoattractants by spacial sensing (Zigmond, 1974), that is, by detecting the difference in concentration across the cell's own length. When cells are close to the source of chemotactic chemicals their movement becomes more random due to the absence of a gradient and, although movement continues, it acts to keep the PMNs moving within the area of injury. In addition to stimulation of chemotaxis, increased adhesiveness of PMNs is seen to occur at this time. This aids the escape of cells through vessel walls and later accumulation at the target site (Wilkinson, 1978). On arrival at the site of injury PMNs begin to phagocytose any particulate matter, such as bacteria, which may be present. It is thought that they may also secrete lysosomal contents directly into the area. Some lysosomal discharge can occur during phagocytosis, however even in the absence of phagocytosis secretion of lysosomal content has been noted. This phenomenon, sometimes referred to as 'reverse endocytosis' (Henson, 1971a, Henson, 1972, Weissmann et al, 1972), may occur as a result of leucocyte exposure to immune complexes on a surface which is not capable of being phagocytosed by the cell (Hawkins, 1971, Henson 1971b). Release of lysosomal enzymes has also been noted when certain materials such as crystals of monosodium urate gain access to the vacuolar system of the cell resulting in cell damage and death (Allison,1971). Such episodes are seen to appear in inflammation associated with acute gout (McCarty and Hollander, 1961). Release of lysosomal enzymes aids the digestion of dead tissue cells and the process of suppuration is the extensive manifestation of this process in one area. In addition to the possible benefits of this process, it is known to cause tissue damage and initiate the formation of tissue destructive free radicals of oxygen which in turn results in the formation of lipid peroxides (Oyanagui, 1976, McCord et al, 1979). These highly reactive substances are thought to stimulate the activity of phospholipases resulting in the release of arachidonic acid from phospholipid (Rainsford 1984a). Material which cannot be disposed of in this way is ingested and removed by macrophages. The overall increase in cells within the extravascular space due to vessel fenestration will also facilitate accumulation of cells at the site of inflammation. Additionally, it has been seen in vitro that damaged cells are able to stimulate chemotaxis of leucocytes by the release of chemotactic factors. Cytotoxins are released from viral infected cells (Ward et al, 1972) and actively phagocytosing leucocytes are known to release substances which attract other leucocytes (Keller and Borel, 1971). A further important function of PMNs is presentation of complement to the immune system (Unanue, 1972).

PMNs have a relatively short life span which has been estimated to be 3-13 days in blood and about 24-48 hours at the site of inflammation (Hurley, 1978).

After the initial acute phase of inflammation in which PMNs are dominant, mononuclear phagocytes (macrophages) and lymphocytes become the characteristic cell types. The

profile of cell type at the site of inflammation is distinct and time related. It is not clear if the delayed arrival of mononuclear phagocytes is due to their slower movement or a response to different chemoattractants. Mononuclear phagocytes are predominant in chronic inflammation or in acute inflammation which is resolving. Acute inflammation may be only a preliminary response followed by chronic changes, for instance in conditions such as rheumatoid arthritis. During chronic inflammation the affected area does not return to normal after the initial acute inflammatory response and the principal significant difference is characterised by a variety of immunological events and the production of fibrous tissue.

The precise mechanisms and triggers which result in the development of chronic inflammation are poorly understood. The three mechanisms considered by Dawson and Willoughby (1985) present the current accepted theories and it may be that chronic inflammation results from any one or a combination of these mechanisms. Firstly, it may be that the initial stimulus may be persistent and poorly removed from the body. Secondly, the regulatory substances normally released during the response to invasion or trauma may become abnormal, this may result in a perpetuating response. Finally, the modification of endogenous proteins by physical or chemical means (such as thermal damage or incomplete degradation during phagocytosis) could result in its perception as a foreign protein.

Large numbers of macrophages and lymphocytes at the site of inflammation are characteristic of chronic inflammation, whatever its cause, and consequent stimulation of the immune system occurs. Antigen is presented to T lymphocytes by the macrophages (Unanue, 1972) and proliferation and differentiation of T lymphocytes occurs. T helper cells thus produced, interact with B lymphocytes to produce plasma cells which are responsible for the production of antibody (Dawson and Willoughby, 1985). These events are regulated by Interleukins 1 and 2 which are produced by macrophages and T lymphocytes respectively. If conventional wisdom that inflammation is essentially a protective mechanism is accepted, then it seems likely that chronic inflammation, in many cases, may be a result of a breakdown of this mechanism.

The tissue and cellular changes seen in inflammation are largely mediated or modulated by chemical substances which are naturally produced within the body. The first discovery of the action of chemical mediators in the process of inflammation was made by Lewis and Grant (1924). These workers produced local irritation with vascular dilation and oedema by intra-dermal injection of histamine. More recently, work by Willis (1969) and Di Rosa et al (1971a) indicated that release of mediators in acute inflammation was a sequential process beginning with the release of the histamine and 5-hydroxytryptamine

(5-HT). These amines are produced by enzymatic catalysis of amino acids, principally in mast cells (histamine) and platelets (5-HT), and are stored in intracellular granules (Bowman and Rand, 1984). The effect of these compounds is short lived and their release is followed by the generation of peptides such as bradykinin. Investigation has shown that antihistamines and 5-HT antagonists have little effect in the treatment of inflammation except during the first 90 minutes (Youlten, 1978). Bradykinin has a more prolonged effect, and Ferreira et al (1974) suggested that this step in the sequence may last for at least six hours after the initial damage has occurred.

There is little doubt that prostaglandins play a positive and direct role in inflammation. Inflammatory effects have been noted after intra-dermal, intravenous or intra-arterial injection of prostaglandin (PG) E_2 and prostacyclin (PGI_2) and large doses of PGE_2 and PGF_{2alpha} when given intramuscularly or subcutaneously cause local pain. A synergistic response to prostaglandins in the presence of bradykinin also has been demonstrated (Ferreira, 1972).

The time course of generation of prostanoids during inflammation has been investigated using equine models (Higgins et al, 1987). Maximum concentrations of PGE_2 were detected at 12 hours, 6-keto-PGF_{1alpha} (the stable derivative of PGI_2) at nine hours and thromboxane B_2 (TxB_2), the stable derivative of thromboxane A_2 (TxA_2) at 6 hours.

In addition to histamine, 5-HT, kinins and prostaglandins a further group of pro-inflammatory substances has been identified. These substances are the leukotrienes (LTs) and LTB_4 is thought to play a major role in the cellular chemotactic response which may lead to chronic inflammation. Leukotrienes are produced de novo by the action of lipoxygenase enzymes on the unsaturated carbons of arachidonic acid (Kitchen et al, 1985) (figure 1.2).
Generation of LTs has been shown to be on a similar time scale to PGs. It has been demonstrated that concentrations of LTB_4 reach a maximum at four to eight hours in inflammatory models using rats and ponies (Simmons et al, 1983; Higgins and Lees, 1984a).

Subsequent to these initial cause and effect discoveries, world wide investigation over the last 20 years has resulted in a clear picture of the chemical structure and relationships of many of these substances and those produced as a result of arachidonic acid metabolism are shown in figure 1.2.

Tissue injury results in the release of arachidonic acid (a 20-carbon unsaturated fatty acid) from cell membranes. The generation of arachidonic acid from cell membrane phospholipid is caused by the activation of membrane bound enzyme phospholipase

(Hong and Levine, 1976a and 1976b; Bills et al, 1977). The cell wall associated enzyme may be activated as a result of changes which cause an alteration in permeability of the membrane to calcium ions (Borgeat and Samuelsson, 1979). Several phospholipase types including A_2 (Hong and Deykin, 1981; McKean et al; 1981, Broekman, 1986) and C (Bell et al, 1979; Daniel et al, 1986) have been implicated in the release process. Much of the evidence suggested that phospholipase C was involved in phospholipase A_2 activation (Lambert et al, 1986; Resink et al, 1987). However, studies by Hong and Deykin (1981) and Kaya et al (1989) have shown that activation of phospholipase A_2 may be independent of phospholipase C in some systems and it now seems likely that phospholipase A_2 activation may be mediated by G protein (Burch et al, 1986 and Burgoyne et al, 1987). Arachidonic acid is a pivotal precursor for the synthesis of a large number of substances (collectively known as eicosanoids) and acts as a substrate for cyclooxygenase and lipoxygenase enzymes. The term eicosanoid is derived from eicosatetraenoic acid (arachidonic acid). The action of cyclooxygenase results in the production of prostaglandins and thromboxane whilst the action of lipoxygenase produces leukotrienes (figure 1.2).

The pharmacological effects of eicosanoids are many and vary between tissues and species. The major effects of the eicosanoids produced by the arachidonic acid pathway are shown in table 1.1.

The biologically active products of the arachidonic acid pathway include the prostanoids (PGs and PGI_2) and thromboxane. The most important pro-inflammatory PGs include PGE and PGF and these display a range of properties which are significant in inflammation (table 1.1). The principal prostanoid found in inflammatory exudate is PGE_2 (Anggard and Jonsson, 1971; Greaves et al, 1971; Gould, 1976; Higgins and Lees, 1984b). Eicosanoids, are not stored in the body and many are highly unstable, for example TxA_2 has a biological half life 32 seconds (Salmon and Flower, 1979).

Evidence exists that of the cyclooxygenase products generated by circulating leukocytes, thromboxane is predominant (Morley et al, 1979). Platelets are a major source of thromboxane in clotting blood but are not thought to contribute significantly to the cyclooxygenase products found in experimentally produced inflammation (Higgs et al 1983). It has been shown however, that the presence of thromboxane in inflammatory exudates relates to the presence of migrating leukocytes which are also a lesser source of PGs (Higgs et al, 1983). PMNs are thought to be a major source of PGs in inflammation (Higgs et al, 1975) and are also thought to be a lesser source of thromboxane production (Higgs et al, 1976). The fact that leukocytes are capable of both leukotriene and prostaglandin production has led Higgins (1985) to suggest that this may indicate some

sort of regulatory and perpetuating role in inflammation by the production of substances from each pathway.

Leukotriene B_4 is the principal biologically active product of the lipoxygenase pathway. It is a highly potent chemotactic agent and has been shown to cause PMN accumulation in vivo following intra-peritoneal, intra-dermal or intra-ocular administration (Bhattacherjee et al, 1981; Higgs et al, 1981a). LTB_4 has also been reported to be equipotent to PGs in enhancing exudation of plasma in the presence of bradykinin (Bray et al, 1981) and to have a direct effects on vascular permeability (Higgs et al, 1981b).

1.3. EARLY ATTEMPTS TO CONTROL PAIN

The relief of pain must have been one of man's earliest therapeutic quests, and opium has long been used to this end. Such drugs act on the central nervous system, being agonists of the opioid receptor types (mu, kappa and delta) which are present at various sites within the spinal and other nervous tissue. The quest for the euphoric state induced by these drugs has, however, led to widespread abuse of opium and other narcotic morphine-like drugs. Serious addictions have resulted from repeated administration and as a result use is now restricted to short term relief of severe acute pain, and to patients who have little prospect of recovery.

The first non-narcotic substances used to control pain were salicylates. In Greek and Roman times tree bark was chewed to give pain relief and by the 19th century salicylic acid had been isolated from various plants including the bark of Willow and Poplar trees. Acetylsalicylic acid (ASA) was the first commercially available non-steroidal anti-inflammatory drug (NSAID) and was produced by Hofmann of the Bayer Pharmaceutical Co. in 1893.

1.4. PRESENT DAY ATTEMPTS TO CONTROL PAIN

1.4.1 NON-STEROIDAL ANTIINFLAMMATORY DRUGS

Following the production of salicylic acid and appreciation of its desirable properties, the search began for more potent analgesics with less toxic side effects. This resulted in the development of pyrazolone derivatives such as phenylbutazone, which was produced in 1946. These drugs were supplemented in the 1960s by indomethacin (an acetic acid derivative) and the propionic acid derivatives such as ibuprofen. Figure 1.3 shows the major groups of NSAIDs available today. The term NSAID or "aspirin

like drug" is used to describe the group of drugs which are mainly organic acids and which possess anti-inflammatory, anti-pyretic and analgesic properties.

Not all NSAIDs are equipotent anti-inflammatory, anti-pyretic and analgesic agents. Paracetamol (acetaminophen) has traditionally been considered to be equipotent to ASA in its ability to control pyrexia and pain, however it had generally been considered to have little or no anti-inflammatory activity (Lee et al, 1976; Flower et al, 1985). More recent studies (Mburu et al, 1988; Mburu, 1991) have cast doubt on this and have demonstrated that paracetamol may be effective in reducing post-operative swelling and pain in dogs. Similar findings have been reported for human subjects after oral surgery (Skjelbred et al, 1977). These discrepancies may well reflect the variety of mechanisms involved in inflammation and it may be that re-evaluation of individual NSAIDs for use in specific situations could allow a better correlation of dose and effect produced.

Although a large number of NSAIDs have been developed few of these are licensed or marketed for use in domestic animal species. Greater affluence within developed countries and increasing concern for animal welfare has resulted in an increased interest in the control of pain, particularly in companion animals. In addition, financial interests motivate the exploitation of animals involved in racing and other pursuits where their athletic ability is important. This has fuelled research on drugs which effectively limit reduced performance associated with inflammatory and painful conditions.

The pharmacokinetics and efficacy of many NSAIDs have been well defined for man. However, it is well known that large inter-species variations exist in pharmacokinetics, efficacy and toxicity and this may affect the usefulness of these drugs in domestic animals (Lees et al, 1991). It is essential that potentially useful drugs are investigated in each new target species to determine the appropriate minimum dose rate and inter-dosing interval to achieve maximum efficacy, whilst avoiding adverse reactions or toxicity.

The non-steroidal agents currently licensed for anti-inflammatory use in animals in the United Kingdom are phenylbutazone, meclofenamic acid, flunixin meglumine, naproxen (until recently), cinchophen, carprofen, ketoprofen, dipyrone, isopyrin and meloxicam (table 1.2.)

Several physiochemical properties are shared by non-steroidal anti-inflammatory drugs. They are acidic compounds, generally with a pKa of 4.5 or lower (Brune, 1974; Brune et al, 1976) and as a result tend to be highly protein bound (95-99%).

The structures of NSAIDs currently licensed or about to be licensed in the United Kingdom are given in figures 1.4a and 1.4b and a brief description of the major drugs now follows.

The pyrazolone group, includes phenylbutazone, dipyrone and isopyrin. Phenylbutazone is a relatively lipophilic NSAID and as a result is extensively metabolised prior to excretion; in the horse less than 2% is excreted unchanged (Maylin, 1974; Lees et al, 1983). Oxyphenbutazone, the major metabolite of phenylbutazone, has also been shown to exhibit some anti-inflammatory activity (Domenjoz, 1960). The half-life of elimination of phenylbutazone has been found to be dose dependent (Piperno et al, 1968) and has been reported to range from 3.5 to 10.9 hours in the horse (Piperno et al, 1968; Norheim et al, 1978). It was first used in human medicine in the late 1940s in the treatment of rheumatic disorders and became available to the veterinary profession in the 1950s. It is thought to be the most widely used NSAID in equine medicine (Tobin, 1981; Lees and Higgins, 1985). Phenylbutazone has been rated amongst the more effective anti-inflammatory and analgesic NSAIDs (Menasse et al, 1978). The principal adverse reaction to phenylbutazone in the horse is the appearance of intestinal lesions (Snow et al, 1981a), however renal papillary necrosis and neutropenia have also been observed (McKay et al, 1983; Snow and Douglas, 1983). Other pyrazolones are marketed in preparations which contain more than one drug. Dipyrone is marketed as Buscopan (Boehringer Ingelheim), a product which also contains the spasmolytic hyoscine and isopyrin is produced in combination with phenylbutazone as Tomanol (Intervet). In addition, a combination of dipyrone and the spasmolytic methindizate was marketed as Isaverin (Bayer), this product has been withdrawn.

Meclofenamic acid is a member of the fenamate group of carboxylic acids, and was synthesised as a result of the search for new NSAIDs following the discovery of the anti-inflammatory activity of phenylbutazone. It is highly ionised at physiological pH and is highly lipophilic. The marketed preparation contains the sodium salt which has good bioavailability and is rapidly absorbed after oral administration (Glazko et al, 1978). Meclofenamic acid is generally considered to be amongst the most potent inhibitors of cyclooxygenase in some animal models (Scherrer, 1985) and is widely used for the treatment of inflammatory conditions in the horse.

Tolfenamic acid is also a member of the fenamic acid group of NSAIDs. Produced in the early 1980s, its anti-inflammatory and analgesic activity was studied by Yamashita et al (1981a, 1981b). These studies found it to be a more potent anti-inflammatory agent than mefenamic acid, phenylbutazone and aspirin and almost

equipotent to diclofenac sodium and indomethacin. Its analgesic activity was estimated to be up to six times greater than phenylbutazone and aspirin and equal to that of mefenamic acid and diclofenac sodium. These authors also found tolfenamic acid to be less ulcerogenic than its comparitors and they suggested that it would be suitable for the treatment of acute and chronic inflammation.

Tolfenamic acid has been licensed in France for use in the dog. It is recommended that it be administered at a dose rate of 4 mg/kg/day, given as a divided dose by the intravenous or subcutaneous route.

There are two features which are thought to be common among but unique to fenamates. Firstly, the ability of to inhibit prostaglandin synthesis is enhanced in the presence of certain enzyme co-factors (Egan et al, 1978). Inhibition is positively correlated to co-factor concentration and can be increased by up to 100 times. Secondly, at physiological concentrations fenamates have been shown to directly inhibit some actions of PGs (Collier and Sweatman, 1968)

Cinchophen is the only quinolone to have been used in veterinary medicine. It was first synthesised by Dobner and Gieseke in 1887 and was developed for use in man during the 1940s for the treatment of gout. It has not been used in man in recent years, and little is known of its pharmacology. It became available for veterinary use in the late 1960s and is still available for use in animals. Cinchophen has been marketed as a combined pharmaceutical preparations containing prednisolone, a steroidal anti-inflammatory (Prednoleucotropin), phenylbutazone (Butaleucotropin), and hexamine and quinine (Leucotropin), although this latter preparation has been withdrawn. At therapeutic dose rates cinchophen and phenylbutazone have been shown to produce a similar clinical response and similar numbers of adverse reactions in dogs with degenerative joint disease (McKellar et al, 1991)

Naproxen is a highly lipid soluble propionic acid derivative which was produced in the late 1960s as a result of research which sought to synthesize a compound with similar anti-inflammatory potency to phenylbutazone but which displayed fewer side effects. The raw product comprises two enantiomers of which d-naproxen displays the greatest anti-inflammatory activity and least adverse effects. The two enantiomers are separated after synthesis, and d-naproxen used to formulate the pharmaceutical product. Naproxen is considerably more effective than phenylbutazone in some inflammatory models; it produces dose related anti-pyresis, and is an effective analgesic (Roszkowski et al, 1971). It has been found to have a long half life of elimination in dogs which may be due to enterohepatic circulation (Runkel et al, 1973; Frey and Rieh, 1981). Gastrointestinal side effects have been noted with daily dose

rates as low as 5 mg/kg (Hallesy et al, 1973).

Flunixin is a comparatively new fenamate belonging to the carboxylic acid group of NSAIDs. The pharmacological preparation which is used for its anti-inflammatory and analgesic properties is the N-methyl-d-glucamine (meglumine) salt. Its efficacy at clinical dose rates has been shown to be equivalent to phenylbutazone for the treatment of canine inflammation and musculoskeletal pain (Kelly and Benitz, 1988). Despite a relatively short half life of elimination in the dog, reported as 3.5 hours (Hardie et al, 1985), the persistence of flunixin in active concentrations in inflammatory exudate, has been demonstrated for 24 hours (Higgins et al, 1986). Clinical effectiveness in lameness models in horses has been demonstrated to persist for 24 hours (Houdeshell and Hennessey, 1977) allowing a daily dosing frequency of 24 hours.

Carprofen is a propionic acid of the carboxylic acid group and was first synthesised during attempts to produce a NSAID which had similar anti-inflammatory activity to, but fewer adverse effects than, indomethacin. It is a racemic mixture in which the S(+) isomer is thought to be more biologically active and less toxic (Gaut et al, 1975). Analgesic activity for carprofen has been shown to occur at similar dose rates to indomethacin, whilst its ulcerogenicity is less than indomethacin but greater than phenylbutazone and acetylsalicylic acid in rats (Maeda et al, 1977). Carprofen obtained a United Kingdom product licence for use in the dog in 1991.
Carprofen is a comparatively poor inhibitor of cyclooxygenase (Strub et al, 1982), and has been shown to be ineffective as an inhibitor of lipoxygenase (Baruth et al, 1986). Despite this it has been demonstrated to have good anti-inflammatory activity using in vivo laboratory models (Maeda et al, 1977).

Developed in the 1970s during attempts to find successor to indomethacin, the propionic acid derivative ketoprofen is an effective inhibitor of PG synthesis (Patrono et al, 1976), but has little or no anti-pyretic activity (Kandasamy et al, 1975). Ketoprofen, a racemic mixture, is highly protein bound and has a $t^1/_2\beta$ of 1.5 hours in man (Delbarre et al, 1976; Ishizaka et al, 1980; Stafanger et al 1981). Recent studies in the horse have examined the pharmacokinetics of the two enantiomers of ketoprofen. The $t^1/_2\beta$ of each enantiomer was found to be similar, approximately 25 minutes (Jaussaud et al, 1993).

1.4.2 STEROIDAL ANTIINFLAMMATORY AGENTS

In addition to NSAIDs a large number of compounds with steroidal structure are used for their anti-inflammatory activity.

The production of these drugs has its basis in the discovery and understanding of the functions of the steroid hormones produced by the adrenal cortex. The secretion of these adrenocorticoids is under the control of adrenocorticotrophic hormone (ACTH), and as a result of their different principal activities these hormones are classified mineralocorticoids, glucocorticoids and adrenal sex hormones. Of these groups the glucocorticoids are of interest in the control of inflammatory disease. In addition to their anti-inflammatory properties, these naturally occurring glucocorticoids play a role in the metabolism of carbohydrate, protein and lipids within the body. They are also known to be inhibitory to immune reactions and to have toxic effects on the gastro-intestinal tract. Naturally occurring glucocorticoids such as hydrocortisone do exhibit some mineralocorticoid activity, and this has led to development of synthetic compounds such as betamethasone and dexamethasone which are largely free of these properties.

1.4.3 OTHER COMPOUNDS

In addition to the drugs described, other compounds exist which are also non-steroidal and anti-inflammatory. The chemistry and mode of action of these compounds in no way resembles the conventional NSAIDs. Examples of these compounds, which occur naturally in the body, include orgotein, hyaluronic acid and glycosaminoglycan polysulphate esters.

1.5 MODE OF ACTION OF DRUGS USED TO CONTROL INFLAMMATION AND PAIN

1.5.1 CONVENTIONAL NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

It was first suggested twenty years ago that the ability of NSAIDs to control inflammation and pain was related to their ability to inhibit the production of prostaglandins within the body (Ferreira et al, 1971; Vane, 1971). Although it is now known that this is an important aspect of their mechanism of action, it is clear that the action of NSAIDs is more complex. Higgs et al, (1980) demonstrated that the dose required to produce a response in 50% of subjects tested (ED_{50}) for treatment of some gross changes seen in inflammation (leucocyte infiltration and swelling) can be higher than the dose required to inhibit cyclooxygenase production by 50% (IC_{50}). In addition, NSAIDs have been shown to inhibit aggregation and movement of neutrophils and the generation of superoxide radicals (Di Rosa et al, 1971b; Kaplan et al, 1984). Some NSAIDs and corticosteroids are able to inhibit the secretion of

neutral proteinases from neutrophils (Perper and Oronsky, 1974). These neutral proteinases are known to be responsible for the activation of complement (Goldstein and Weissmann, 1974) and kinin systems (Movat et al 1972) and to be capable of directly causing degeneration of tissue (Oronsky et al,1973).

Stimulated lymphocytes have been shown to generate substances which result in the release of inflammatory mediators, such as acid hydrolases and collagenase, from macrophages (Perper and Davies, 1977). Since the ability to induce release of acid hydrolases and the production of chronic inflammation have been shown to be related (Davies and Allison, 1976), it has been suggested that inhibition of acid hydrolysis release may be a site of action for some NSAIDs.

The actions of NSAIDs which result in suppression of production of prostaglandins are a result of their inhibitory actions on the conversion of arachidonic acid to the endoperoxide PGG_2. This reaction is catalysed by the cyclooxygenase enzyme system. A variety of inhibition mechanisms exist, some being of a competitive nature, others irreversible. Aspirin is known to acetylate a serine which interferes with the site of cyclooxygenation (Roth and Siok, 1978). Platelets, being anucleate, are unable to regenerate cyclooxygenase resulting in permanent acetylation, thus inhibiting the activity of the enzyme for the life of the platelet (about 10 days).

Some members of the fenamate group of NSAIDs, including tolfenamic acid have been shown to inhibit the synthesis of, and be antagonistic to, the actions of PGs (Collier and Sweatman, 1968; Vapaatalo et al, 1977). This may explain the poor correlation which has been noted for inhibition of prostaglandin synthetase and in vivo anti-inflammatory activity by fenamates.

The search for new NSAIDs has recently led to the production of a group of compounds which are known as dual inhibitors. These substances are able to inhibit the activity of both the cyclooxygenase and lipoxygenase enzyme systems, and it may be that this property will result in even greater anti-inflammatory activity than is possessed by current NSAIDs. One such compound, BW 755C (3-amino-1-[m-(trifluoromethyl)-phenyl]-2-pyrazoline) has been shown to inhibit both enzyme systems and to produce a reduction in experimentally produced oedema. It is thought that inhibition of leukocyte migration may result from the ability of these compounds to reduce the production of chemotactic hydroxy acids. Although Higgs et al (1979), have shown this compound to be highly effective in reducing the PG concentration in inflammatory exudate, it was less effective than a conventional NSAID (indomethacin). However, it did have significantly greater effect on leukocyte migration. These workers also compared BW755C with a steroidal anti-inflammatory

drug (dexamethasone). The dual inhibitor was found to be equipotent to the steroid in its ability to reduce PG concentration and reduce leukocyte migration.

Research has also shown tolfenamic acid and sodium meclofenamate, two widely utilised NSAIDs, to be dual inhibitors in some biological systems (Alanko, 1990; Boctor et al, 1986).

1.5.2 ALTERNATIVE NON-STEROIDAL ANTIINFLAMMATORY DRUGS

Orgotein (superoxide dismutase) is a metalloprotein which exists as an intracellular enzyme with a role in the protection of the body against naturally produced superoxide radicals and has been shown to be a potent anti-inflammatory agent in the horse (Ahlengard et al, 1978). Superoxide radicals are produced naturally within the body by neutrophils and macrophages as a defensive mechanism. Any excess which is produced may result in damage to tissue and the phagocytes themselves. Orgotein scavenges these excess superoxide radicals, reducing damage and extending phagocyte viability (Salin and McCord, 1975).

Hyaluronic acid is a component of normal synovial fluid and articular cartilage. It comprises variable length chains of glucuronic acid or glucuronate sodium with N-acetyl glucosamine and is used as an intra-articular therapy. It has lubricant properties within the joint and has been shown to be effective in the treatment of osteoarthritis in the dog (Schiavinato et al, 1989). It is also thought to inhibit chemotaxis and phagocytosis of leukocytes (Wigren et al, 1976; Swanstrom, 1978), thereby reducing the potential for joint damage by neutral proteases which may be released after the ingress of leukocytes.

Glycosaminoglycan polysulphate esters (GAGPs) stimulate synthesis of glycosaminoglycan in chondrocytes (Nevo and Dorfman, 1972) and inhibit enzymes (such as hyaluronidase and collagenase) which are important in the degeneration of cartilage (May et al, 1988). Their use has been shown to be effective as a protection for cartilage exposed to experimentally induced osteoarthritis (Altman et al, 1988; Altman et al, 1989).

1.5.3 STEROIDAL DRUGS

The anti-inflammatory mechanisms of steroidal drugs are not yet fully understood. Some possible actions summarised by Malseed (1990) include: stabilisation of lysosomal membranes resulting in decrease in tissue damage by lysosomal enzymes, reduction of leukocyte migration and phagocytosis and decreased permeability and dilation of small blood vessels.

Despite their potent anti-inflammatory activity glucocorticoids have been shown to be without effect on cyclooxygenase (Ferreira et al, 1971; Vane, 1971; Lewis and Piper, 1975). The discovery by Flower and Blackwell (1979) that these drugs stimulated the production of a substance which inhibits phospholipase A_2, and thereby prevents prostaglandin generation resulted in further investigation of this mechanism. Research by Danon and Assouline (1978) led to the discovery of the role of RNA and protein synthesis in the action of corticosteroids and is the basis of our current understanding of their principal mode of anti-inflammatory action.

Corticosteroids circulate in plasma and enter cells to bind with specific intra-cellular receptors. This results in a change in configuration of the receptors, revealing sites to which nuclear material is able to bind. On entry into the cell nucleus this complex interacts with sites on specific genes resulting in an increase in transcription and mRNA synthesis. Production of specific proteins (lipocortins) is stimulated by this process, resulting in the inhibition of the enzyme phospholipase A_2 and consequently, arachidonic acid release. Prevention of the release of arachidonic acid blocks the synthesis of a large number of the identified mediators of inflammation, significantly, those produced by the action of cyclooxygenase (eg PGE_2 and PGI_2) and lipoxygenase (leukotrienes). These biochemical actions result in the changes that are seen in response to steroidal anti-inflammatory therapy including reduction of PMN migration and phagocytic activity, and inhibition of deposition of fibrin.

1.6 ADVERSE REACTIONS TO ANTI-INFLAMMATORY DRUGS

1.6.1 NON-STEROIDAL ANTIINFLAMMATORY DRUGS

Prolonged use of NSAIDs in human subjects has led to the discovery of a wide range of adverse effects. The most widely reported side effects, both in man and animals, affect the gastrointestinal tract. These include gastric discomfort, emesis, ulceration and gastrointestinal bleeding. It has been shown that in animals duodenal ulceration is common. In man gastric ulceration appears to be the more common result of chronic NSAID therapy (Piper et al, 1981) while ulceration of the large intestine is a more common feature in horses (Brodie et al, 1970; Snow et al, 1981a). This may be caused by a number of factors relating to species or drug studied. Lees and Higgins (1985) have suggested that drug may be bound to components of the diet such as the cellulose and fibre in hay and therefore be less available to cause damage in the proximal regions of the gut. As digestion and fermentation occurs, drug may be released in the more distal sections of the gut where ulceration results. This would indicate that similar problems might be expected to occur in all herbivore species

where the dietary components and gut fermentation are similar.

There is no clear picture of the exact mechanism of gastrointestinal ulceration which results from NSAID therapy. Many factors are thought to contribute, however, the temporal nature of their action and interactions between these factors is as yet poorly defined. Those mechanisms currently considered to be relevant are now considered.

The majority of NSAIDs are acidic drugs with pKa values of 3 - 6. Local effects have been observed in the gut which are likely to be a result of the direct physical properties of the drug, notably damage to and loss of the gastrointestinal mucosal surface (Cooke, 1976; Rainsford, 1984b). The damaged mucosa has also been shown to allow increased back diffusion of H^+ ions which may contribute to this damage (Cooke, 1976).
It is likely that one action of this trauma is an increase in the local release of histamine resulting from degranulation of mast cells (Johnson, 1966; Johnson and Overholt, 1967; Parmar and Ghosh, 1981). In addition, it is known that PGE and PGA can inhibit basal and stimulated gastric secretion (Classen et al, 1971; Wilson et al, 1971). If this is the basis of an inbuilt control mechanism, inhibition of prostaglandins may result in an abnormally large response to stimulation by histamine coupled with an increased basal level of secretion. Several workers (Ashley et al, 1985; Gana et al, 1987; Kitahora and Guth, 1987) have suggested that a NSAID induced reduction in blood flow occurs in areas of mucosa which subsequently ulcerate. Ischemia reperfusion experiments using animal gastrointestinal tracts have shown that endothelial injury occurred after neutrophil adherence and activation which followed the production of inflammatory mediators (Hernandez et al, 1987; Granger et al, 1989). Wallace and Granger (1992) have summarised a range of work which suggests that a similar sequence of events occurs following NSAID administration. It may be that ulceration occurs due to physical occlusion of the microcirculation by adhering neutrophils, however, the protective effect of antioxidant enzymes (Vaananen et al, 1991) indicates that damage may be as a result of tissue-destructive proteases and highly reactive oxygen metabolites released by neutrophils.

In general, NSAIDs inhibit the metabolic pathways which lead to the production of adenosine triphosphate (ATP) (Smith, 1966; Whitehouse, 1968; Smith and Dawkins, 1971; Glenn et al, 1979). Inhibition of ATP may influence regulation of xanthine dehydrogenase by adenine nucleotide resulting in its conversion to the oxidase form which is capable of producing superoxide radicals (Granger et al, 1981), thereby creating the potential for greater tissue damage. Borg (1965) has also shown that free radicals may be generated from salicylate at an acidic pH such as that

which occurs in the stomach.

Lowered ATP levels may also lead to reduced synthesis of gut mucosa (Rainsford, 1984c) and one study has shown that aspirin, indomethacin and phenylbutazone are capable of decreasing the rate of gastric mucus secretion in the dog (Menguy, 1969).

Prostaglandin E_2 and PGI_2 are the principal prostaglandins synthesised by gastric mucosa and are responsible for the inhibition of acid secretion and production of cytoprotective mucus. It has been shown experimentally that these eicosanoids play a protective role in preventing mucosal damage produced by NSAIDs (Robert, 1981). In addition, it has been suggested that accumulation of drug may occur in acid secreting parietal cells (Rainsford, 1984c). This may be due to the acidic nature of NSAIDs which are highly unionised within the acidic environment of the stomach, providing suitable conditions for passage into the mucosal membranes and beyond. Following entry into the less acidic environment within the mucous and parietal cells the drugs loose protons and remain ionised and are therefore, trapped. Any concentration within these cells may lead to an amplification of the range of effects already described. Blood loss into the interstitial tissues or gut lumen may be exacerbated by impaired platelet aggregation resulting from the inhibition of thromboxane production.

Non-steroidal anti-inflammatory drugs are known to cause inhibition of the cytoprotective prostanoids - prostaglandins and PGI_2 (Konturek et al, 1981; Cifone et al, 1982). Rainsford (1985) has suggested that this may result in impaired blood flow through the stomach. It is known that hydrogen ions diffuse back into the mucosa (Kivilaasko and Silen, 1979), any decrease in blood flow in this area will result in a reduction in the removal of these hydrogen ions resulting in damage to the mucosa. A change in mucus synthesis (Rainsford, 1978) may also occur.

Elevation of AMP levels following tissue injury results in conversion of xanthine dehydrogenase, an enzyme normally abundant in the gastro-intestinal tract, to xanthine oxidase. The oxidase form of this enzyme is capable of producing free radicals of oxygen which are known to be tissue destructive (Granger et al,1981; McCord, 1982; Parks et al 1982). It has been suggested (Rainsford,1984c) that a similar enzyme conversion may occur if AMP is elevated following NSAID disruption of oxidative phosphorylation and that the resultant free radical production may contribute to mucosal injury.

Sensitivity to NSAID induced gastrointestinal lesions has been shown to vary widely between species (Hallesy et al 1973; Adams et al, 1970; Joulou et al, 1976). The

minimal ulcerogenic dosage of indomethacin was found to range from 0.5 mg/kg/day in dogs to 20 mg/kg/day in rabbits (Peck, 1968). Sensitivity to drug induced intestinal lesions has also been shown to be linked to the extent of biliary excretion of drug (Hucker et al, 1966; Yesair et al, 1970).

Pharmacokinetic and plasma drug profiles which suggest enterohepatic cycling of NSAIDs in dogs have been reported for indomethacin (Duggan et al, 1975), diclofenac (Tsuchia, 1980), naproxen (Frey and Rieh, 1981) and ibuprofen (Rubin and Papich, 1989). Biliary excretion of drug results in exposure of the gut mucosa to drug or metabolite even after parenteral administration. Gut ulceration has been observed after parenteral administration of NSAIDs and this has been linked to the inhibition of synthesis of prostaglandins by gastric mucosa (Whittle and Vane, 1983; Meschter et al, 1984).

Brodie et al (1970) found that intestinal sensitivity resulting from orally administered drug was greatly reduced or almost abolished after fasting and bile duct ligation respectively. It may be that a reduction of ulcerogenic effect could be achieved by administration of drug on an empty stomach and by withholding food for a period afterwards, thereby reducing biliary flow which is stimulated by consumption of food. Pierson et al (1961) have demonstrated that gastric bleeding was not significantly different in patients given aspirin alone, with food, milk or if buffered or as an enteric coated preparation.

In addition to the gastrointestinal side effects seen after administration of NSAIDs, a wide range of other toxic effects of varying severity have been noted in human subjects. Many of these are not predominant, or have not been identified in the dog.

One other major toxic side effect which has been noted in the dog is renal toxicity (Nash et al, 1987). Renal toxicity is generally associated with higher doses of NSAID (Shelly, 1978). For aspirin it has been found that the drug accumulates in the kidney, especially the outer cortical regions (Rainsford et al, 1981; Rainsford et al, 1983), and it has been suggested that this may be due to normal mechanisms which concentrate anions within the kidney. Some NSAIDs cause acetylation of proteins, lipids and glycoproteins in the kidney (Rainsford et al, 1981; Rainsford et al, 1983) and stimulate the production of tissue destructive lysosomal enzymes. Recent work by Mathews et al (1990) suggests that there may be a risk of renal toxicity at lower NSAID dose rates when used in combination with some general anaesthetics. These workers reported nephrotoxicity in dogs after administration of flunixin meglumine at a dose rate of 1 mg/kg during anaesthesia with methoxyflurane. It has been established that methoxyflurane is nephrotoxic in man (Hook, 1971). Free fluoride

ions produced during the metabolism of methoxyflurane have adverse effects on ADH receptor sites in distal tubules. Toxicity has been directly related to concentrations of these free fluoride ions in serum (Hook, 1971). Mathews et al (1990) suggested that the nephrotoxic effects reported for methoxyflurane and flunixin co-administration may be the result of any one or a combination of effects. Firstly, the reduction in production of vasodilatory prostaglandins (PGE_2 and PGI_2), resulting from the inhibition of cyclooxygenase. This would suppress the protective role of these substances which act to oppose vasoconstrictor effects which may occur during anaesthesia. Secondly, there may be competition between methoxyflurane and flunixin for plasma protein binding sites. As a result the circulating concentration of unbound flunixin and methoxyflurane would be higher than when either drug is present alone. This may result in a more rapid metabolism of methoxyflurane and a higher concentration of free fluoride. Also, a higher concentration of unbound flunixin may result in a greater inhibition of cyclooxygenase and a further reduction in prostaglandin production. Finally, it has been suggested that release of free fluoride may occur during metabolism of flunixin (which contains three fluoride atoms per molecule), and that the total concentration of free fluoride may cause greater toxicity than has been described for methoxyflurane alone. McNeil (1992) has also highlighted the increased risks associated with the use of flunixin after general anaesthesia when reporting the death of a dog which was administered flunixin and antibiotics post-operatively. These studies are consistent with studies of Stoff and Clive (1983) who suggested that high concentrations of angiotensin II and catecholamines produced during anaesthesia had the potential to produce vasoconstriction. They considered that this effect would be counteracted by the production of the vasodilatory prostanoids, the beneficial effects of which would be lost if the action of cyclooxygenase inhibitors reduced or abolished the production of these substances. There is little evidence to suggest that renal toxicity is likely to be a problem in normal patients not requiring general anaesthesia.

Non-steroidal anti-inflammatory drugs inhibit platelet cyclooxygenase (Gallus, 1979; Roth et al, 1975), with consequent reduction in cyclic endoperoxides and therefore TxA_2 (Roth and Majerus, 1975). Thromboxane A_2 is a potent vasoconstrictor and platelet aggregator (Hamberg et al, 1975). Prolonged bleeding time has been widely reported for acetylsalicylic acid (Ivy et al, 1941; Meilke et al, 1969) and results from this inhibition of cyclooxygenase (and reduction of Tx production). This side effect can be of major significance in individuals with bleeding disorders (haemophilia) or in cases where NSAID use has resulted in gastric bleeding. It is interesting to note that this property of aspirin has been used constructively in the prevention of thrombosis in heart disease (Patrono, 1989).

NSAIDs are known to uncouple oxidative phosphorylation (Adams and Cobb, 1958; Whitehouse and Haslam, 1962). It is thought that uncoupling is a result of the ability of these drugs to defeat the proton gradient which must exist across the mitochondrial membrane for oxidative phosphorylation to occur. Uncoupling results in a reduced production of ADP and ATP in the cell. While normal hydrolysis and pyrophosphate cleavage continues in the cell, the quantity of ATP and ADP decline and the AMP present in the cell increases. In addition, reduction in the release of ADP from platelets, which is normally stimulated by substances such as adrenaline and collagen, may result in inhibition of platelet aggregation. This has implications for the prolongation of bleeding time as described previously.

A wider range of toxic effects of NSAIDs have been reported in man. In addition to gastrointestinal irritation and renal toxicity, hepatic damage has been reported after administration of various NSAIDs including phenylbutazone and naproxen (Juul, 1965; Bass, 1974), and a wide range of NSAIDs are thought to cause bone marrow aplasia (Davies, 1985).

1.6.2 STEROIDAL DRUGS

There are numerous side effects produced by steroidal anti-inflammatory drugs which are related to both their mineralocorticoid and glucocorticoid activities. These include water and electrolyte retention, muscle wasting, delayed wound healing and with prolonged usage, osteoporosis as a result of alteration of metabolism of calcium. In addition, the use of these drugs results in suppression of the secretion of ACTH from the pituitary gland which may cause atrophy of the adrenal cortex after prolonged use. Sudden termination of administration may result in adrenal insufficiency.

1.7 STUDY OBJECTIVES

The objectives of this study were, firstly to determine the pharmacokinetics of tolfenamic acid, flunixin, cinchophen and piroxicam in dogs after administration by various routes. Secondly, to assess the pharmacodynamics of these NSAIDs by measurement of TxB_2 inhibition, clotting time and detection of occult blood in faeces. Thirdly, to develop an in vitro method for assessing cyclooxygenase inhibition, and to use this to compare in vitro TxB_2 inhibition when examined as response curves produced by NSAIDs in different species. Finally to determine the extent of protein binding of NSAIDs used in the in vitro assessment of inhibition of TxB_2 in different species.

1.8 TABLES AND FIGURES

Eicosanoid	Function
Thromboxane A_2 (TxA_2)	Platelet aggregation, Smooth muscle contraction, Vasoconstriction, Thrombosis
Prostacyclin (PGI_2)	Inhibits platelet aggregation, Smooth muscle relaxation, Vasodilation, Acid Secretion, Cytoprotection
Prostaglandins (PGs)	
PGE	Smooth muscle contraction, Vasodilation, Bronchodilation, Inhibition of gastric secretion, Cytoprotection
PGF	Smooth muscle contraction, Vasoconstriction
PGD	Bronchoconstriction, Vasodilation.
Leukotriene B_4 (LTB_4)	Vasodilation, Bronchoconstrction, Increased vascular permeability, Leukocyte chemotaxis.
Leukotriene C_4 (LTC_4)	Smooth muscle contraction, Increased vascular permeability,

Table 1.1 Some functions of major eicosanoids.

ANTIINFLAMMATORY AGENT	LICENSED SPECIES
Phenylbutazone	Horses, Dogs
Dipyrone	Horses, Dogs, Cats
Isopyrin	Horses, Dogs, Cats
Meclofenamic Acid	Horses
Flunixin	Horses, Dogs, Cattle
Cinchophen (in combination with steroidal anti-inflammatory)	Dogs, Cats
Naproxen	Horses
Carprofen	Dogs, Horses
Ketoprofen	Horses, Dogs
Meloxicam	Dogs

Table 1.2 Non-steroidal anti-inflammatory drugs licensed in the United Kingdom for use in animals.

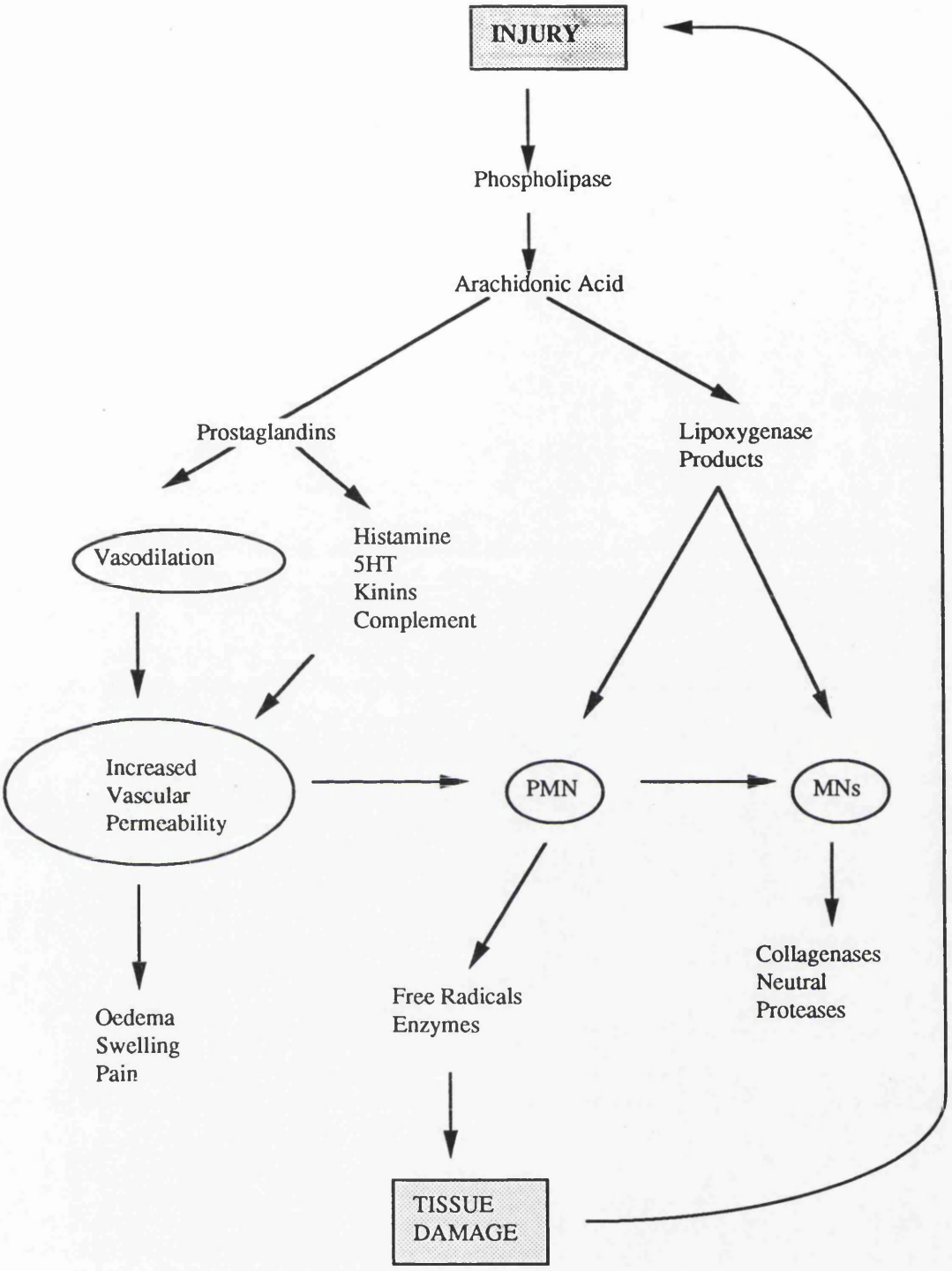
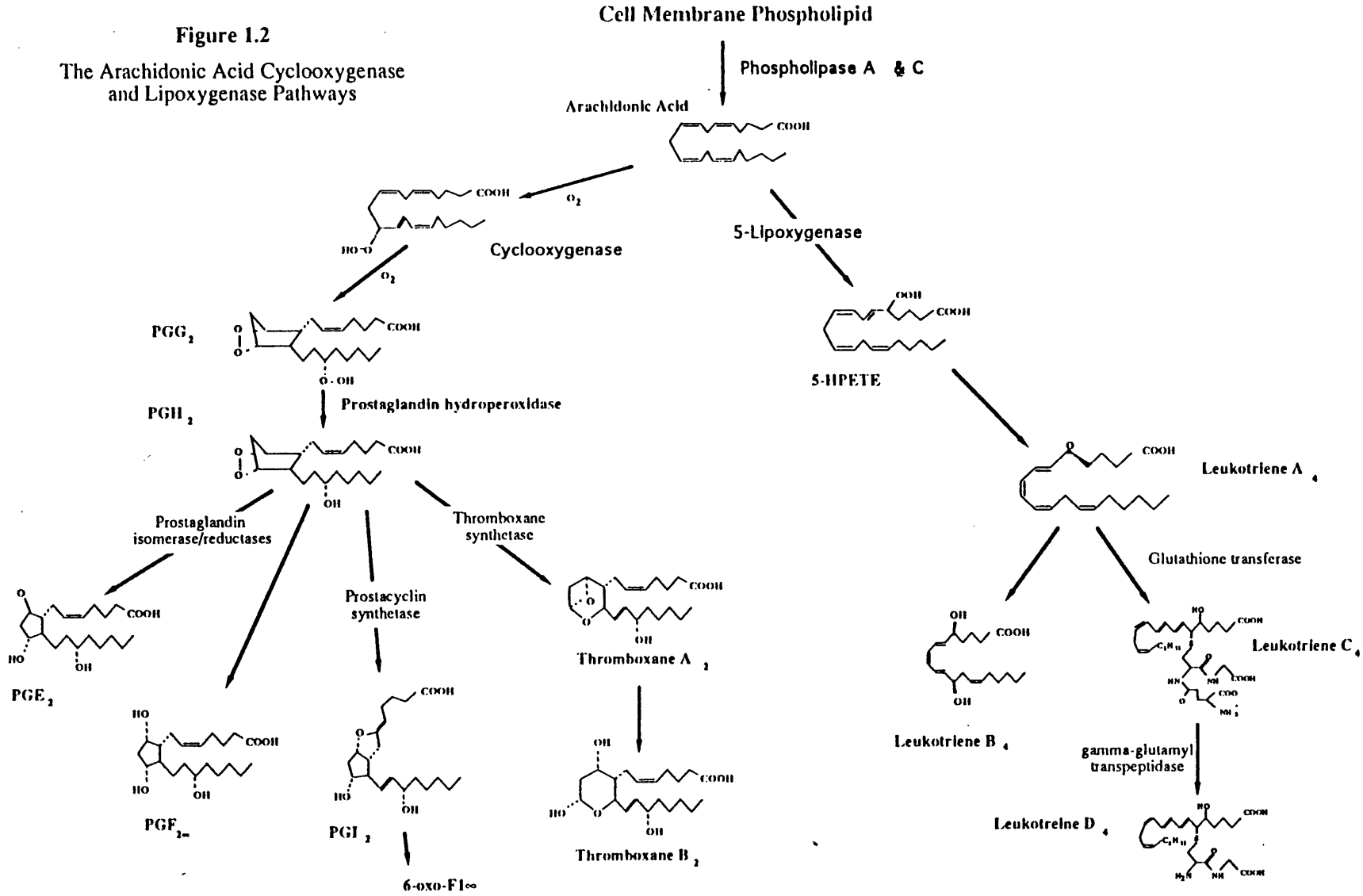


Figure 1.1 The body's response to injury at the cellular level.

Figure 1.2

The Arachidonic Acid Cyclooxygenase and Lipoxygenase Pathways



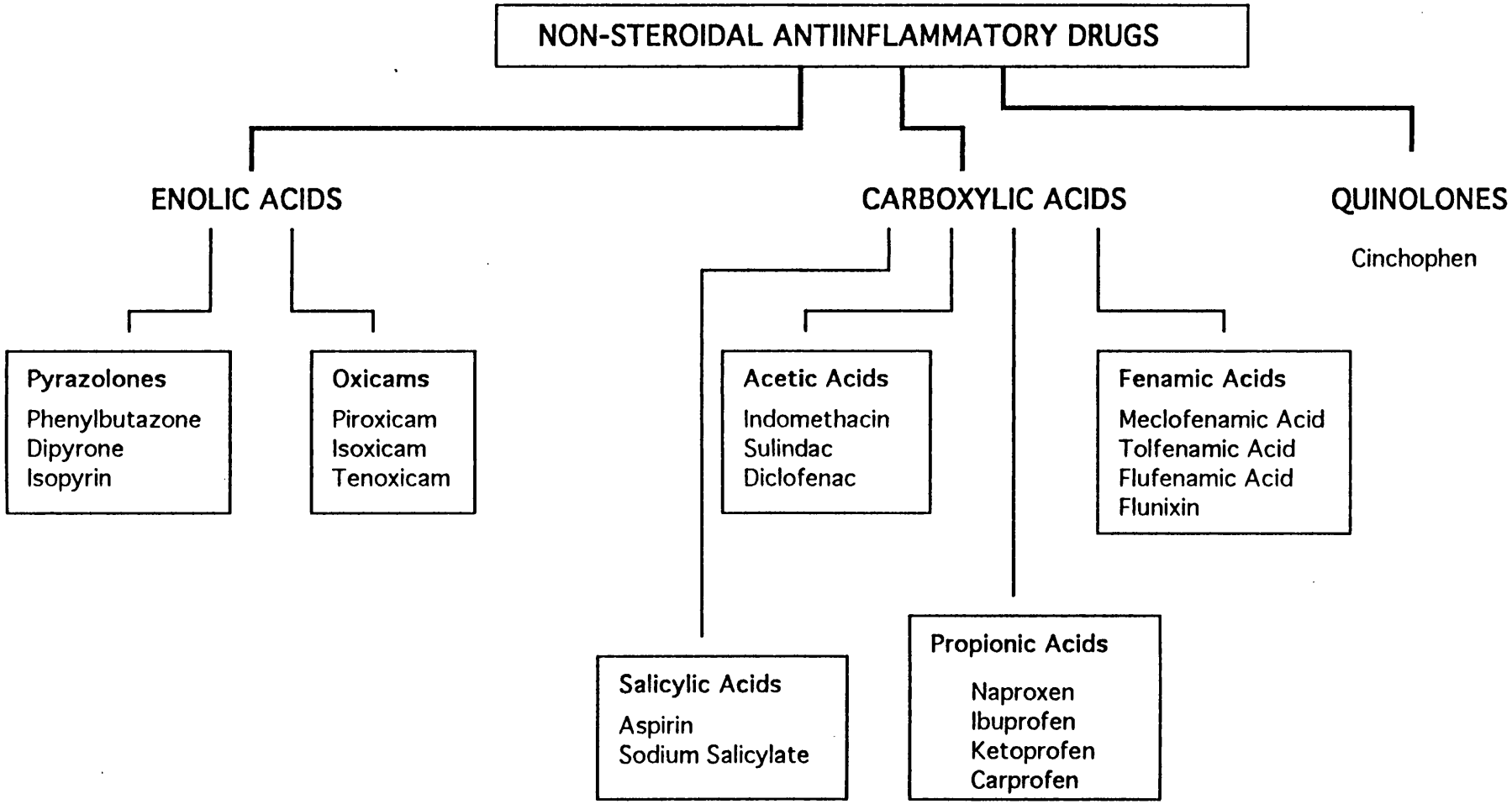
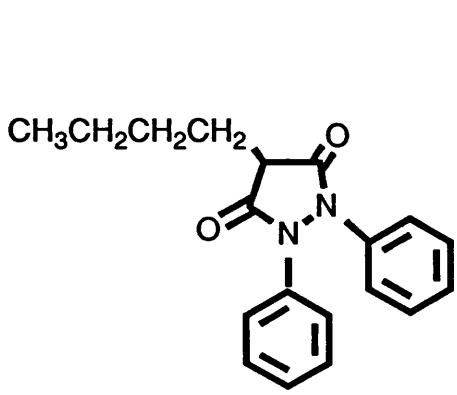
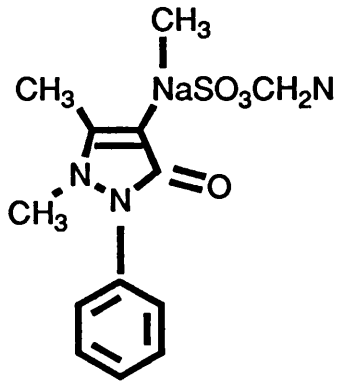


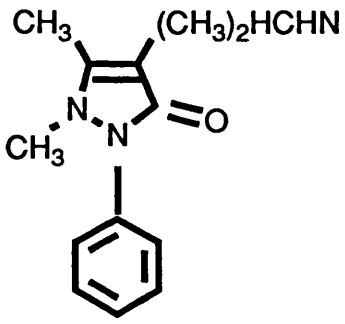
Figure 1.3 Major groups of non-steroidal anti-inflammatory drugs currently available.



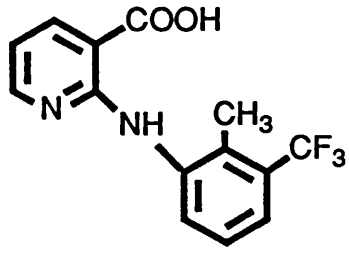
PHENYLBUTAZONE



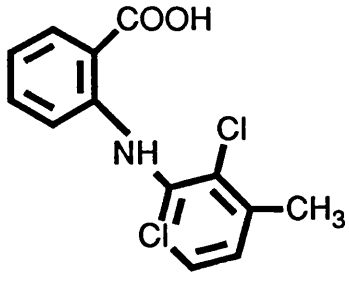
DYPYRONE



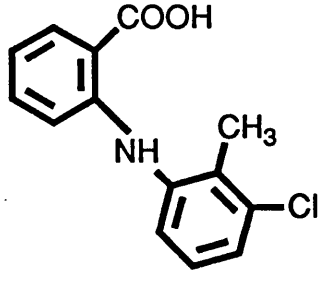
ISOPYRIN



FLUNIXIN

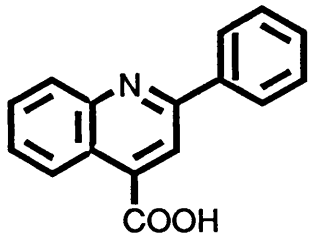


MECLOFENAMIC ACID

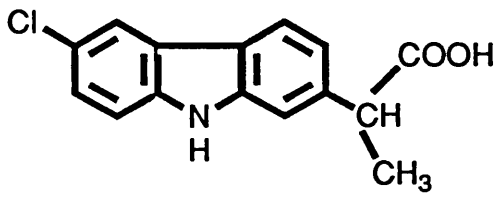


TOLFENAMIC ACID

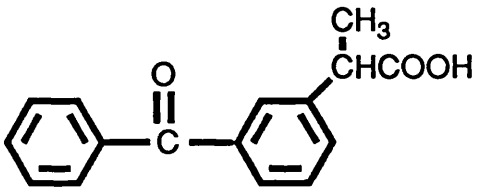
Figure 1.4a Structure of some non-steroidal anti-inflammatory drugs



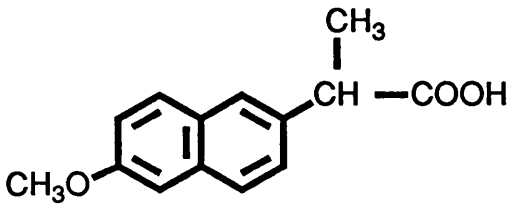
CINCHOPHEN



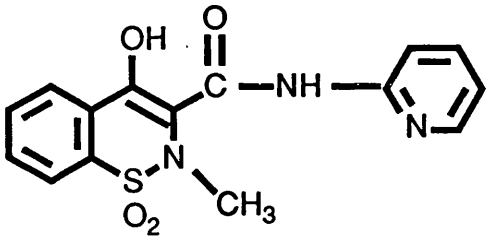
CARPROFEN



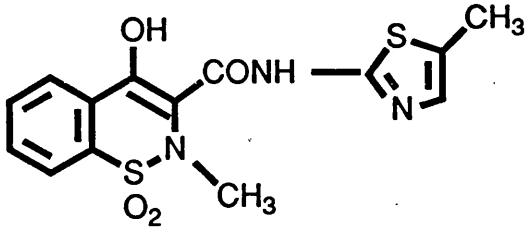
KETOPROFEN



NAPROXEN



PIROXICAM



MELOXICAM

Figure 1.4b Structure of some non-steroidal anti-inflammatory drugs

Chapter 2

GENERAL MATERIALS AND METHODS

2.1 COLLECTION OF BLOOD SAMPLES, PREPARATION AND STORAGE

2.1.1 SAMPLES FOR DRUG ANALYSIS

Samples for drug analysis were collected into 10 ml Monovettes (Sarstedt Ltd) containing 150 i.u. lithium heparin. They were then centrifuged at 1500 g and 4°C for 15 minutes, the plasma recovered and stored at -20°C until analysis.
Prior to analysis samples were placed in a refrigerator at 4°C overnight and allowed to defrost. They were then brought to room temperature by standing on the bench for one hour before use.

2.1.2 SAMPLES FOR ESTIMATION OF PLATELET NUMBERS

Samples for the estimation of the number of platelets in blood were collected into a 10 ml sterile disposable syringe using a 20 g 1 inch needle (Beckton-Dickenson Ltd). The syringe was removed from the needle and a small volume of blood was immediately added to vials containing potassium-EDTA (Sarstedt Ltd.) to produce a concentration of 2 mg EDTA per millilitre of blood. Care was taken to ensure that the maximum recommended volume of blood was not exceeded and that the sample was thoroughly but gently mixed with the anticoagulant.

Platelet numbers were estimated microscopically after the addition of one percent ammonium oxalate. It was possible in most cases to carry out platelet determination within a few hours of collection of the sample. However, for a few samples which were taken late in the day or outwith normal working hours, it was necessary to carry out the procedure on the following day. When required, the samples were stored at 4°C until counted. All platelet counts were carried out within 24 hours of sample collection.
Platelets were counted in the haematology laboratory of the department of Veterinary Pathology at Glasgow University Veterinary School.

2.1.3 SAMPLES FOR THE ESTIMATION OF CYCLO-OXYGENASE INHIBITION

The blood remaining in the syringe after the collection of sample for platelet counts (approximately 9 ml) was retained and placed in a 15 ml sterile glass test tube. The tube was stoppered and immediately placed in a water bath at 37°C where it was allowed to incubate without agitation for 90 minutes. After this time the tube was

centrifuged at 900 g and 4°C for 15 minutes. The serum supernatant was collected and stored in glass screw-top vials at -20°C until thromboxane analysis.

2.1.4 SAMPLES FOR THE ESTIMATION OF CLOTTING TIME

The needle used for blood collection was retained in situ. After ensuring that the blood was flowing freely three unheparinised glass capillary tubes were filled and immediately placed in a container suspended in a water bath at 37°C. The time was noted and each tube was tested by gentle inversion at intervals of 30 seconds to determine if the blood was free flowing or clotted. The time taken for each tube to clot was noted and the mean clotting time of the three tubes was considered to be the clotting time for that animal on that sampling occasion.

2.2 COLLECTION OF FAECAL SAMPLES AND TESTING OF OCCULT BLOOD

Whenever available, faecal samples were gathered daily throughout each experiment from each pen in which the dogs were housed. As each pen contained two dogs it was not possible to determine the individual dog from which the faecal stool originated. On occasions where two separate faecal stools were found in a single pen it was assumed that they were from different animals.

Testing of faeces for occult blood was carried out at the time of collection using a Colo-rectal test system (Hoffman la Roche). The peroxidative activity of haemoglobin present in the faecal sample oxidises a chromatographic paper impregnated with 'gum guaiac' in the presence of alcoholic hydrogen peroxide. Appearance of a blue colour indicates a positive result. The test is sensitive to 2 ml of blood mixed homogeneously with 100 g faeces. Since intestinal bleeding would not result in a homogeneous mixture the test must be considered qualitative, not quantitative.

2.3 ESTIMATION OF THROMBOXANE B_2 IN SERUM BY RADIOIMMUNOASSAY (RIA)

Estimation of TxB_2 in serum samples collected from dogs after oral administration of flunixin was carried out at London University by Professor Peter Lees using an in-house radioimmunoassay validated for dogs. The technique (Higgins and Lees , 1984b) was based on a modification of the methods of Salmon (1978) and Higgs and Salmon (1979)

.

Thromboxane estimation for serum samples collected during all other experiments was performed using a commercial radioimmunoassay test kit (TRK 780 test system, Amersham International (UK) Ltd.). The Technical Services Department of Amersham International confirmed that although intended for use in humans, the kit was suitable for use in animal species. Nevertheless, prior to use, the system was validated by comparison with the in-house method of Professor Lees (see 2.3.3).

2.3.1 ASSAY METHOD

Competition for binding between unlabelled TxB_2 and a fixed amount of tritium labelled TxB_2 to a fixed quantity of an antibody provides the basis of the assay. Quantification (by scintillation counting) of the radioactivity of antibody bound TxB_2 allowed calculation of the amount of unlabelled TxB_2 in the sample.
The amount of radioactive TxB_2 (^3H) bound to the antibody is inversely proportional to the concentration of non-radioactive TxB_2 in the system as the quantities of ^3H TxB_2 and antibody are fixed (see figure 2.1).

2.3.2 MODIFICATIONS OF THE METHOD

The TRK 780 test kit is designed for use with human samples and is therefore suitable for samples containing 0.05 - 3.0 ng TxB_2 per millilitre of sample. The mean serum TxB_2 concentration in normal dogs is 0.89 µg/ml (SEM 0.12 µg/ml) (McKellar et al, 1990) and therefore direct measurement of the samples was often not possible.

Three possible modifications to the assay method were considered:

Firstly, dilution of the sample with buffer prior to assay. Secondly, dilution of the sample with serum from which TxB_2 had been remove ('stripped serum'), and finally reduction of the volume of serum used in the assay.

The following system was used to assess dilution with buffer -

A Tris : HCl buffer (pH 7.4) was prepared as follows :-

To 800 ml of distilled water were added -

6.61 g Trizma HCl (Sigma Chemical Co.)

0.97 g Trizma Base (Sigma Chemical Co.)

1.00 g Gelatine (B.D.H. Ltd.)

0.10 g Sodium Azide (B.D.H. Ltd.)

The mixture was stirred and gentle heat applied until all ingredients had dissolved. After cooling to room temperature the solution was made up to one litre with distilled water. If necessary the pH was adjusted to pH 7.4 using 1M sodium hydroxide. This buffer was used as a diluent for samples tested during the validation of the Amersham test system. The dilution required varied from sample to sample but for normal dog samples a standard dilution of 1 in 1000 was found to be appropriate.

Stripped serum was prepared as follows -

Flunixin meglumine was administered orally to dogs at a dose rate of 1.65 mg/kg. Blood was collected after one hour and serum harvested as described earlier. To 100 ml of the serum 10 g of charcoal was added. The mixture was stirred overnight at 4°C then centrifuged for one hour at 5000 g. The resulting supernatant was passed through a 3μm filter (Millipore Ltd.) to remove any remaining charcoal. This procedure was designed to produce serum which was free of TxB_2. Thromboxane present in the serum collected after administration of flunixin was absorbed onto the charcoal.This 'stripped serum' was used as a diluent for samples tested in the validation of the Amersham test system.

The process of preparing 'stripped serum' was lengthy and required withdrawal of blood from animals. In addition, the effectiveness of the stripping process would have been difficult to standardise. As there appeared to be little advantage in the use of 'stripped serum' as a diluent when compared to assay buffer (table 2.1) the use of assay buffer as a sample diluent was adopted.

Reduction of sample volume was not tested since it was calculated that in some cases the volume of serum would require to be reduced by 1000 times to permit assay of TxB_2 concentrations. It was considered impractical to accurately measure such volumes.

2.3.3 VALIDATION

Prior to use of the Amersham test system it was necessary to establish that results obtained by this method were comparable to those obtained previously. A comparison of results obtained for samples analysed by both methods showed good correlation (table 2.1). Small variations in results between methods were considered to be of little importance since the difference in concentration of thromboxane between samples

was important in the present series of experiments, rather than the absolute concentration in any one sample.

2.4 PHARMACOKINETIC ANALYSIS

Pharmacokinetic analysis of plasma drug concentration versus time data was carried out using a computer programme, CSTRIP, (Sedman & Wagner, 1976) using an ICL main frame computer. This programme is designed to determine the best fit theoretical curve to suit the observed data, and uses up to three exponents (B1, B2, B3) and y-axis intercepts (A1, A2, A3) to generate the equation. A correlation coefficient (r^2) is calculated for each theoretical curve generated, and in addition the goodness of fit for each of the theoretical curves (using one, two or three exponents) was verified by means of Akaike's Information Criterion (AIC) (Yamaoka et al, 1978).

Care was taken to examine the number of observations used to compute each exponent and to disregard any fitted curve which contained an exponent which had been calculated on the basis of only two observations. Having applied this criterion in the selection of the best fit theoretical curve it was found that the correlation coefficient and AIC were generally in agreement. If AIC and r^2 values were contradictory the best fit curve as indicated by AIC was used.

In general, mathematical modelling of plasma concentration versus time data for intravenous experiments produced theoretical curves which were closely correlated to the observed values. The number of exponents in the computed curve which best described the data was not always in agreement for all animals within each experiment.

The values of slope and intercept for each animal derived from the mathematical modelling of intravenous data were used to calculate the disposition kinetics of each drug in the plasma of dogs. Equations used to generate this information were obtained from Baggot (1977), Gibaldi and Perrier (1982), Sams (1987), Rowland and Tozier (1989) and Gibaldi (1991).

Where possible pharmacokinetic parameters were also calculated from observed data and these figures are included.

Mathematical models of plasma concentration versus time data following administration of drugs by routes other than intravenous were generally not well correlated with observed data therefore kinetic parameters for these routes have been calculated from observed data.

Pharmacokinetic parameters were calculated as follows, and the equations are given in table 2.2.

The theoretical concentration of a drug in plasma at time zero after intravenous administration (Cp0, μg/ml) was calculated as the sum of zero time y-intercepts ('equation' 1).
Area under the plasma concentration time curve (AUC_{obs}, μg/ml.h) was determined for observed data by the trapezoidal rule and was calculated for time zero to infinity. The area under the curve described by the triangle between zero time and the first measured concentration (x_1) was determined from the mean of the estimated concentration at zero time (Cp0) and the first measured concentration multiplied by the time of the first measurement (t_1) ('equation' 2). The area under the curve described by the triangle between the last measured concentration (xw) (if not zero) and zero concentration was determined from the last measured concentration divided by the slope of the final exponent (B1) ('equation' 3). The AUC (μg/ml.h) for computed data was calculated as the sum of the ratio of appropriate y-axis intercepts and slopes ('equation' 4).

The area under the first moment curve (AUMC) is the area under the product of the plasma concentration and time plotted against time (μg.h^2/ml). This was also calculated from observed and computed data. For observed data this was calculated by means of the trapezoidal rule as in the AUC calculation. AUMC for computed data was calculated by substituting the square of the appropriate slope in equation 4 ('equation' 5).

Calculation of mean residence time (MRT) allowed an estimate of the time taken to eliminate 63.2% of a drug from the body. This was determined from the ratio of the AUMC and AUC and was calculated for both observed and computed values ('equation' 6). The mean absorption time (MAT) was calculated as the difference between the MRT following extravascular administration and the MRT following intravenous administration ('equation'7). The time taken (hours) for the plasma concentration to fall to 50% of the concentration at time zero, the half life of elimination ($t^1/_2ß$) was calculated using the equation 0.693/slope of the terminal exponent (B1) ('equation' 8)
Having calculated Cp0 it was possible to calculate the volume of the central compartment, Vc (ml/kg) from the ratio of the dose of drug administered and Cp0 (mg/kg) ('equation' 9).
The volume of distribution (Vd_{area}) of a drug (ml/kg) is the direct measurement of the distribution of a drug in the body and was calculated from the dose divided by the product of the AUC and the slope of the final intercept (B1) ('equation' 10). The apparent volume of distribution can be directly influenced by the rate of elimination of a drug from plasma. A drug having a very rapid elimination rate will have undergone significant elimination during the period normally attributed to distribution in the body. In addition, if elimination from plasma is rapid drug will be removed from plasma at a rate greater

than that required for re-equilibration. These factors may result in an apparently high volume of distribution. This anomaly can be addressed by determining the volume of distribution at steady state (Vd_{ss}). Vd_{ss} (ml/kg) is purely a measure of distribution since it reflects the state in the body when the arrival of drug in the body matches the rate of elimination and this can be calculated after intravenous administration of the drug. Estimates of Vdss were calculated for both observed and computed data from the ratio of the product of dose and AUMC to the square of the appropriate AUC ('equation' 11).

The rate at which a drug is removed from the body by metabolism or excretion is termed the rate constant for elimination k_{el} (hours) and was estimated from the ratio of the clearance rate (determined from the ratio of AUC and dose, ('equation' 12)) and the volume of the central compartment ('equation' 13). The rate constants for drug leaving the central compartment and entering the peripheral compartment / leaving peripheral compartment and entering the central compartment are k_{12} and k_{21} respectively and are also measured in hours. These parameters were calculated from the ratio of the sum or product of the exponents B1 and B2 and the rate constant of elimination. ('equations' 14 and 15) as appropriate.

The bioavailability (F) of a drug was determined as a percentage of the ratio of the AUC for observed data after intravenous and oral, intramuscular or subcutaneous administration after correction for dose ('equation' 16). It was assumed that the clearance rate remained constant after administration by each route.

2.5 STATISTICAL ANALYSIS

Statistical analysis of data was performed using Statworks (v 1.1), a statistical package for micro-computers (Cricket Software, Pensylvania, USA.).

Wilcoxon matched-pairs signed-ranks test, a non-parametric test for two related samples was used to identify significant differences in TxB_2 inhibition between intravenous administration and oral, intramuscular or subcutaneous administration of each drug in individual animals. Comparison of TxB_2 inhibition in each animal after administration of all drugs by all routes was carried out by Friedman two-way analysis of variance for three or more related samples.
Samples were considered to be significantly different when $p \leq 0.05$.

For validation of methods, drug peak height and concentration data were plotted using a computer programme (Cricket Graph, v1.3.2, Cricket Software, Pensylvania, USA.) which allowed computation of lines of best fit and correlation coefficients

Drug concentration versus serum TxB_2 inhibition data obtained from in vitro studies were adjusted using a logistic transformation. This data was then also computed to produce best-fit straight lines and correlation coefficients as for method validation.

2.6 TABLES

Sample Number	Method of Lees & Co-workers	Amersham Buffer Method	Amersham Stripped Serum Method
1	0.02	0.03	0.03
2	6.40	5.82	ND
3	0.04	0.06	0.05
4	8.00	9.06	9.10
5	0.04	0.03	ND
6	8.00	7.56	ND
7	0.05	0.03	ND
8	6.40	5.76	5.88

ND - Concentration of TxB_2 not determined.
Amersham Buffer Method - samples pre-diluted in assay buffer.
Amersham Stripped Serum Method - samples pre-diluted in 'stripped serum'.

Table 2.1 Concentration (μg/ml) of TxB_2 in serum of dogs analysed by three different methods.

1. $Cp0 = A1 + A2$

2. $\text{AUC first triangle} = \frac{(X1+Cp0)}{(2)}\, t1$

3. $\text{AUC last triangle} = \frac{X\omega}{B1}$

4. $AUC = \frac{A2}{B2} + \frac{A1}{B1}$

5. $AUMC = \frac{A2}{(B2)^2} + \frac{A1}{(B1)^2}$

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

7. MAT = MRT (extravascular) - MRT (intravenous)

8. $t^1/_2\beta = \frac{0.693}{B1}$

9. $V_c = \frac{dose}{Cp0}$

10. $Vd_{area} = \frac{dose}{AUC.B1}$

11. $Vd_{ss} = \frac{dose.AUMC}{(AUC)^2}$

12. $Cl = \frac{dose}{AUC}$

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

14. $K_{12} = (k_{21}+k_{el}) - (B1+B2)$

15. $K_{21} = \frac{(B1.B2)}{K_{el}}$

16 $F = \frac{\text{AUC (non intravenous)}}{\text{AUC (intravenous)}} \times 100$

Table 2.2 Pharmacokinetic equations

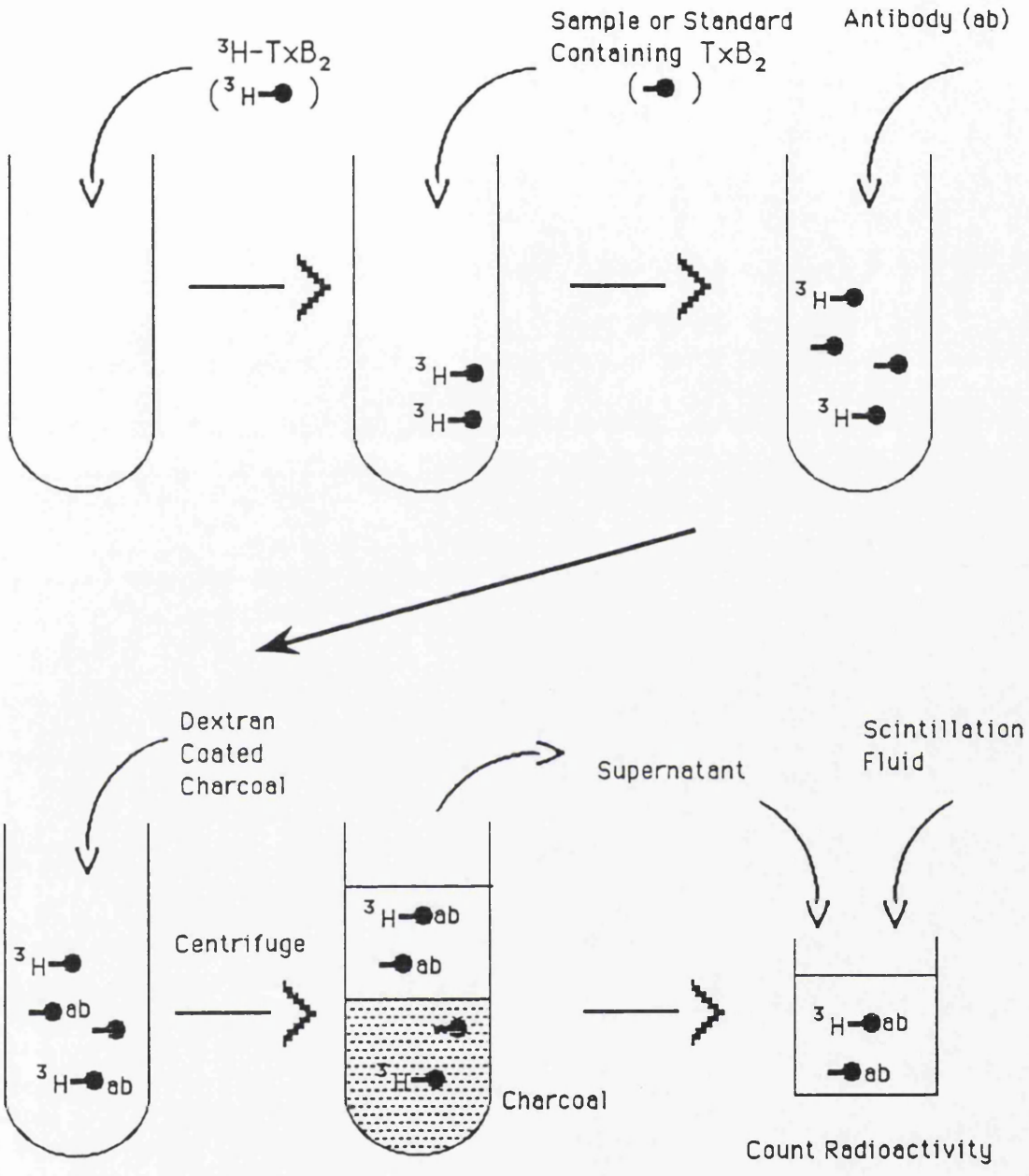


Figure 2.1 Representation of radioimmunoassay for measurement of TxB_2 in serum.

Chapter 3

STUDIES WITH FLUNIXIN

3.1 INTRODUCTION

Flunixin (3-pyridine carboxylic acid, 2[(2-methyl-3-trifluoromethyl) phenyl] amine) is a carboxylic acid belonging to the fenamate group of anti-inflammatory drugs. It has found clinical use as the meglumine (N-methyl-d-glucamine) salt, and is available as preparations suitable for intravenous, intramuscular and oral administration. The recommended dose rate in the dog is 1.1 mg/kg for up to three days, and the dose can be repeated weekly.

Flunixin has been established as an effective analgesic for horses (Houdeshell and Hennessey, 1977). When used in the treatment of equine colic a good response was recorded for 93 % of flatulent and 72 % of spastic cases. A feature of this treatment was the rapid onset of action with 40 % of cases having some improvement within 15 minutes and a duration of action of up to eight hours (Vernimb and Hennessey, 1977). Flunixin was found to be four times more effective than phenylbutazone in the treatment of lameness and swelling in horses; 30 hours after flunixin treatment, lameness was improved by 71 % and swelling by 31 % (Houndeshell and Hennessey, 1977).

Endotoxic shock is the clinical manifestation of the body's response to harmful lipopolysaccarides (endotoxins) which are released by gram-negative bacteria. The damage caused to vascular endothelium by these substances results in an activation of the arachidonic acid cascade and a release of eicosanoids. Experiments by Templeton et al (1983) have shown that flunixin prevented the increase of PGI_2 and thromboxane normally associated with endotoxic shock in ponies, its use increased the survival rate from 25 to 75 % seven days after exposure to endotoxin. Pre-treatment with flunixin has also been shown to prevent the onset of some changes which are a feature of this condition (eg hypoxaemia, lactic acidosis and colic) in the horse (Moore at al, 1981). Similar results have also been reported for flunixin treatment of experimentally induced endotoxic shock in ponies (Bottoms et al, 1981; Fessler et al, 1982).

The elimination half life of flunixin in the horse has been shown to be around 2 hours (Houdeshell and Hennessey, 1977; Semrad et al, 1985; Lees et al, 1987). Onset of action, assessed by improvement in lameness and reduction in swelling, occured by 2 hours with maximum effects between 2 and 16 hours and a duration of up to 30 hours (Houdeshell and Hennessey, 1977). This long duration of action is somewhat surprising in view of the short half life. Lees and Higgins (1987a) were unable to detect drug in plasma after 12 hours following intravenous administration of flunixin at a dose rate of 1.1 mg/kg, although inhibition of TxB_2 was recorded for up to 30 hours. However values had returned to normal by 48 hours indicating that this was not caused by irreversible inhibition.

The fact that the duration of clinical efficacy of flunixin exceeds its measurable duration in plasma has been attributed to accumulation and persistence of drug in inflammatory exudate (Lehmann et al, 1981; Lees et al, 1986).

Flunixin caused dose related inhibition of equine and canine neutrophils when examined in vitro (Dawson et al, 1987; Strom et al, 1990). In each case flunixin was found to be a more potent inhibitor than phenylbutazone and indomethacin. Persistence of this activity was reported by Strom et al (1990). These workers noted that flunixin continued to cause inhibition of PMN migration at 24 hours when drug was not detected in plasma, and have suggested that prolonged duration of action may result from flunixin binding irreversibly to PMN in blood and bone marrow.

After intravenous administration at a dose rate of 1.1 mg/kg to horses, 13.8 % of the total administered dose had been excreted in urine by 24 hours, and at one hour, the concentration in urine was 37 times that present in plasma.

Flunixin toxicity has been reported in ponies (Trillo et al, 1984; Webbon and Wooliscroft, 1984). Administration of 3 mg/kg flunixin twice daily for five days resulted in anorexia, oral ulceration, depression, hypoproteinaemia and neutropenia. Gastric and colonic ulcers were found to be present at the time of post-mortem. This was a dose of more than five times the recommended dose rate. Houdeshell and Hennessey (1977) observed no clinical or biochemical signs of toxicity after intravenous administration at three and five times the normal dose rate for 10 and five days respectively.

Other work has examined the pharmacokinetic and pharmacodynamic characteristics of flunixin in the dog (Hardie et al 1985a), the use of which is now approved in the U.K. The elimination half life of flunixin was found to be approximately three hours, irrespective of dose rate. Similar kinetics were observed in dogs suffering septic peritonitis (Hardie et al, 1985b). Its use has been shown to increase the survival time in these cases (Hardie et al, 1983) although the significance of these studies is questionable since long term survival was not assessed.

Flunixin has also been shown to be effective in the treatment of endotoxic shock in this species (Bottoms et al, 1983).

3.2 EXPERIMENTAL OBJECTIVES

The objectives of this work were, to investigate suitable oral dose rates for flunixin meglumine treatment of dogs by examination of the pharmacokinetic profile and the pattern of serum thromboxane inhibition.

To compare the kinetics and serum thromboxane inhibition of flunixin when the established dose rate was administered to dogs by the intravenous, subcutaneous and oral routes.

3.3 MATERIALS AND METHODS

3.3.1 ANALYSIS OF FLUNIXIN

Analysis of flunixin meglumine was carried out by high performance liquid chromatography (H.P.L.C.) using spectrophotometric detection.

3.3.1.1 REAGENTS

1. Flunixin meglumine (Schering Inc., U.S.A.)

2. Diethyl ether, redistilled grade (Rathburn Chemicals)

3. Methanol 'Analar' (B.D.H. Ltd), redistilled prior to use

4. Disodium hydrogen orthophosphate 'Analar' (B.D.H.Ltd)

5. Citric acid 'Analar' (B.D.H.Ltd)

6. Citrate/phosphate buffer pH 5.0 was produced by mixing the following solutions :

49.3 ml M/5 Na_2HPO_4 + 50.7 ml M/10 citric acid, to give 100 ml of buffer.

7. Perchloric acid 70 % 'Analar' (B.D.H.)

8. Water, redistilled in the presence of potassium permanganate prior to use.

9. H.P.L.C. mobile phase comprised 85 : 15, methanol : water, containing 50 µl of 1 in 55 aqueous solution of perchloric acid per 100 ml mobile phase.

3.3.1.2 H.P.L.C. EQUIPMENT

Pump :- Gilson 301

Detector :- Spectraphysics 100

Column :- 100 mm X 8 mm containing 5 µ O.D.S. Hypersil (Shandon Southern).

Wavelength :- 320 nm

Absorbance :- 0.05 AUFS

Flow rate of mobile phase :- 1 ml/min.

Retention time of flunixin under these conditions was 3.5 minutes.

3.3.1.3 SAMPLE PREPARATION

A 2 ml aliquot of each plasma sample was placed in a 50 ml ground glass tube. To this was added 0.6 ml of citrate / phosphate buffer (pH 5.0), followed by 20 ml diethyl ether. The tube was stoppered and the mixture shaken for 10 minutes by inversion on a slow rotary mixer. Fifteen millilitres of ether were removed from the upper layer and placed in a 50 ml glass test tube. A further 20 ml diethyl ether was added to the sample mixture prior to shaking for a second period of 10 minutes, after which 20 ml of the upper ether fraction was removed and combined with the 15 ml previously recovered.

The tube containing the combined ether extracts was placed in a Dri-Block (Techne Ltd) at 50 ^{o}C under a stream of air and allowed to evaporate until approximately 5 ml remained. This remaining diethyl ether was transferred to a 10 ml conical glass tube. The 50 ml glass tube was rinsed three times with approximately 1 ml of diethyl ether which was then added to the conical tube. The ether extract was evaporated to dryness at 50 ^{o}C under a stream of air. A final 1 ml of ether was run down the walls of the conical tube to ensure that all residue present was in the base of the tube. The extract was returned to the Dri-Block and allowed to evaporate to dryness. Sample tubes were covered with clingfilm and stored overnight at 4 ^{o}C if necessary. Prior to injection into the HPLC system the residues were reconstituted in methanol. An appropriate known volume (not less than 150 µl) of methanol was added to the bottom of the conical tube which was then tilted and rotated in an ultrasonic bath for 1 minute. A 20 µl injection loop was used for injection, the loop was loaded with 15 µl of sample.

The concentration of flunixin in each sample was determined by reference to a calibration curve prepared for each analysis. This was generated by fortifying blank plasma with known amounts of flunixin to produce the appropriate range of concentrations. A number of fortified plasma aliquots treated in this way were processed in an identical manner to that described for unknown samples. In addition, standard solutions of flunixin were injected directly onto the HPLC. These standard solutions allowed equipment performance to be monitored and were also used to calculate the concentration of flunixin measured in the

fortified samples. From these figures it was possible to calculate the percentage recovery of flunixin after extraction.
Unknown sample concentrations were calculated by reference to the standard drug solutions. These concentrations were then adjusted to 100 % to allow for extraction losses by comparison to the recoveries obtained for the fortified samples.

A typical chromatogram is shown in figure 3.1

3.3.1.4 RECOVERY, PRECISION, LINEARITY AND SENSITIVITY

Recovery of drug from plasma samples was approximately 90 %. Typical recovery of flunixin, inter-assay and intra-assay variations are shown in appendix 1.1.
Calculation of the inter-assay coefficient of variation for recovery of flunixin from fortified plasma allowed an assessment of the precision of the assay to be made (appendix 1.1). The mean intra-assay variation was found to be 3.2 % mean inter-assay variation was 5.8 %.
Simple regression was used to determine the linearity of concentration with respect to peak height for fortified samples. The correlation coefficient (r) for concentrations ranging from 0.1 - 5.0 μg/ml of flunixin in plasma was 0.999.
The sensitivity of the assay was determined with respect to the background fluctuation recorded on the trace produced from the HPLC system. The minimum peak height which could be reliably recorded was estimated from standard solutions in replicate analyses. This was determined as being at least twice the maximum overall background fluctuation and was set at one unit. A peak height of one unit of flunixin, under the HPLC conditions described, corresponded to 0.05 μg drug per millilitre of plasma.

3.3.1.5 OTHER ANALYSES AND METHODS USED.

Methods for the estimation of serum TxB_2 concentration, platelet numbers in blood, blood clotting time and detection of occult blood in faeces are described in the general material and methods section. Statistical and pharmacokinetic analysis including equations are also outlined in that section.

The estimation of serum TxB_2 in samples taken after oral administration of flunixin was carried out by Professor Peter Lees and colleagues at the University of London.

3.4 EXPERIMENTS WITH FLUNIXIN

Three male and three female Beagle dogs were used for all experimental work. All animals were approximately 1-2 years of age at the beginning of experimental work.

Animals were housed in pairs and fed a complete cereal diet once daily, water was available ad libitum.

3.4.1 EXPERIMENTAL DESIGN

Flunixin was administered to each of the six dogs by oral intravenous and subcutaneous routes.

Oral administration of flunixin was carried out using a three way crossover experimental design. Animals were allocated to groups of two and each group was given a different dose rate on each occasion. Dose rates used were 0.55, 1.10 and 1.65 mg/kg body weight. Intravenous and subcutaneous administrations were performed as separate experiments and in these studies each dog was administered flunixin at a dose rate of 1.10 mg/kg by the appropriate route. There was a minimum wash out period of seven days between each drug administration. Dogs were weighed immediately prior to each experiment and the dose of flunixin to be administered calculated from this weight.

3.4.2 DRUG ADMINISTRATION

Flunixin meglumine was administered orally as tablets (Banamine; Schering Inc., USA). Tablets containing 5 and 20 mg of drug were used and when an exact dose could not be prepared from whole tablets they were supplemented with powdered tablet administered in a gelatine capsule. The dose rate administered to each animal on each occasion is shown in appendix 2.1.

A 50 mg/ml injectable solution of flunixin meglumine (Finadyne, Kirby-Warrick), was used for intravenous administration. The drug was administered into the right cephalic vein over a period of 30 seconds.

Finarine (Rigaux Galena, France), a 10 mg/ml solution of flunixin meglumine was used for subcutaneous administration. This was administered under the loose skin at the back of the animal's neck.

In each experiment, administration of drug was carried out one hour prior to feeding

3.4.3 SAMPLING REGIMEN

Blood was collected from the jugular vein into suitable tubes prior to and at: 0.5, 1, 2, 4, 8, 12, 24, 48, 72, 96 and 168 hours after oral administration; 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 48 and 72 hours after intravenous administration and 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48, 72, 96 and 168 hours after subcutaneous administration. The appropriate sample was used for drug analysis, haematology and serum TxB_2 estimation, as described previously.

In addition, samples for estimation of clotting time were collected into capillary tubes at 1, 2, 4, 6, 8, 24, 48 and 72 hours after intravenous administration and at 0.25, 0.5, 1, 2, 4, 6, 8, 48, 72 and 168 hours after subcutanous administration of drug.

Faecal samples were collected throughout the experiment and an estimate of the presence of occult blood was made.

3.5 RESULTS OF ORAL EXPERIMENTS WITH FLUNIXIN

The concentration of flunixin in the plasma of each dog after oral administration of flunixin meglumine at a dose rate of 0.55, 1.10 and 1.65 mg/kg body weight are shown in appendix 2.2 to 2.4. Mean (± SEM) concentrations after each dose rate are shown in table 3.1 and figure 3.2. The main pharmacokinetic variables for each dose rate are shown in table 3.2.

3.5.1 FLUNIXIN CONCENTRATION AFTER ORAL ADMINISTRATION

3.5.1.1 ORAL ADMINISTRATION OF 0.55 mg/kg FLUNIXIN

Flunixin was detected in the plasma of all animals with the exception of dog 1 at 0.5 hours post drug administration. In three of the animals (dogs 4, 5 and 6) this was also the maximum concentration detected in plasma. Concentrations measured at this sample time varied widely over the range 0.11 - 3.69 μg/ml. In two animals, the maximum plasma concentrations were measured at one hour (dogs 2 and 3). No drug was detected in any sample from dog 1 until four hours, this was the only time at which drug could be measured in this animal. The inter-animal variation in maximum concentration was large (0.67 - 5.23 μg/ml).

3.5.1.2 ORAL ADMINISTRATION OF 1.10 mg/kg FLUNIXIN

Plasma from all animals was found to contain flunixin at 0.5 hours post drug administration. In dog 1 this was also the time at which the maximum concentration of drug was detected in plasma. Concentrations at this sample time varied more widely than after 0.55 mg/kg and were in the range 0.06 - 4.39 µg/ml. Maximum plasma concentrations of flunixin in plasma were measured at one hour in four animals (dogs 2, 4, 5 and 6). One animal (dog 3) had the maximum plasma concentration of drug at 2 hours. Inter-animal variation in maximum concentration was large with values ranging from 2.15 to 9.21 µg/ml.

3.5.1.3 ORAL ADMINISTRATION OF 1.65 mg/kg FLUNIXIN

Plasma from all animals was found to contain flunixin at the first sample time of 0.5 hours. Concentrations measured at this sample time varied widely over the range 0.40 - 7.20 µg/ml. In four animals the maximum plasma concentrations were measured at one hour (dogs 3, 4, 5 and 6). Maximum concentrations for dogs 1 and 2 were detected at four hours and two hours respectively. The inter-animal variation in maximum concentration was large (1.67 - 16.59 µg/ml).

3.5.2 PHARMACOKINETICS OF FLUNIXIN AFTER ORAL ADMINISTRATION

The mean C_{max} values of flunixin in plasma of dogs following oral administration at dose rates of 0.55, 1.10 and 1.65 mg/kg were 2.77 (± 0.63), 5.03 (± 0.99) and 8.17 (± 2.02) µg/ml respectively and these were observed at 1.25 (± 0.56), 1.08 (± 0.20) and 1.67 (± 0.49) hours.

The area under the plasma concentration versus time curves (AUC) showed a large inter-animal variation after all dose rates the ranges being 2.01 - 19.78 (mean 9.01), 5.58 - 25.16 (mean 14.62) and 9.32 - 37.98 µg/ml.h (mean 25.98) after 0.55, 1.10 and 1.65 mg/kg respectively. These figures are approximately linearly related to the dose rate administered.

Wide inter-animal variations in AUMC and MRT were also noted. Persistence of flunixin as measured by the MRT value was between 1.37 and 5.05 hours after 0.5 mg/kg, 1.95 and 6.86 hours after 1.1 mg/kg and 3.13 and 8.20 hours after 1.65 mg/kg. Estimated $t^1/_2\beta$ calculated from the MRT for each dose rate gave mean values of 3.06, 2.66 and 3.87 hours for 0.55, 1.10 and 1.65 mg/kg dose rates respectively. The relative differences between lowest and highest AUMC measured after each dose rate was calculated. This difference was smallest after

administration of 1.65 mg/kg (seven times) and highest after 0.55 mg/kg (18 times). Mean absorbance time values were found to be negative in several animals after oral administration of flunixin, mean values being -0.62, -1.12 and 0.38 hours after 0.55, 1.10 and 1.65 mg/kg dose rates.

3.5.3 SERUM THROMBOXANE INHIBITION AFTER ORAL ADMINISTRATION OF FLUNIXIN.

Serum TxB_2 concentrations for each animal at each time point are shown in appendix 2.5 and percentage inhibition of TxB_2 in table 3.3.

At the first sample time (0.5 hours), inhibition of serum TxB_2 was found to be between 52.50 and 98.96 % after a dose rate of 0.55 mg/kg, between 8.43 to 99.80 % after 1.10 mg/kg and ranged from zero to more than 99.92 % after the 1.65 mg/kg dose rate.

After oral administration of 0.55 mg/kg, maximum inhibition was greater than 95 % in five animals (dogs 2-6) and occurred at 0.5 - 1 hour. In one animal (dog 1) the maximum inhibition was 84.38 % and this was measured at 4 hours.

In three animals, inhibition decreased to zero at one sample time, and was measured at approximately 30 % at the next sample time (24 hours later). In these three dogs (numbers 2, 4 and 6), inhibition continued thereafter and was 25 % or greater in the final sample (168 hours). In one animal (dog 5) no inhibition of serum TxB_2 was detected in any sample after 24 hours. The maximum mean inhibition of serum TxB_2 was 91.47 % and occured at 1 hour.

At 0.5 hours after oral administration of flunixin at a dose rate of 1.10 mg/kg inhibition of 97 % or greater was measured in samples from four dogs (dogs 1, 2, 5 and 6). In dog 3, the inhibition in this first sample was 69 %, the sample from dog 4 showed only 8.4 % inhibition.

The maximum inhibition was greater than 99 % in five dogs, and serum from all animals had a maximum inhibition greater than 97 %. In four animals (dogs 2,3,4 and 6), the inhibition of serum TxB_2 decreased to zero at one sample time prior to rising again in the next sample. No inhibition was detected in any sample from dog 4 after 8 hours. In all other animals some degree of inhibition was measured in the final sample (168 hours). The maximum mean inhibition was greater than 98.84 % and was measured at 1 hour.

After oral administration of flunixin at 1.65 mg/kg, inhibition of serum TxB_2 ranging from 69.05 to more than 99.92 % was detected in serum from five dogs at the first sample time. In the other animal (dog 6) no inhibition was detected at this time, although inhibition of serum TxB_2 was measured as 99.84 % at the next sample time (1 hour). In one animal (dog 1) inhibition was zero at 12 hours, however at 24 hours (the next sample time) inhibition was found to be 41.67 % This animal also had erratic levels of inhibition at later time points. In dog 2, no inhibition was measured after 24 hours and in dog 6, no inhibition was measured after 12 hours. The maximum mean inhibition of serum TxB_2 was 97.31 % and was measured at 2 hours.

The mean maximum inhibition of serum TxB_2 was 99.11, 99.24 and 99.67 % after 0.55, 1.10 and 1.65 mg/kg respectively. The area under the serum TxB_2 inhibition versus time curves were 5238, 5952 and 6169 %.h respectively.

3.5.4 PLATELET NUMBERS AFTER ORAL ADMINISTRATION OF FLUNIXIN.

Platelet numbers for each dog on each sampling occasion are given in appendix 2.6. Throughout the experiments platelet counts were frequently found to be above the normal range expected for dogs. This was particularly a feature of dogs 1 and 2 and appeared to occur irrespective of dose rate.

3.5.5 OCCULT BLOOD IN FAECES AFTER ORAL ADMINISTRATION OF FLUNIXIN.

Gastrointestinal blood loss determined throughout each experiment by Colo-Rectal test is shown in appendix 2.7.

Traces of occult blood were detected in faeces from dog 1, 48 hours after administration of the 0.55 mg/kg dose rate and from dog 5, 72 hours after administration of the 1.65 mg/kg dose rate.

A definitive positive reaction indicated the presence of blood in faeces from dog 1 at 48 hours after administration of 1.65 mg/kg flunixin

3.6 RESULTS OF INTRAVENOUS EXPERIMENTS WITH FLUNIXIN

3.6.1 FLUNIXIN CONCENTRATION AFTER INTRAVENOUS ADMINISTRATION.

The concentration of flunixin in the plasma of each dog after intravenous administration of flunixin meglumine at a dose rate of 1.10 mg/kg is shown in appendix 3.1. Mean (± SEM) concentrations are shown in table 3.4 and figure 3.3. The main pharmacokinetic variables are shown in table 3.5.

The mean concentration of flunixin measured in plasma of dogs at 0.25 hours post drug administration was 12.80 ± 1.05 μg/ml. As shown by the standard error the inter-animal range in concentration was quite large (9.91 - 16.78 μg/ml) and this was generally the case at each sampling time. The concentration of drug declined rapidly and in one dog (dog 1) no drug could be detected at 8 hours. Flunixin was detected in plasma from two dogs at 48 hours (dogs 4 and 6), and at 72 hours was only detected in plasma from dog 4. Mean concentration of flunixin in plasma was 0.22 (± 0.11) μg/ml at 12 hours and 0.09 (± 0.03) μg/ml at 24 hours.

The $t^1/_2\beta$ of flunixin after intravenous administration at a dose rate of 1.1 mg/kg was found to be in the range 0.90 - 25.10 hours. The mean $t^1/_2\beta$ was 2.97 hours. Two animals (dogs 4 and 6) had particularly large $t^1/_2\beta$ values (25.10 and 23.18 hours respectively). These variable values for $t^1/_2\beta$ were matched by the values for MRT calculated from computed data. Examination of MRT values calculated from observed data shows a much smaller inter-animal variation (1.13 - 9.95 hours). When $t^1/_2\beta$ values were calculated from these data a mean figure of 3.56 hours was obtained.

The inter-animal variation of persistence of flunixin in plasma is also reflected in the large inter-animal variation in computed and observed AUCs. These ranged from 13.93 - 34.70 μg/ml.h for computed data and 11.23 - 29.31 μg/ml.h for observed data . The mean observed AUC after administration of flunixin at a dose rate of 1.10 mg/kg by the intravenous route was 20.47 μg/ml.h.

The area under the first moment curve for observed values had an extremely wide inter-animal variation. The mean observed AUMC was 117 (± 39) $\mu g/ml.h^2$. In the case of two animals (dogs 4 and 6) the AUMC value was much greater if calculated from computed best fit curve values. Inclusion of the estimated last triangle did not greatly affect the AUMC values. Mean body clearance of drug was 47.85 ± 7.34 ml/kg.h when calculated from computed values and 48.83 ± 6.37 ml/h.kg when calculated from observed values for the same period. The volume of distribution at

steady state (Vd_{ss}) was 326 ± 105 ml/kg for values from the computed best fit curves and 189 ± 46 ml/kg for observed data.

3.6.2 SERUM THROMBOXANE INHIBITION AFTER INTRAVENOUS ADMINISTRATION OF FLUNIXIN.

Serum TxB_2 concentrations for each animal at each time point are shown in appendix 3.2, and percentage inhibition of TxB_2 in table 3.6.

Inhibition of TxB_2 was greater than 99 % in all animals at 0.25 and 0.5 hours after drug administration. In one dog (dog 1) inhibition had decreased to 97.9 % at one hour, however the mean inhibition at this time was still greater than 99 %. Mean inhibition remained above 90 % at four hours and still exceeded 70 % at 12 hours. At 24 hours TxB_2 concentrations had returned to near pre drug administration values. The area under the serum TxB_2 inhibition versus time curve after intravenous administration at a dose rate of 1.1 mg/kg was 2148 ± 283 %.h.

3.6.3 PLATELET NUMBERS AFTER INTRAVENOUS ADMINISTRATION OF FLUNIXIN.

Platelet numbers for each dog on each sampling occasion and mean (±SEM) platelet numbers are given in appendix 3.3. On several occasions throughout the experiment samples were noted to contain lower than normal platelet counts. This was not seen to be a consistent feature and was not considered to be associated with drug administration.

3.6.4 CLOTTING TIMES AFTER INTRAVENOUS ADMINISTRATION OF FLUNIXIN.

Clotting times for each animal at each sampling time and mean clotting times are shown in appendix 3.4. These values remained within the expected range at all sample times with the exception of the eight hour sample from dog 1 which was lower than expected (0.59 minutes).

3.6.5 OCCULT BLOOD IN FAECES AFTER INTRAVENOUS ADMINISTRATION OF FLUNIXIN.

Gastrointestinal blood loss determined throughout the experiment by Colo-Rectal test is shown in appendix 3.5. Blood was detected in one faecal sample collected from pen 3 at 24 hours and pen 1 at 168 hours.

3.7 RESULTS.OF SUBCUTANEOUS EXPERIMENTS WITH FLUNIXIN.

3.7.1 FLUNIXIN CONCENTRATION AFTER SUBCUTANEOUS ADMINISTRATION.

The concentration of flunixin in the plasma of each dog after subcutaneous administration of flunixin meglumine at a dose rate of 1.10 mg/kg is shown in appendix 4.1. Mean (± SEM) concentrations are shown in table 3.4 and figure 3.4. The main pharmacokinetic variables are shown in table 3.7

The mean concentration of flunixin measured in plasma of dogs at 0.25 hours post drug administration was 3.15 ± 0.35 μg/ml. A small inter-animal range in concentration (1.94 - 4.14 μg/ml) was found to be typical of this and other sample times until 12 hours. The maximum mean concentration (6.31 ± 0.63 μg/ml) occured at one hour after drug administration and flunixin was detected in the plasma of all animals at 12 hours (mean concentration 0.34 ± 0.13 μg/ml). Flunixin could be detected in the plasma of two dogs (dogs 4 and 6) at 48 hours and in plasma of one animal (dog 6) 0.14 μg/ml was measured at 96 hours after administration. Examination of the drug concentration versus time profile for dog 6 shows that after declining to a concentration of 0.11 μg/ml at 48 hours a higher concentration of 0.21 μg/ml was measured at 72 hours. The mean maximum concentration of 6.36 μg/ml was measured at 0.92 hours.

The inter-animal variation for persistence of flunixin in plasma is reflected in the large intra-animal variation in AUCs which ranged from 9.95 - 41.23 μg/ml.h.and in the MRT which ranged from 2.48 hours in dog 5 to 32.64 hours in dog 6. Calculation of an estimated $t^1/_2\beta$ from MRT values gave a figure of 6.9 hours. The mean AUC after administration of flunixin at a dose rate of 1.10 mg/kg by the subcutaneous route was 26.30 μg/ml.h indicating that drug was well absorbed from the injection site. Examination of MAT values showed a large inter-animal variation with several animals having negative values, the mean value was 4.13 hours. The mean value for AUMC was 304 (± 210) $\mu g/ml.h^2$.

3.7.2 SERUM THROMBOXANE INHIBITION AFTER SUBCUTANEOUS ADMINISTRATION.

Serum TxB_2 concentrations for each animal at each time point are shown in appendix 4.2, and percentage inhibition of TxB_2 in table 3.6.

Inhibition of TxB_2 was greater than 98 % in all animals at 0.25 and 0.5 hours after drug administration. Mean inhibition remained above 90 % at four hours and still exceeded 70 % at 12 hours.

In four animals (dogs 1, 2, 4 and 6) the inhibition of TxB_2 decreased to zero, after which some degree of inhibition was measured at the next sample time, 24 hours later.

At 48 hours mean serum TxB_2 concentrations had returned to near control values.The area under the serum TxB_2 inhibition versus time curve after subcutaneous administration at a dose rate of 1.1 mg/kg was 2741 ± 540 %.h.

3.7.3 PLATELET NUMBERS AFTER SUBCUTANEOUS ADMINISTRATION

Platelet numbers for each dog on each sampling occasion and mean (±SEM) platelet numbers are given in appendix 4.3. On several occasions throughout the experiment samples were noted to contain lower than normal platelet numbers. This was seen to be a consistent feature in one animal (dog 4), however the platelet count in the pre drug administration sample was also somewhat low for this animal. Platelet counts were not considered to be sufficiently low on a consistent basis to affect concentrations of thromboxane produced in serum.

3.7.4 CLOTTING TIMES AFTER SUBCUTANEOUS ADMINISTRATION

Clotting times for each animal at each sampling time and mean clotting times are shown in appendix 4.4. These values remained within the expected range at all sample times with the exception of the 0.5 hour sample from dog 1 which was lower than expected (0.96 minutes).

3.7.5 OCCULT BLOOD IN FAECES AFTER SUBCUTANEOUS ADMINISTRATION.

Gastrointestinal blood loss determined throughout the experiment by Colo-Rectal test is shown in appendix 4.5.
No blood was detected in any sample collected during this experiment.

3.8. DISCUSSION

No adverse effects were seen in any animal after oral administration of flunixin. With only one exception (dog 1 at 0.55 mg/kg) drug was detected in the plasma of all animals at 0.5 hours after administration thus indicating that this oral preparation of flunixin is rapidly absorbed. Examination of results for individual animals shows wide variations in concentrations and this was particularly apparent at the 0.55 mg/kg dose rate where drug was only detected in plasma at 4 hours for one animal (dog 1). It is likely that this variation reflects the innate inter-animal variation in absorption rate as it was not a consequence of concurrent feeding. Each dog was in a similar state of fasting prior to administration of the drug and food was not available to any animal until one hour after drug administration. The AUC for individual animals after oral administration also reflects this variation. Persistence of drug in plasma as measured by MRT was significantly different amongst the dose rates ($p = 0.01$). This was shown to be due to the MRT being significantly higher after administration of the 1.65 mg/kg dose rate than after the 1.10 mg/kg dose rate. A visual examination of the plasma concentration versus time curves produced after each oral dose rate also indicated that a general similarity existed in the pharmacokinetics of flunixin at the three dose rates. This is in agreement with the findings for the pharmacokinetics of flunixin in sheep after intravenous administration at dose rates of 1.0 and 2.0 mg/kg (Welsh et al, 1993). As expected the C_{max} increased with increasing dose rates, however similar values for t_{max} were seen after each dose rate thus indicating that rate of oral absorption is not affected by dose rate within the range 0.55-1.65 mg/kg.

The maximum mean inhibition of TxB_2 exceeded 90 % after each oral dose rate, however, after the 1.10 and 1.65 mg/kg dose rates the mean maximum inhibition achieved was greater than 99 %. A significant difference was found ($p = 0.039$) when these maxima were compared statistically using Friedman's test. However, differences were not found to be significant on examination of data as pairs using Wilcoxon Signed Ranked Test. This may have been a result of small sample numbers. At the higher oral dose rates of 1.10 and 1.6 mg/kg, each animal had a TxB_2 inhibition of 97 % or greater on at least one sampling occasion after drug administration. The maximum inhibitions achieved with the 0.55 mg/kg dose rate were more variable and in dog 1 the maximum achieved was as low as 84 %. Flunixin is known to be highly bound to plasma proteins (perhaps 99 %) (Soma et al, 1988). In several samples where drug concentrations were as low as 0.1 μg/ml, inhibition of TxB_2 to a level of around 50 % was detected. Since this concentration represents the total concentration of flunixin in plasma the concentration of unbound drug could be estimated to be in the order of 0.001 μg/ml, indicating that the drug is extremely potent in producing inhibition of platelet

cyclooxygenase. In all cases at 24 hours the inhibition of TxB_2 had greatly declined, thus indicating that flunixin is a reversible inhibitor of platelet cyclooxygenase and confirming the findings of Lees et al, (1987).
The the area under the TxB_2 inhibition versus time curve was 5238, 5952 and 6169 %.h after 0.55, 1.10 and 1.65 mg/kg respectively. The increase in the area under the TxB_2 inhibition versus time curve between the 0.55 and 1.10 mg/kg dose rates was 13 %, whereas the increase between 1.10 and 1.65 mg/kg was 3 %. This, together with the fact that the maximum TxB_2 inhibition was greater after 1.10 and 1.65 mg/kg than after 0.55 mg/kg, but was similar after 1.10 and 1.65 mg/kg, led to the conclusion that there was little apparent benefit in the use of the highest dose rate. Accordingly, a dose rate of 1.10 mg/kg was used in the intravenous and subcutaneous studies with flunixin.

Lees and Higgins (1984) have shown, for cattle and horses, that the concentration of flunixin in plasma may not be an accurate indicator of its clinical efficacy, since the drug accumulates in inflammatory exudate. Concentrations of up to four times those in plasma have been measured in inflammatory exudate from horses after intravenous administration of flunixin (Lees and Higgins, 1984 ; Higgins et al, 1986), and inhibition of TxB_2 and PGE_2 production in exudates is more persistent than in plasma. It seems likely that this situation will also apply for the dog.

Platelet numbers were within the normal range for beagle dogs throughout these experiments and the changes in TxB_2 concentrations were not associated with changes in platelet numbers. In addition, although occult blood was detected in faeces from dogs on some occasions this was not persistent. Monitoring of dogs outwith experimental periods indicated that blood was passed in faeces occasionally and was not associated with flunixin administration. It is likely that in NSAID toxicity the amount of blood passed in faeces would be much greater and that it would be a consistent feature.

The plasma concentration versus time curve for flunixin after intravenous administration was best described by a biexponential equation in all animals, however, the second exponent of the equation for data from dog 2 contained only two data points indicating that this description was unsound. These findings are in agreement with studies on dogs (Hardie et al, 1985a), horses (Chay et al, 1982) and cattle (Benitz, 1984; Hardee et al, 1985). However, a recent study on the pharmacokinetics of flunixin in sheep at dose rates of 1.0 and 2.0 mg/kg dose rates found the plasma concentration versus time curve to be best described by a triexponential equation (Welsh et al, 1993). It is possible that the identification of a third exponent is a result of the greater sensitivity of the assay method used by Welsh. The low background absorbance of plasma samples from sheep allowed an increase in the sensitivity for the measurement

of flunixin of approximately five times, and in the study in sheep, flunixin was detected in plasma at 33 hours after administration. In the present study the concentration of flunixin in plasma remained above 0.3 μg/ml for eight hours in all but one animal (dog 1), but was below the limit of detection in two animals at 12 hours after administration.

No adverse effects were seen in any animal after intravenous administration of flunixin.

The half life of elimination of flunixin was seen to vary widely between animals. These values may be associated with enterohepatic cycling . Half lifes of elimination can be greatly influenced by sampling times and limit of detection for a drug. A low limit of detection and appropriately spaced sampling times can result in an apparently longer half life. There is no way of determining the exact time at which drug can no longer be detected in plasma, this time point may occur at any time between the last positive sampling time, and the first negative sampling time and as a result these figures may also have a marked effect on the calculated half life of elimination. The $t^1/_2\beta$ in the present study was approximately 3 hours. This is similar to the values previously reported for dogs (3.7 hours, Hardie et al, 1985), sheep (3.4 - 3.8 hours, Welsh et al, 1993) and lactating cattle (3.1 hours, Anderson, 1990). Other studies in cattle have reported $t^1/_2\beta$ values which are considerably longer (approximately 8 hours, Hardee et al, 1985) and the $t^1/_2\beta$ reported by Chay et al (1982) for horses was considerably shorter (1.6 hours). A factor which may hamper the comparison of $t^1/_2\beta$ values is the method of expression. In the present study all values for $t^1/_2\beta$ have been expressed as harmonic mean values. Calculation of the harmonic mean requires that values are expressed as the inverse of the sum of the inverse individual values divided by the number of observations, and is considered to be the most appropriate method of describing rates (Moroney, 1963). Conversion of this value to an arithmetic mean produces a $t^1/_2\beta$ of approximately 10 hours. Without full knowledge of the method of calculation of $t^1/_2\beta$ and the ability to express data in the same way it is impossible to make accurate comparisons of these figures.

The mean Vdss calculated from observed data was 189 ml/kg and the Clb was 48.83 ml/h/kg. These figures, together with the other main pharmacokinetic parameters, are in good agreement with the findings of Hardie et al (1985a) who studied mixed breed dogs. This indicates that there is likely to be little difference in pharmacokinetics between breeds of dogs although differences between individuals are large.

It is difficult to draw conclusions from the data obtained for MAT as in many cases these values were negative, indicating that theoretically drug was more rapidly available after oral administration than intravenous administration. As this is not possible these results must be due to the errors inherent in comparing the intravenous

and oral concentration versus time curves. This does, however suggest that absorption is very rapid after oral administration.

The AUC values calculated for each animal reflect the inter-animal variation in persistence of drug concentrations in plasma. Enterohepatic cycling has been noted in dogs by several workers after the administration of NSAIDs (Duggan et al, 1975; Tsuchia, 1980; Frey and Rieh, 1981; Rubin and Papich, 1989) and it may be that although the characteristic peaks and troughs in drug concentrations were not seen, the persistence of drug in some animals was due to this phenomenon.

Bioavailability of drug after oral administration was calculated when the intravenous experiment had been performed. In the case of each dose rate the AUC after oral administration was compared with the intravenous AUC at the dose rate of 1.10 mg/kg. Observed values plus the theoretical last triangle were used in this calculation and results were in good agreement with those reported for mixed breed dogs by Hardie et al (1985a). In general, bioavailability at each dose rate was approximately equivalent when adjusted for the dose administered. However, at the lowest dose rate the inter-animal variation was such that the lowest bioavailability was 27.94 % whilst the highest was 197.40 %. Inter-animal variation at the higher dose rates was smaller, the range being 50.42 - 113.23 % and 43.18 - 101.50 % after 1.10 and 1.65 mg/kg respectively. It is of interest to note that when these values for bioavailability are adjusted for a dose rate of 1.10 mg/kg the mean values would be 74.8, and 69.8 % and are similar to the value of 60.7 % obtained after oral administration of 1.10 mg/kg, thus reinforcing the theory that absorption is not related to dose rate. These estimates of bioavailability are somewhat lower than that reported for the horse by Soma et al (1988), who found the bioavailability of flunixin to be 85.8 % after administration to mares at a dose rate of 1.1 mg/kg.

The maximum inhibition of serum TxB_2 production was generally higher after the administration of flunixin by the intravenous route than after any of the three oral dose rates. The high levels of inhibition were also maintained over a longer time scale. following intravenous administration. However, when the decline in inhibition occured it was rapid and by 72 hours only one animal (dog 3) had any inhibition and the area under the serum TxB_2 inhibition versus time curve was 2148 %.h after intravenous administration, less than half that measured after oral administration at the same dose rate. As in the oral experiment the reversible nature of the cyclooxygenase inhibition was confirmed by the return to near normal values for the concentration of TxB_2 in some dogs by 24 hours, after administration of flunixin.

No adverse effects were seen in any animal after subcutaneous administration of drug and the administration did not appear to cause undue discomfort to any animal. Concentrations ranging from 1.94 - 4.14 μg/ml (mean 3.15 ± 0.35 μg/ml) of flunixin were detected in plasma at 0.25 hours after drug administration indicating that a rapid absorption occurs after subcutaneous administration. Drug concentrations were well maintained in most animals until 12 hours. A large inter-animal variation in persistence of drug was noted. Examination of the plasma drug concentration versus time profile for dog 6 showed a trough, followed by increased concentrations during the period 24 - 96 hours and in this animal drug was detected in plasma until 96 hours. This profile would suggest that the drug may have undergone enterohepatic circulation. The persistence of flunixin in the plasma of dog 6 is also reflected in the larger AUC. This value is four times the smallest measured AUC (Dog 1, 9.95 μg/ml.h) and almost twice the mean AUC calculated for the other five animals. Mean residence times calculated after subcutaneous administration showed a large inter-animal variation, the mean value being 8.55 hours. An estimated $t^1/_2\beta$ calculated from this figure is 6.9 hours. The large difference in AUCs may also reflect the large inter-animal differences in absorbance of the drug (MAT 4.13 ± 3.82 hours).

Good inhibition of serum TxB_2 occured after the subcutaneous administration of flunixin and this inhibition also mirrored the concentration of flunixin in plasma. At 12 hours, the last sampling time at which flunixin could be detected in all animals, the mean inhibition was 78 %. At 24 hours, when drug could be detected in plasma from only two animals (dogs 4 and 6) this mean inhibition had decreased to 29 %. Maximum inhibition was similar to that measured after intravenous and oral administration of flunixin at the 1.10 mg/kg dose rate. High levels of inhibition were maintained for longer periods than after oral administration, however, the decline when it occured was rapid. The area under the serum TxB_2 inhibition versus time curve was slightly larger than after intravenous administration and approximately half of that measured after oral administration at the 1.10 mg/kg dose rate.

Platelet numbers were, generally, within the normal range for beagle dogs throughout this experiment, samples from dogs 1 at two hours, dog 3 at eight hours and dog 4 at one and 24 hours being the exception to this. These samples contained low numbers of platelets, however since these occurrences were not part of a trend but were individual exceptions it seems likely that these figures may have resulted from clotting which had occured but was undetected in the sample. Clotting times were also generally considered to be normal with the exception of a single sample from dog 1 at 0.5 hours. Occult blood in faeces was not a consistent feature for any animal after the

subcutaneous administration of flunixin. This further confirms the observations that dogs did not respond adversely to subcutaneous administration of the drug.

General

The dose rates and routes of administration of flunixin produced no adverse reactions in any dog, and this observation was borne out by the results obtained for platelet counts, clotting times and the occult blood test. Examination of the data produced by the oral study resulted in the selection of a dose rate of 1.10 mg/kg for the intravenous and subcutaneous experiments.

Comparison of a single oral dose of flunixin administered at three dose rates, showed that absorption and bioavailability are approximately linear through the range 0.55 to 1.65 mg/kg. Comparison of the data from the administration of 1.10 mg/kg flunixin by the oral route with that produced after administration of the same dose rate by the intravenous and subcutaneous routes showed that $t^1/_2\beta$ estimates were slightly longer after oral than intravenous administration (3.12 and 2.97 hours respectively). The value estimated after subcutaneous administration was considerably longer at 6.91 hours. These observations would be expected, as the elimination phase of any plasma concentration versus time curve for a drug given by an extravascular route is a composite of absorption and elimination factors. The possibility of enterohepatic circulation having occured in these experiments has been discussed and it seems possible that the variations in $t^1/_2\beta$ may have resulted from this phenomenon. Large inter-animal variations in plasma concentration and $t^1/_2\beta$ were noted in all experiments and this was particularly a feature after the 0.55 mg/kg oral administration and the intravenous administration. As all animals were in a similar state of fasting prior to the experiment, it is likely that the differences arose as a result of normal individual differences in absorption, distribution and excretion of drug. The extent of this variation may have serious implications for the inter-animal variation in efficacy and toxicity of flunixin if dosage regimens are based on mean data, or in cases of multiple administration over a period of days or weeks. Mean absorbance time was extremely short in all experiments and in some cases was calculated as a negative value. Theoretically this would only be possible only if a proportion of drug had not been delivered directly into the vein during the intravenous administrations. As there is no other evidence to support this it seems likely that a normally short MAT was augmented by extremely large inter-experimental variation in MRT which could result from enterohepatic circulation.

Flunixin was found to be a potent, reversible inhibitor of cyclooxygenase activity. Examination of all available information suggests that a single daily administration of 1.10 mg/kg flunixin should provide relatively safe and effective anti-inflammatory activity. The greater area under the serum TxB_2 inhibition versus time curve after oral administration, coupled with the convenience of this route of administration indicates that this may be the route of administration of choice.

3.9. TABLES AND FIGURES

Time	Dose Rate Administered (mg/kg)		
(h)	0.55	1.10	1.65
Pre	0	0	0
0.50	1.93 ± 0.63	1.95 ± 0.77	2.94 ± 1.30
1.00	2.40 ± 0.70	4.57 ± 1.12	7.42 ± 2.07
2.00	1.44 ± 0.62	2.83 ± 0.57	4.99 ± 1.11
4.00	0.55 ± 0.13	1.33 ± 0.39	1.64 ± 0.16
8.00	0.32± 0.18	0.44 ± 0.16	0.60 ± 0.13
12.00	0.17 ± 0.08	0.22 ± 0.16	0.31 ± 0.10
24.00	0	0.02 ± 0.02	0.07 ± 0.04
48.00	0	0	0.02 ± 0.02
72.00	0	0	0
96.00	0	0	0
168.00	0	0	0

Table 3.1 Mean concentration (μg/ml) of.flunixin in plasma of dogs after administration of a single dose of flunixin by the oral route at a dose rate of 0.55, 1.10 and 1.65 mg/kg body weight.

Parameter	Units	Animal Number 1	2	3	4	5	6	Mean	±	SEM
0.55 mg/kg										
Cmax	µg/ml	0.67	2.25	5.23	2.52	3.69	2.24	2.77	±	0.63
tmax	hours	4.00	1.00	1.00	0.50	0.50	0.50	1.25	±	0.56
AUC	µg/ml.h	2.01	5.14	19.78	3.98	14.89	8.29	9.01	±	2.83
AUMC	µg/ml.h^2	8.04	14.51	100.00	5.43	74.51	37.76	40.04	±	15.98
MRT	hours	4.00	2.82	5.05	1.37	5.00	4.56	3.80	±	0.59
MAT	hours	2.87	-2.58	3.13	-1.83	0.06	-5.39	-0.62	±	1.35
F	%	27.94	46.26	197.40	29.22	90.24	57.98	74.84	±	26.22
1.10 mg/kg										
Cmax	µg/ml	4.39	9.21	3.42	2.15	5.62	5.37	5.03	±	0.99
tmax	hours	0.50	1.00	2.00	1.00	1.00	1.00	1.08	±	0.20
AUC	µg/ml.h	7.66	25.16	10.59	5.58	24.34	14.42	14.62	±	3.43
AUMC	µg/ml.h^2	14.9	81.41	30.27	13.10	166.42	36.71	57.13	±	24.08
MRT	hours	1.95	3.24	2.86	2.35	6.86	2.55	3.31	±	0.73
MAT	hours	0.82	-2.16	0.94	-0.85	1.90	-7.40	-1.12	±	1.39
F	%	53.23	113.23	52.84	20.48	73.76	50.42	60.66	±	12.61
1.65 mg/kg										
Cmax	µg/ml	1.67	9.80	5.60	7.45	7.91	16.59	8.17	±	2.02
tmax	hours	4.00	2.00	1.00	1.00	1.00	1.00	1.67	±	0.49
AUC	µg/ml.h	9.32	33.83	20.27	19.31	35.20	37.98	25.98	±	4.64
AUMC	µg/ml.h^2	33.74	105.91	74.16	61.21	248.00	311.25	139.04	±	46.19
MRT	hours	3.62	3.13	3.66	3.17	7.03	8.20	4.80	±	0.91
MAT	hours	2.49	-2.27	1.74	-0.03	2.09	-1.75	0.38	±	0.84
F	%	43.18	101.50	67.43	47.26	71.11	88.53	69.83	±	9.27

Table 3.2 Main pharmacokinetic variables in dogs after administration of flunixin at dose rates of 0.55, 1.10 and 1.65 mg/kg by the oral route.

0.55mg/kg

Time	Animal Number						
(h)	1	2	3	4	5	6	Mean
Pre	0	0	0	0	0	0	0
0.50	52.50	64.52	98.96	98.54	98.04	96.36	84.82
1.00	58.75	99.19	99.58	96.34	98.59	96.36	91.47
2.00	40.00	>82.26	99.24	98.21	92.83	78.18	>81.79
4.00	84.38	72.58	91.67	95.61	90.00	56.36	81.77
8.00	76.25	29.03	95.42	26.83	82.17	41.82	58.59
12.00	30.00	43.55	91.94	47.97	80.43	43.64	56.26
24.00	17.50	46.77	58.33	0	39.13	5.45	26.10
48.00	35.00	0	22.22	36.59	0	0	15.63
72.00	45.00	29.03	55.55	65.85	0	34.54	30.16
96.00	62.50	19.35	0	72.36	0	52.73	34.49
168.00	0	25.81	0	72.36	0	43.64	23.63

1.10 mg/kg

Time	Animal Number						
(h)	1	2	3	4	5	6	Mean
Pre	0	0	0	0	0	0	0
0.50	99.12	97.73	69.23	8.43	99.80	99.64	78.99
1.00	97.43	99.70	99.94	97.11	>99.95	98.92	>98.84
2.00	88.82	97.80	99.10	93.25	99.75	93.42	95.36
4.00	67.65	>99.24	92.82	0	98.70	98.14	>76.09
8.00	48.53	71.21	71.79	10.84	97.90	88.57	64.81
12.00	11.76	48.48	0	0	93.40	32.86	31.08
24.00	11.76	12.12	33.33	0	57.0	54.29	28.08
48.00	23.53	0	33.33	0	69.0	0	20.98
72.00	41.18	37.88	47.43	0	66.0	17.14	34.94
96.00	70.59	65.16	42.31	0	63.0	2.96	40.65
168.00	58.82	51.52	7.69	0	54.0	21.43	32.24

1.65 mg/kg

Time	Animal Number						
(h)	1	2	3	4	5	6	Mean
Pre	0	0	0	0	0	0	0
0.50	69.05	98.91	97.25	88.50	>99.92	0	>70.23
1.00	83.93	98.80	99.69	99.94	>99.92	99.84	>97.02
2.00	99.05	99.57	98.81	91.50	99.60	95.32	97.31
4.00	92.86	97.93	95.87	76.25	>99.19	91.61	>92.29
8.00	78.57	82.61	83.75	78.13	93.71	61.29	79.68
12.00	0	43.48	50.00	57.50	79.03	3.22	38.87
24.00	41.67	4.35	51.25	65.00	79.84	0	40.35
48.00	7.14	0	35.00	55.00	50.00	0	24.52
72.00	61.90	0	38.75	76.25	50.00	0	37.82
96.00	0	0	62.50	56.25	66.13	0	30.81
168.00	19.05	0	70.00	56.25	72.58	0	36.31

Table 3.3 Inhibition (%) of thromboxane B_2 in serum of dogs after oral administration of flunixin at three dose rates.

Time	Route of Administration	
(h)	Intravenous	Subcutaneous
Pre	0	0
0.25	12.80 ± 1.05	3.15 ± 0.35
0.50	8.71 ± 0.59	5.31 ± 0.58
1.00	5.28 ± 0.35	6.31 ± 0.63
2.00	2.16 ± 0.21	4.20 ± 0.78
4.00	1.12 ± 0.24	1.56 ± 0.36
6.00	0.51 ± 0.09	NS
8.00	0.34 ± 0.08	0.39 ± 0.07
12.00	0.22 ± 0.11	0.34 ± 0.13
24.00	0.09 ± 0.03	0.04 ± 0.03
48.00	0.04 ± 0.03	0.03 ± 0.02
72.00	0.02 ± 0.02	0.03 ± 0.03
96.00	NS	0.02 ± 0.23
168.00	NS	0

Table 3.4 Mean (±SEM) concentration (μg/ml) of.flunixin in plasma of dogs after administration at a dose rate of 1.10 mg/kg by the intravenous and subcutaneous routes

Parameters		Units	Animal Number Dog1	Dog2	Dog 3	Dog 4	Dog 5	Dog 6	Mean	±	SEM
AUC	(obs)	µg/ml.h	11.23	19.52	16.06	22.26	29.31	24.43	20.47	±	2.60
+ Δ	(obs)	µg/ml.h	14.39	22.22	20.04	27.24	33.00	28.60	24.25	±	2.73
AUC	(cal)	µg/ml.h	13.93	22.11	19.63	34.70	30.63	30.78	25.30	±	3.26
+ Δ	(cal)	µg/ml.h	17.09	24.82	23.61	39.68	34.33	34.95	29.08	±	3.49
AUMC	(obs)	µg/ml.h^2	15.43	119.42	37.41	85.85	162.10	283.52	117.29	±	39.75
+ Δ	(obs)	µg/ml.h^2	16.22	120.10	38.41	87.10	163.02	284.56	118.23	±	39.77
AUMC	(cal)	µg/ml.h^2	16.18	122.45	44.94	745.98	143.69	529.56	267.13	±	122.05
+ Δ	(cal)	µg/ml.h^2	16.97	123.13	45.93	747.23	144.61	530.60	268.08	±	122.11
MRT	(obs)	hours	1.13	5.40	1.92	3.20	4.94	9.95	4.42	±	1.30
MRT	(cal)	hours	1.16	5.54	2.29	21.50	4.69	17.21	8.73	±	3.47
Cp0	(cal)	mg/ml	14.91	11.74	19.38	23.09	16.82	18.83	17.40	±	1.60
Vd_{ss}	(obs)	ml/kg	86.16	267.57	105.21	129.12	164.67	382.68	189.23	±	46.70
Vd_{ss}	(cal)	ml/kg	91.94	275.48	128.26	681.69	168.47	614.94	326.80	±	105.10
Vd a	(cal)	ml/kg	102.10	453.07	175.20	1148.31	246.48	1195.32	553.41	±	201.45
Clb	(obs)	ml/h/kg	76.44	49.50	54.89	40.38	33.33	38.46	48.83	±	6.37
Clb	(cal)	ml/h/kg	78.97	49.75	56.03	31.70	35.91	35.74	47.85	±	7.34
$t^1/_2ß$	(cal)	hours	0.90	6.31	2.17	25.10	4.76	23.18	2.97*		--
V_c	(cal)	ml/kg	73.76	93.70	56.75	47.63	54.40	58.43	65.94	±	6.60
k_{el}	(cal)	hours	1.07	0.53	0.99	0.67	0.55	0.61	0.74	±	0.09
k_{12}	(cal)	hours	2.12	0.43	1.02	0.87	0.53	0.56	0.92	±	0.26
k_{21}	(cal)	hours	0.52	0.22	0.81	0.07	0.34	0.06	0.34	±	0.12

(obs) = calculated from observed values
(cal) = calculated from computed values
+ Δ = computed values for 1st and last triangle added
* = harmonic mean

Table 3.5 Main pharmacokinetic variables in dogs after administration of flunixin at a dose rate of 1.10 mg/kg by the intravenous route.

Intravenous

Time (h)	1	2	3	4	5	6	Mean	±	SEM
0	0	0	0	0	0	0	0		-
0.25	99.86	99.92	99.93	99.91	99.64	99.80	99.84	±	0.04
0.50	99.90	99.95	99.88	99.86	99.93	99.73	99.87	±	0.03
1.00	97.93	99.69	99.66	99.16	99.88	99.60	99.32	±	0.29
2.00	96.56	96.51	98.87	96.05	98.47	98.86	97.55	±	0.54
4.00	96.90	93.10	94.37	95.37	96.66	82.29	93.11	±	2.24
6.00	83.91	86.39	92.48	86.07	92.43	71.61	85.48	±	3.13
8.00	57.33	79.22	87.54	66.67	94.43	75.84	76.84	±	5.52
12.00	13.53	94.44	87.68	72.96	95.21	94.22	76.34	±	13.02
24.00	5.87	0	30.74	25.18	46.44	0	18.04	±	7.78
48.00	0	30.87	0	31.25	17.13	4.77	14.00	±	5.97
72.00	0	0	2.84	0	0	0	0.47	±	0.47

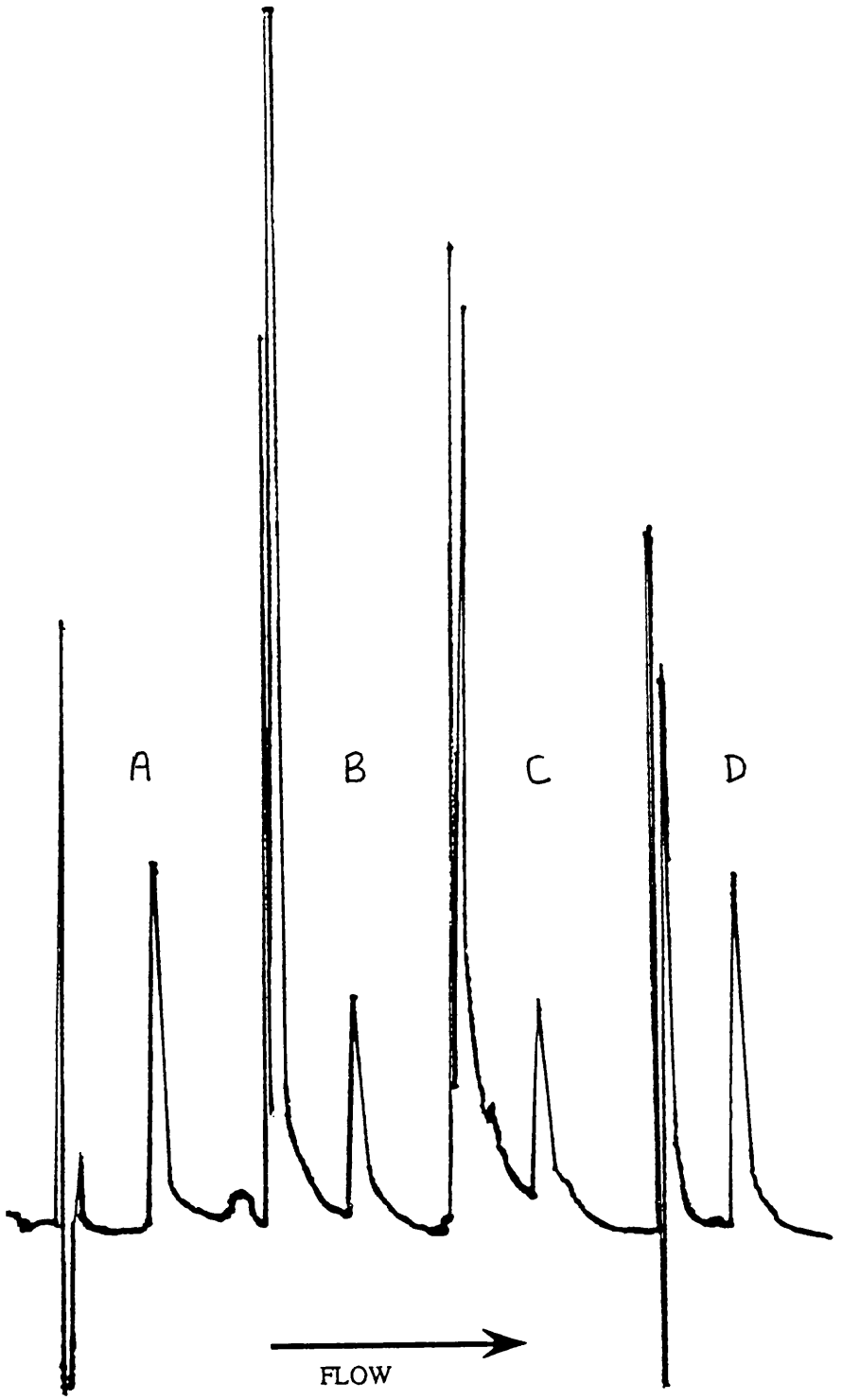
Subcutaneous

Time (h)	1	2	3	4	5	6	Mean	±	SEM
0	0	0	0	0	0	0	0		-
0.25	98.86	99.80	99.84	99.60	99.26	99.43	99.46	±	0.15
0.50	98.77	99.90	99.93	99.86	99.57	99.85	99.65	±	0.18
1.00	99.85	99.76	99.73	99.60	99.72	99.74	99.73	±	0.03
2.00	93.94	98.81	99.88	99.70	98.80	99.33	98.41	±	0.91
4.00	92.88	96.90	99.29	98.89	93.62	77.52	93.18	±	3.31
8.00	86.74	87.19	82.88	88.98	59.84	82.39	81.34	±	4.42
12.00	74.62	79.90	86.28	84.37	47.76	95.51	78.07	±	6.69
24.00	27.27	29.13	8.71	48.51	4.41	58.88	29.48	±	8.74
48.00	5.05	0	0	16.88	0	35.44	9.56	±	5.82
72.00	10.38	8.57	0	0	0	0	3.16	±	2.01
96.00	0	0	0	4.73	0	10.99	2.62	±	1.84
168.00	10.76	0	0	7.72	0	25.71	7.36	±	4.12

Table 3.6 Inhibition (%) of thromboxane B_2 in serum of dogs after administration of flunixin at a dose rate of 1.10 mg/kg by the intravenous and subcutaneous routes.

Parameter	Units	Animal Number 1	2	3	4	5	6	Mean	±	SEM
Cmax	µg/ml	4.12	6.37	6.96	8.55	6.50	5.67	6.36	±	0.60
tmax	hours	0.50	1.00	1.00	1.00	1.00	1.00	0.92	±	0.08
AUC	µg/ml.h	9.95	24.72	30.17	34.87	16.84	41.23	26.30	±	4.73
AUMC	$\mu g/ml.h^2$	29.41	86.93	106.57	215.46	41.73	1345.58	304.28	±	210.04
MRT	hours	2.96	3.52	3.53	6.18	2.48	32.64	8.55	±	4.85
MAT	hours	1.83	-1.88	1.61	2.98	-2.46	22.69	4.13	±	3.82
F	%	69.14	111.25	150.55	128.01	57.45	144.16	101.09	±	15.89

Table 3.7 Main pharmacokinetic variables in dogs after administration of flunixin at a dose rate of 1.10 mg/kg by the subcutaneous route.



A. Standard solution containing 1 µg/ml of drug.

B. Plasma sample estimated to contain 0.94 µg/ml of drug.

C. Blank dog plasma fortified with 1.0 µg/ml drug.

D .Standard solution containing 1 µg/ml of drug.

Figure 3.1 Typical chromatogram of flunixin in dog plasma

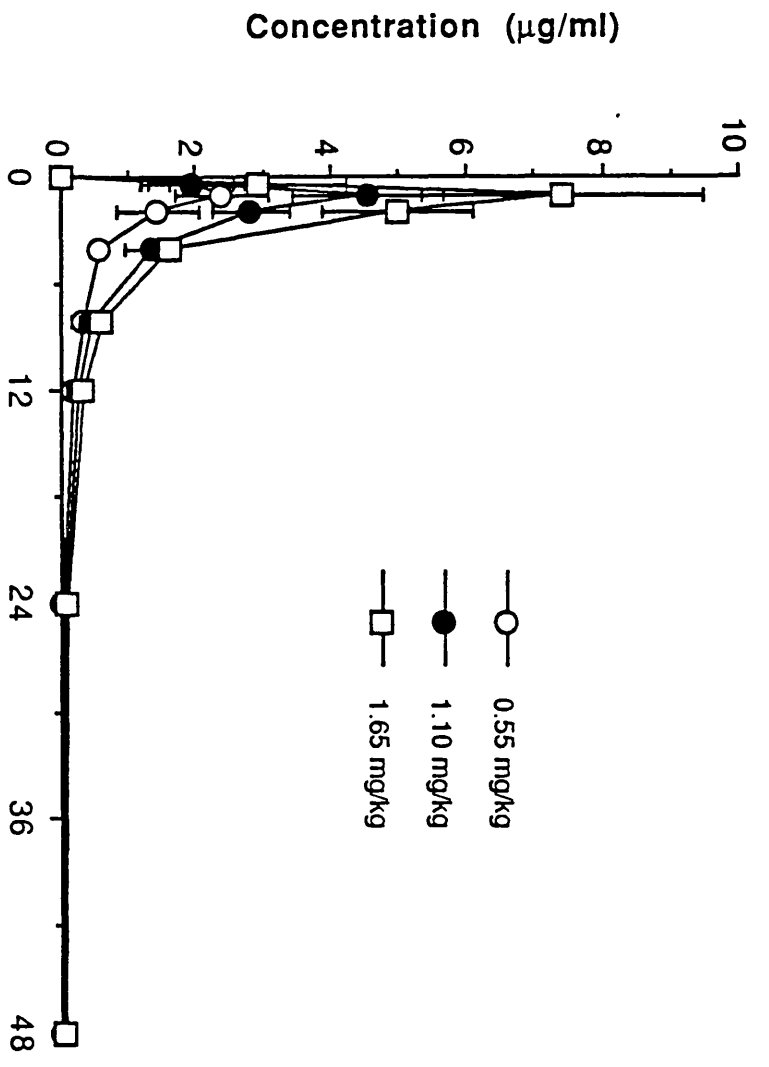


Figure 3.2 Mean concentration (µg/ml) of flunixin in plasma of dogs after oral administration at three dose rates .

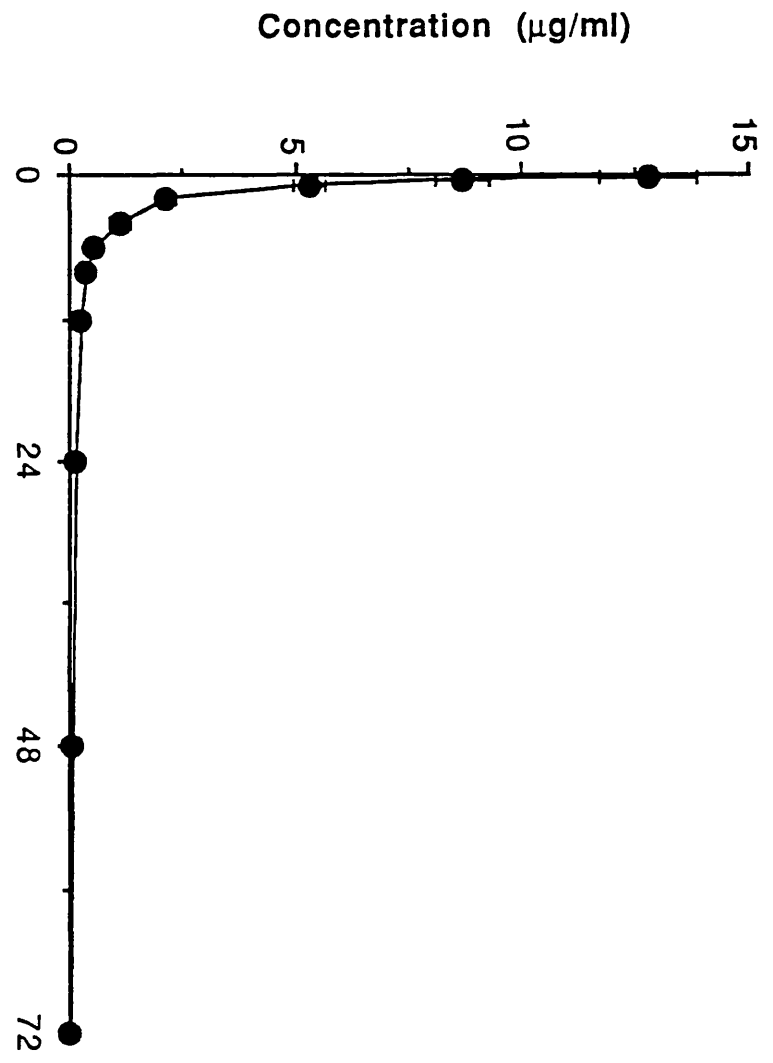


Figure 3.3 Mean (±SEM) concentration (µg/ml) of flunixin in plasma of dogs after intravenous administration at a dose rate of 1.10 mg/kg.

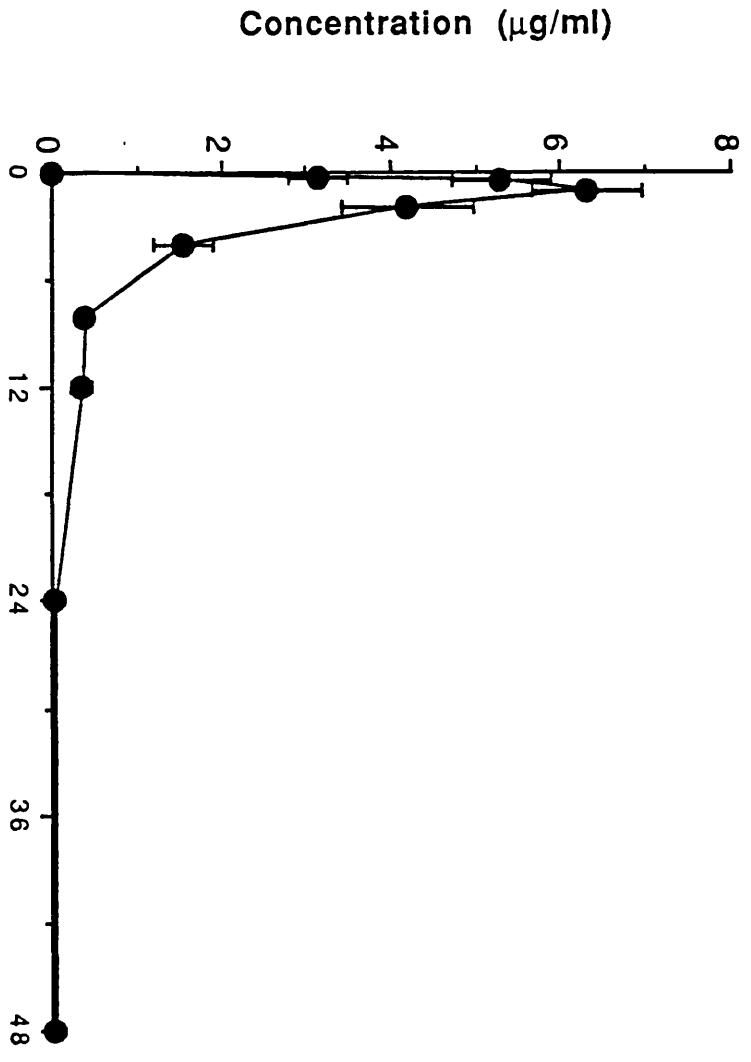


Figure 3.4 Mean (±SEM) concentration (µg/ml) of flunixin in plasma of dogs after subcutaneous administration at a dose rate of 1.10 mg/kg .

Chapter 4

STUDIES WITH PIROXICAM

4.1. INTRODUCTION

Piroxicam (4-hydroxy-2-methyl-N-2-pyridlyl-2H-1,2-benzothiazine-3-carboximide-1,1-dioxide) is a member of the oxicam group of NSAIDs which was first synthesised in the 1970s. It is currently only available for use in humans. The normal daily dose rate is 20 mg per adult, and this has been established to be effective and safe (Wiseman, 1985).

Piroxicam exhibits no opioid like analgesic activity, in hot plate and tail-flick tests (Brogden et al, 1981). However, in other experimental models such as the phenylquinone-induced writhing test it has shown substantial analgesic activity. At doses of 10 - 32 mg/kg orally, piroxicam inhibited phenylquinone-induced writhing in mice to a similar extent to the reduction observed after 320 mg/kg aspirin (Wiseman et al 1976). When using this pain model Milne and Twomey (1980) found 1.85 mg/kg piroxicam to be less effective than indomethacin (0.39 mg/kg) but more effective than ibuprofen (4.9 mg/kg), naproxen (201 mg/kg) and phenylbutazone (32 mg/kg) in reducing writhing frequency by 50 %. Schiantarelli and Cadel (1981) found piroxicam to be 70 times more effective in this model than phenylbutazone and Wiseman (1978) found it to be 11 times more effective than naproxen and 64 times more effective than aspirin. Piroxicam has been shown to give relief of post-operative pain one hour after oral administration (Jain et al, 1978) and to produce some improvement in pain from gout after 2 hours (Widmark, 1978). Other studies have shown that administration of 20 mg/kg orally produces true analgesic effects in man within 1-2 hours of administration, and maximal effects were observed at 3-4 hours (Pitts, 1982). With repeated daily doses, pain relief increases over a 6-8 day period (Tausch, 1982), and it is likely that this is due to increasing plasma drug concentrations until steady state is achieved.

Pitts (1982) reported that there was a difference in blood concentration of drug which produces analgesic and anti-inflammatory effects. Clinical analgesia was seen at 2 μg/ml, however optimal anti-inflammatory effects in man were seen at blood concentrations of 5 μg/ml.

Use of 10 mg/kg piroxicam was found to be as effective as 56 mg/kg aspirin in the inhibition of experimental pyrexia produced by the injection of Eschericha coli lipopolysaccharide.

Piroxicam has been shown to be effective in the treatment of inflammatory conditions such as rheumatoid arthritis and osteoarthritis. In osteoarthritis a dose of 20 mg daily was found to produce maximal improvement (Zizic et al, 1978), and this dose was found to as effective as the standard dose of phenylbutazone in the treatment of ankylosing spondylitis (Pitts, 1982). In the treatment of rheumatoid

arthritis, piroxicam was equal to or superior to aspirin, naproxen, diclofenac, ketoprofen and indomethacin (Rohde et al, 1980; Finstad, 1981; Sydness, 1981; Villiaumey, 1981; Willkens et al,1982), and in the treatment of oesteoarthritis its effectiveness has equalled or exceeded that of aspirin, naproxen and indomethacin (Goldie, 1981; Abruzzo et al,1982). Siegmeth (1980), found the clinical response to be related to plasma drug concentrations in rheumatoid arthritis.

Use in experimentally produced inflammation has shown piroxicam to be effective both topically and orally (Kandil et al, 1980; Larson and Lombardino, 1980).

Radiographs of inflamed joints of arthritic rats indicated that piroxicam therapy resulted in large decreases in the deposition of granuloma tissue and a reduction in bony erosions (Otterness et al, 1982).

Piroxicam has been shown to have a $t^1/_2\beta$ in man of approximately 45 hours (Nuotio and Makisara, 1978; Wiseman and Hobbs, 1982). Conflicting reports of 45 hours (Wiseman and Hobbs, 1982) and 17.3 hours have been given for the $t^1/_2\beta$ in dogs (Esteve et al, 1986). The markedly shorter $t^1/_2\beta$ found in rats (4-19 hours) (Esteve et al, 1986) and monkeys (4.6 hours) (Wiseman, 1978), indicates that this value varies widely with species, and it has been demonstrated that sex related differences also occur in rats where the $t^1/_2\beta$ was found to be longer in the female of the species (Hobbs and Twomey,1979; Esteve et al, 1986). In man, it has been shown that $t^1/_2\beta$ is not related to dose rate (Wiseman and Chiaini, 1972; Hobbs and Twomey, 1979) and that concentrations in blood remain within the therapeutic range during the 24 hour period following a single dose of 10-40 mg/kg (Wiseman, 1978). Steady state in blood is reached within 7 days when piroxicam is given once daily, and the long half life results in a slow decay of plasma concentrations when the drug is withdrawn (Nuotio and Makisara, 1978).

Piroxicam is rapidly absorbed after oral administration in man (Hobbs and Twomey, 1979). Measurable concentrations were found in blood at 1-2 hours and maximum concentrations occured at 8 hours. It was also found to be rapidly absorbed after rectal administration, and bioavailability by this route was found to be similar to that after oral administration in rats, rabbits and man (Schiantarelli et al, 1981). A plasma concentration profile containing multiple drug peaks was found after administration of piroxicam in man (Hobbs and Twomey, 1979), this suggested that piroxicam may undergo enterohepatic recycling.

Patients with rheumatoid arthritis have been shown to have synovial fluid concentrations of drug which were approximately 40% of those in plasma (Bachmann, 1980).

It is likely that the anti-inflammatory activity of piroxicam is, at least in part, a result of its ability to inhibit cyclooxygenase enzymes and it has been established that it is a

potent and reversible inhibitor of prostaglandin synthesis (Carty et al, 1980a and b). Estimation of serum PGE_1, PGE_2 and PGF_{2alpha} concentrations in asthmatic patients showed reductions within 1-3 hours of administration of piroxicam (Spector et al, 1979).

The anti-inflammatory properties exhibited by piroxicam may also result from its effect on cells in inflamed tissue. A dose of 5 mg/kg piroxicam was reported to inhibit the increase in synovial pressure, and reduce the number of leukocytes in inflamed joints produced by injection of urate crystals into the knee joint of dogs (Wiseman et al, 1976). When administered at a dose rate of 20 mg/kg after the experimental induction of pleural inflammation in rats, there was an inhibition of pleural exudate production. The total leukocyte migration and the monocyte component of this was also reduced (Otterness et al, 1982). In addition, it has been shown that migration of leukocytes into sponges implanted in rats was significantly reduced after oral administration of piroxicam at dose rates of 5 and 10 mg/kg, and that both mononuclear and polymorphonuclear leukocyte numbers were reduced (Schiantarelli et al, 1981). It has also been demonstrated that aggregation, secretion of lysosomal enzymes and generation of tissue destructive superoxide radicals by human neutrophils are inhibited by low concentrations of piroxicam (ID_{50} 50 μM, approximately 0.2 μg/ml) (Edelson et al, 1982).

Piroxicam is extensively metabolised prior to excretion. Less than 10% of the administered dose was found to be excreted unchanged in urine (Wiseman, 1978; Brogden et al, 1981). A variety of metabolites are produced depending on species. However, for all species hydroxylation of the pyridyl ring is an important pathway (Wiseman, 1978), and metabolites thus produced have been found to be 1000 times less inhibitory to the production of prostaglandins than the parent drug (Wiseman and Boyle, 1980). In addition, metabolites have little or no anti-inflammatory activity in animal models studied (Spector et al, 1979).

Studies on piroxicam have shown it to be well tolerated, however, LD_{50} values have shown wide inter-species variation. Reported LD_{50} values after oral administration are 270 mg/kg in rats, 360 mg/kg in mice and more than 700 mg/kg in dogs, indicating that piroxicam has a good therapeutic index. Chronic administration at daily dose rates of 2.5, 5 and 10 mg/kg to primates over a 12 month period resulted in no drug related toxicity (Wiseman, 1978). Gastrointestinal lesions and renal papillary necrosis were, however, seen in dogs, rats and mice after administration over periods of 12-18 months. At higher dose rates, these side effects were seen to be more severe in female compared to male rats (Wiseman, 1978). In clinical studies, 32 % of 3000 patients being treated for inflammatory disorders manifested side effects. Of this total, 19 % were related to the gastrointestinal tract. However, unlike indomethacin

there was no other single major group of side effects (Pitts, 1982). No evidence of organ toxicity was found by these workers although small reversible blood urea nitrogen elevations were measured. Such changes in blood urea nitrogen are thought to be common with NSAID administration, and Pitts (1982) has suggested that they may be a result of inhibition of renal prostaglandin biosynthesis. Piroxicam was found to be one third as ulcerogenic in the gastrointestinal tract as indomethacin in rats (Ohtsuki et al, 1981) and ibuprofen in man (Pitts, 1982) at their respective anti-inflammatory dose rates. Although gastrointestinal side effects were the most common intolerance associated with piroxicam, such episodes were less frequent than after administration of therapeutically equivalent doses of aspirin, indomethacin and naproxen. Studies on reproductive effects in rats and rabbits have shown piroxicam to be non embryo or foeto-toxic, although in rats a dose related delay or inhibition of parturition was observed (Wiseman, 1978). Overall, Pitts (1982) has reported fewer side effects after administration of piroxicam than after aspirin, indomethacin and phenylbutazone and a comparable number to that produced after ibuprofen or naproxen administration.

Protein binding of piroxicam is high in all species. In dogs, binding was 93 % at serum drug concentrations of 5 μg/ml and in man was 99 % at concentrations of 5 to 50 μg/ml indicating that the degree of protein binding is not concentration related (Hobbs and Twomey, 1979). Co-administration of aspirin was found to have no effect on the kinetics of piroxicam and did not cause displacement of bound drug (Hobbs and Twomey, 1979). This lack of displacement is not common in extensively protein bound drugs where competition for binding sites is high. In man, aspirin has been shown to reduce the plasma concentration of other NSAIDs such as naproxen (Segre et al, 1974) diclofenac (Muller et al, 1977) and indomethacin (Kaldestad et al, 1975). It may be that the low doses used and resultant low concentrations of piroxicam do not result in the occupation of a significant number of binding sites.

4.2 EXPERIMENTAL OBJECTIVES

The objectives of this work were to examine the kinetics of piroxicam after administration at the recommended dose rate of 0.3 mg/kg body weight by the intravenous and oral routes and to quantify the inhibition of TxB_2 in serum following administration of piroxicam by each route.

4.3 MATERIALS AND METHODS

4.3.1 ANALYSIS OF PIROXICAM

Analysis of piroxicam was carried out by high performance liquid chromatography (H.P.L.C.) using spectrophotometric detection.

4.3.1.1 REAGENTS

1. Piroxicam (Sigma Chemical Co.)

2. Acetonitrile, redistilled grade (Rathburn Chemicals)

3. Orthophosphoric acid (BDH Ltd)

4. Potassium dihydrogen phosphate 'Analar' (BDH Ltd)

5. 1-Heptane sulphonic acid (Sigma Chemical Co.).

6. Water, redistilled in the presence of potassium permanganate prior to use.

7. H.P.L.C. mobile phase comprising 427.5 ml acetonitrile, 5.1 ml phosphoric acid, 1.72 g potassium dihydrogen phosphate and 0.564 g 1-heptane sulphonic acid per litre of water.

4.3.1.2 H.P.L.C. EQUIPMENT

Pump :-	Gilson 301
Detector :-	Spectraphysics 100
Column :-	100 mm X 8 mm containing 5 μ O.D.S. Hypersil (Shandon Southern).
Wavelength :-	354 nm
Absorbance :-	0.01 AUFS

Flow rate of mobile phase :- 1.0 ml/min.

Retention time of piroxicam under these conditions was 2.3 minutes.

4.3.1.3 SAMPLE PREPARATION

One millilitre of plasma was pipetted into a conical centrifuge tube and 1 ml of acetonitrile added. The tube was stoppered and the contents mixed by vortexing for 30 seconds. The sample was then centrifuged at 1500 g and 4 ^{o}C for 15 minutes and the resultant clear supernatant recovered into a clean stoppered tube prior to chromatography. Samples were chromatographed within five hours of preparation. A 100 µl stainless steel loop was used for injection, the loop was loaded with 50 µl of sample.

The concentration of piroxicam in each sample was determined by reference to a calibration curve for each analysis. This was prepared by fortifying blank plasma with known amounts of piroxicam to produce the appropriate range of concentrations. A number of fortified plasma aliquots treated in this way were processed in an identical manner to that described for unknown samples. In addition, standard solutions of piroxicam were injected directly into the HPLC. These standard solutions allowed equipment performance to be monitored and were also used to calculate the concentration of piroxicam measured in the fortified samples. From these figures it was possible to calculate the percentage recovery of piroxicam after extraction.

Unknown sample concentrations were also calculated by reference to the standard drug solutions. These concentrations were then adjusted to 100 % to allow for extraction losses by comparison to the recoveries obtained for the fortified samples.

A typical chromatogram is shown in figure 4.1

4.3.1.4 RECOVERY, PRECISION AND LINEARITY

Recovery of drug from plasma samples was approximately 70 %. Appendix 1.2 shows typical recoveries of piroxicam, inter-assay and intra-assay variation.

Calculation of the inter-assay coefficient of variation for recovery of piroxicam from fortified plasma allowed an assessment of the precision of the assay to be made. The mean inter-assay variation was found to be 10.3 % mean intra-assay variation was 2.5 %.

Simple regression was used to determine the linearity of concentration with respect to peak height for fortified samples. The correlation coefficient (r) for concentrations ranging from 0.1 µg/ml to 2.0 µg/ml of piroxicam in plasma was 0.998.

4.3.1.5 OTHER ANALYSES AND METHODS USED.

The following techniques and methods were employed during the studies carried out on piroxicam. Estimation of serum TxB_2 concentration, estimation of platelet numbers in blood, blood clotting time and detection of occult blood in faeces, pharmacokinetic and statistical analysis.These methods were also used in studies on other drugs included in this thesis and are included in the Materials and Methods section.

4.4 EXPERIMENTS WITH PIROXICAM

Three male and three female Beagle dogs aged between 1 and 2 years were used for all experimental work.
Animals were housed in pairs and fed a complete cereal diet once daily, water was available ad libitum.

4.4.1 INTRAVENOUS ADMINISTRATION OF PIROXICAM

4.4.1.1 EXPERIMENTAL DESIGN

The six dogs were administered piroxicam at a dose rate of 0.3 mg/kg on a single occasion. The dogs were weighed immediately prior to the experiment and the dose of piroxicam to be administered calculated according to body weight. Weights ranged from 11.5 - 16.3 kg.

4.4.1.2 DRUG ADMINISTRATION

No intravenous preparation of piroxicam is commercially available therefore piroxicam powder (Sigma Chemical Co.) dissolved in ethanol was administered. A solution containing 1.33 mg/ml of piroxicam in ethanol was prepared and administered into the right cephalic vein at a dose rate of 0.3 mg/kg over a period of thirty seconds. Administration of piroxicam was carried out one hour prior to feeding.

4.4.1.3 SAMPLING REGIMEN

Blood was collected from the jugular vein into suitable tubes for drug analysis, haematology and serum TxB_2 estimation as described previously. Sampling was carried out prior to and at 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 48 and 72 hours post drug administration.

In addition samples of blood were taken into capillary tubes prior to drug administration and at 0.5, 1, 2, 4, 6, 8, 24,48 and 72 hours for estimation of clotting times.
The presence of occult blood was investigated in faecal samples which were collected throughout the experiment.

4.4.2 ORAL ADMINISTRATION OF PIROXICAM

4.4.2.1 EXPERIMENTAL DESIGN

The six dogs were administered piroxicam at a dose rate of 0.3 mg/kg on a single occasion. The dogs were weighed immediately prior to the experiment and the dose of piroxicam to be administered calculated according to body weight. Weights ranged from 11.7 - 16.5 kg.

4.4.2.2 DRUG ADMINISTRATION

Piroxicam (Feldene; Pfizer, UK) was administered orally in capsules at a dose rate of 0.3 mg/kg. In order to accurately achieve this dose rate, the contents of the commercial preparation were removed from their capsules and re-weighed into gelatine capsules thus providing the correct drug dosage for each animal. Administration of piroxicam was carried out one hour prior to feeding.

4.4.2.3 SAMPLING REGIMEN

Blood samples were collected from the jugular vein, prior to and at 0.5, 1, 2, 4, 8, 12, 24, 48, and 72, hours following drug administration using suitable tubes for drug analysis, haematology and serum TxB_2 estimation.
Blood samples were also collected in capillary tubes before drug administration, and at 0.5, 1, 2, 4, 8, 24, 48 and 72 hours after drug administration.
Faecal samples, which were collected throughout the experiment, were tested for the presence of occult blood.

4.5 RESULTS OF EXPERIMENTS WITH PIROXICAM

4.5.1 INTRAVENOUS ADMINISTRATION

4.5.1.1 PIROXICAM CONCENTRATIONS AND PHARMACOKINETICS AFTER INTRAVENOUS ADMINISTRATION

Appendix 5.1 shows the concentration of piroxicam in the plasma of each dog after intravenous administration of piroxicam at a dose rate of 0.3 mg/kg. Individual concentrations are also given in figure 4.2, and mean (±.SEM) concentrations are shown in table 4.1 and figure 4.3. The main pharmacokinetic variables are shown in table 4.2.

The mean concentration of piroxicam measured in plasma of dogs at 0.25 hours after drug administration was 1.11 ± 0.05 μg/ml and the inter-animal range in concentration was comparatively small (0.99 - 1.25 μg/ml). The concentration of drug had declined in all animals at 0.5 hours. However, plasma concentrations of drug rose again in all animals. These second peaks of concentration were similar to those measured at 0.25 hours for dogs 1, 2 and 6 and exceeded the earlier concentrations for the other animals. The second peaks occured between 2 and 8 hours. A further small peak in concentration was noted at 24 hours in samples from all dogs, with the exception of dog 1. Piroxicam was detected in the plasma of all animals at 72 hours (mean concentration 0.30 ± 0.04 μg/ml).

The inter-animal variation in computed $t^1/_2$ ß was large, values ranged from 30.54 to 53.72 hours, however, estimates of $t^1/_2$ ß calculated from MRT values for observed data were somewhat lower than this (range 18.61 to 23.24 hours). Similarly, the MRT value calculated from computed data (58.27 ± 7.92) was approximately double the value calculated from observed data (26.78 ± 0.79 hours). The inter-animal variation in persistence of piroxicam in plasma was also greater for MRT values calculated from computed data, especially if the estimated last triangle of the AUC and AUMC were included.

Observed AUCs ranged from 34.63 to 63.69 μg/ml.h. The mean observed AUC after administration of piroxicam at a dose rate of 0.3 mg/kg by the intravenous route was 47.39 ± 4.52 μg/ml.h.

The area under the first moment curve for observed values had a wide inter-animal variation. The mean observed AUMC was 1259 (±100) hours, with a range of 912 to 1499 $\mu g/ml.h^2$. As with the MRT this value was much larger if calculated from computed best fit curve values and also if the calculated last triangle was included.

Mean body clearance of drug was 6.62 ± 0.62 ml/h.kg when calculated from observed values and 4.04 (± 0.58) hours when calculated for computed values for the same time period. The volume of distribution at steady state was 178

(± 18) ml/kg, for observed data and 235 (± 38) ml/kg when calculated from parameters for computed theoretical best fit curves.

4.5.1.2 SERUM THROMBOXANE INHIBITION AFTER INTRAVENOUS ADMINISTRATION

Serum TXB_2 concentrations for each animal at each time point are shown in appendix 5.2 and percentage inhibition of TxB_2 in table 4.3.

Inhibition of TxB_2 was greater than 90 % in all animals at 0.25 hours after drug administration (mean >96.85 %). The time at which maximum inhibition was detected varied widely between animals (range 0.25 - 4 hours). The mean maximum inhibition was greater than 98 % and was found to occur at 1.04 hours. Inhibition of serum TxB_2 exceeded 40 % in five animals at 72 hours (mean 50.21 %). The area under the serum TXB_2 inhibition versus time curve after intravenous administration of piroxicam was 5310 %.h.

4.5.1.3 PLATELET NUMBERS AFTER INTRAVENOUS ADMINISTRATION

Appendix 5.3 shows platelet numbers for each dog on each sampling occasion. Samples from all dogs showed a marked decrease in platelet numbers 0.25 hours after administration of piroxicam and counts were found to be below pre drug administration levels throughout the experiment. Dogs 3, 4, 5 and 6 had platelet counts of 30 x 10^9 per litre of blood or less at 0.25 hours and dogs 4, 5 and 6 had counts of around 50 x 10^9 per litre at 0.5 hours.

4.5.1.4 CLOTTING TIMES AFTER INTRAVENOUS ADMINISTRATION

Clotting times for each animal at each sampling time and mean clotting times after administration of piroxicam by the intravenous route are shown in appendix 5.4. Several samples from dog 4 (at 2, 6 and 8 hours) and one sample from dogs 3 (4 hours), 5 (24 hours) and 6 (4 hours) were found to be slightly outwith the range established as normal for these dogs. No apparent pattern of altered clotting time was detected throughout the experiment.

4.5.1.5 OCCULT BLOOD IN FAECES AFTER INTRAVENOUS ADMINISTRATION

Gastrointestinal blood loss determined throughout the experiment by Colo-Rectal test are contained in appendix 5.5. Blood was not detected in faecal samples with the exception of a single sample from pen 2 (dogs 3 and 4) at 72 hours, in which blood was detected.

4.5.2 ORAL ADMINISTRATION

4.5.2.1 PIROXICAM CONCENTRATION AND PHARMACOKINETICS AFTER ORAL ADMINISTRATION

Appendix 6.1 contains the concentration of piroxicam in the plasma of each dog after oral administration at a dose rate of 0.3 mg/kg. Mean (± SEM) concentrations are shown in table 4.1 and figure 4.4. The main kinetic variables are shown in table 4.4.

The first sample time after oral administration of piroxicam was 0.5 hours and at this time the mean concentration of drug in plasma was 0.77 ± 0.14 μg/ml. As shown by the standard error the inter-animal range in concentration was large (0.27 - 1.27 μg/ml). The maximum mean concentration (1.27 ± 0.14 μg/ml) occured at two hours after drug administration. Piroxicam was detected in the plasma of all animals at 72 hours (mean concentration 0.39 ± 0.06 μg/ml) and the C_{max} was 1.35 μg/ml. There was a large inter-animal variation in time of maximum concentration (t_{max} 3.33 ± 1.09 hours). In one animal (dog 6), the maximum concentration of drug occured at 1 hour and this concentration of drug was also detected in the 2 hour sample. In four animals, (dogs 2, 3, 5 and 6), the plasma concentration versus time graph showed a multi-peak profile

There was good inter-animal agreement in the persistence of piroxicam (MRT) after oral administration, the range being 26.31 to 29.62 hours for observed values (mean 28.42 ± 0.63 hours). The mean observed AUC after administration of piroxicam at a dose rate of 0.3 mg/kg by the oral route was 46.77 μg/ml.h. After addition of the estimated last triangle this figure was 100.72 μg/ml.h and the inter-animal variation was greatly increased. The area under the first moment curve for observed data ranged from 941 to 1751 $\mu g/ml.h^2$, and this value, and the inter-animal variation, was also greatly increased by the addition of the estimated last triangle.
Bioavailability (calculated for the period 0 to 72 hours) showed a very wide inter-animal variation, range 76 to 160 μg/ml.h (mean 103 ± 12 μg/ml.h).

4.5.2.2 SERUM THROMBOXANE INHIBITION AFTER ORAL ADMINISTRATION

Serum TxB_2 concentrations for each animal at each time point are shown in appendix 6.2, and percentage inhibition of TxB_2 in table 4.5.

Inhibition of TxB_2 at the first sample time of 0.5 hours was greater than 70 % in only three animals (dogs 2, 3 and 4) and in one animal (dog 5) was found to be less than 45 % (mean inhibition 64.71 ± 5.34 %). In all but two animals (dog 2 and 3), inhibition had increased at 1 hour and the mean inhibition at this time was 72.45 ± 3.38 %. Maximum mean inhibition (74.35 ± 2.91%) occured at 8 hours, however the time at which maximum inhibition occured in individual animals varied widely (range 0.5 - 8 hours) and the mean maximum inhibition of 73.74 (± 1.66) % occured at 3.25 hours. The serum TxB_2 inhibition versus time graphs showed a multi-peak profile, and one or more secondary peak in inhibition occured for each animal. In all but one animal (dog 1) inhibition remained greater than or close to 40% at 72 hours. The area under the serum TXB_2 concentration versus time curve was 4067 %.h.

4.5.2.3 PLATELET NUMBERS AFTER ORAL ADMINISTRATION

Appendix 6.3 gives platelet numbers for each dog on each sampling occasion after oral administration of piroxicam at a dose rate of 0.3 mg/kg. A large inter and intra-animal variation in platelet counts was observed throughout the experiment, however, in no animal was a consistent trend apparent.

4 5.2.4 CLOTTING TIMES AFTER ORAL ADMINISTRATION

Clotting times for each animal at each sampling time and mean clotting times are shown in appendix 6.4. These values remained within the expected range at all sample times with the exception of the 1 hour sample from dog 3.

4.5.2.5 OCCULT BLOOD IN FAECES AFTER ORAL ADMINISTRATION

Faecal samples collected throughout the experiment were tested for the presence of gastro-intestinal blood by Colo-Rectal and the results are shown in appendix 6.5. No blood was detected in any faecal sample collected during the experiment.

4.6. DISCUSSION

Piroxicam was well tolerated after intravenous and oral administration to dogs. However, clinical observations consistent with the administration of alcohol were recorded during the first 0.5 hours after intravenous administration. Peripheral vasodilation was noted and it is likely that some lowering of blood pressure would have been associated with this. The actions of alcohol are similar to those of general anaesthetics as it causes irregularly descending depression of the higher centres of the central nervous system. It is not surprising, therefore, that slight depression was noted in all animals after intravenous administration of the drug solution.

The plasma concentration versus time curves for the drug were characterised by successively lower peaks and troughs after both intravenous and oral administration. The intra-animal variation in plasma concentration of drug for each route of administration was generally small. In three animals (dogs 3, 4 and 5) the maximum concentration of drug in plasma after intravenous administration occured at 4 hours and not at the first sample time (0.25 hours) as is normally observed after intravenous administration and this may indicate that some of the drug dose was injected peri-venously. However, concentration of drug was high in the first sample from these dogs (0.98 - 1.25 μg/ml) and was lower at the next sampling occasion (0.5 hours) indicating that it was unlikely that drug was being absorbed from surrounding tissue.

Concentrations of piroxicam detected in plasma at 0.5 hours after oral administration indicated that the drug had been rapidly absorbed. A pattern of secondary peaks and troughs, similar to that seen after intravenous administration was also seen. Plasma drug concentrations were generally well maintained for 24 hours at which time a mean concentration of 0.8 μg/ml was detected.

The pattern of peaks and troughs in plasma drug concentration seen generally after each route of administration may be due to enterohepatic circulation. This is in agreement with the evidence presented by Dupont et al (1982) and Hobbs and Twomey (1979) who reported that a plasma profile consistent with enterohepatic cycling was seen after administration of piroxicam to human subjects. The occurrence of enterohepatic cycling can result in difficulty in producing reliable pharmacokinetic data.

Mathematical modelling of the drug concentration versus time curves produced after intravenous administration of piroxicam demonstrated that the data were best described by one exponent. This was confirmed by application of AIC and is in

agreement with the findings of Esteve et al (1986). In the case of only one animal (dog 1), was the correlation (r^2) of these best fit curves greater than 0.9, as a result there was considerable variation between kinetic values calculated from these best fit curves and those calculated from observed data. Mathematical modelling of the concentration versus time data obtained after oral administration was only possible in one animal (dog 1) for this animal the correlation was 0.97, accordingly all pharmacokinetic parameters are calculated from observed data.

Drug was detected in the plasma of all animals at 72 hours after administration of piroxicam in each experiment and this was reflected in the large AUC, AUMC and long $t^1/_2\beta$ (40.16 hours) calculated for these animals. The $t^1/_2\beta$ of piroxicam which was calculated after intravenous administration (40.16 hours) is similar to that previously reported in man and dogs (Wiseman and Hobbs, 1982). In the present study, the group of six dogs comprised three male and three female animals. Comparison of the $t^1/_2\beta$ from male and female animals showed no significant difference. The arithmetic mean obtained for males was, however, higher than that for females (46.31 vs 37.25 hours). This difference is the converse of the findings of Esteve et al (1986), who reported $t^1/_2\beta$ values of 4.66 and 19.52 hours (respectively) for piroxicam in male and female rats. The overall $t^1/_2\beta$ value from this study has been expressed as the harmonic mean. Comparison of the harmonic mean $t^1/_2\beta$ values for male and female dogs in this study also shows good agreement between sexes (35.36 hours for male and 36.40 hours for female).

Piroxicam was found to have an excellent oral bioavailability (mean 102.69 %). In three animals (dogs 1, 2 and 6), this was found to be greater than 100% and in the case of one dog (animal 6), the bioavailability was calculated to be approximately 160%. The cause of this overestimate remains unexplained. However, good oral absorption coupled with intra-animal experimental error seem unlikely explanations for such a large overestimate.

Mean absorption times (MAT) calculated from observed data varied widely amongst animals. The maximum value was calculated for dog 5 (6.08 hours) and a negative value was calculated for dog 1 (-0.01 hours). This negative value is theoretically impossible as it implies that drug entered the bloodstream faster after oral administration than after intravenous administration. It is likely that this phenomenon is a result of rapid absorption coupled with intra-animal variation which may have occured between the two experiments.

Comparison of bioavailability and MRT values calculated from observed data indicate that, in terms of availability of piroxicam in circulating plasma, there would

be little to be gained from intravenous administration. However, comparison of oral data with data derived from the best fit mathematical model, which makes allowance for the section of the intravenous curve between Cp0 and the first sample time indicate that both mean AUC and mean MRT were considerably larger after intravenous administration.

The maximum inhibition of TxB_2 after intravenous administration was greater than 90 % for all animals and inhibition of greater than 80 % was observed in all but one sample for 8 hours. Platelet numbers were considerably lower than pre-treatment values throughout this experiment and since platelets are the principal source of TxA_2 in the circulation it is possible that the reduction in TxB_2 concentrations is related to reduced platelet numbers rather than cyclooxygenase inhibition. The cause of the thrombocytopenia observed during this experiment is unknown. All animals appeared clinically healthy throughout the experiment. Although thrombocytopenia has been recorded as a side effect of piroxicam (Bjornstad and Vik, 1986) it was not observed in these animals after administration of piroxicam at the same dose rate by the oral route. In addition, some increase in platelet numbers was observed after 0.5 hours and on this time scale it is unlikely that recovery could be explained by the production of new platelets. The presence of ethanol in the circulation is a complicating factor. The likely concentration of ethanol in blood of these animals would have been around 200 milligrams per one hundred millilitres of blood (approximately equivalent to a human subject having consumed six pints of beer), a figure which is frequently exceeded in man, in whom thrombocytopenia (and other blood disorders) have been linked with alcoholism (Lindenbaum and Hargrove, 1968; Post and Desforges, 1968a; Cowan and Hines, 1971) even in the absence of nutritional deficiency (Lindenbaum and Leiber, 1969). However, such studies have observed the onset of thrombocytopenia over a longer period of exposure to alcohol, for example 20 days at blood alcohol levels up to 125 mg/100 ml (Lindenbaum and Leiber, 1969). In addition, it has been observed that mice exposed to alcohol vapour (10-25 mg/litre air) for 20 or more days also developed blood disorders, including thrombocytopenia and at higher vapour concentrations (25-38 mg/litre), similar abnormalities were observed after 24 hours.(Malik and Wickramasinge, 1986). Thrombocytopenia was not detected by Cowan and Hines (1971) after the intravenous administration of ethanol to man and in a similar study by Haut and Cowan (1974) which produced blood alcohol concentrations of around 200 mg/100 ml blood no thrombocytopenia was reported. This is in contrast to an earlier study reported by Post and Desforges (1968a) where patients were administered 2,000 ml of 5 % ethanol in dextrose by intravenous infusion at a rate of 11 ml/minute. These workers recorded a fall in platelet count at 4 - 6 hours after the start of infusion, a

recovery in platelet numbers began to be apparent several hours after administration was discontinued. The thrombocytopenia associated with alcohol consumption is generally characterised by a reduction in platelet lifespan. In one case the longevity of the platelets was found to be reduced by 50 % or more (Cowan, 1973). However, the speed of recovery of platelet counts noted by Post and Desforges (1968b) indicates that the mechanism of recovery is not replacement by new platelets. These workers also investigated the possibility of splenic or hepatic sequestration of platelets after intravenous administration of ethanol. Platelets carrying a radioactive label were introduced into the patient prior to alcohol administration and the radioactivity present in the spleen and liver subsequently monitored. No increase in the number of labelled platelets was found in these organs.
In the present experiment, the platelet counts varied widely between sample times and did not appear to follow a time related course. It is not possible to determine if this effect was due to piroxicam or ethanol administration or to some other unidentified cause.

The maximum inhibition of serum TxB_2 was considerably less after administration of piroxicam by the oral route. The maximum mean inhibition was 74.3 % and occured at 8 hours, at no time did the inhibition exceed 82 % in any animal. In addition, there was wider inter and intra-animal variation in inhibition after oral than after intravenous administration. No inhibition of TxB_2 was measured in the serum of dog 1 at 72 hours.
Comparison of the area under the serum TxB_2 inhibition versus time curve after intravenous and oral administration of piroxicam showed that the AUC after oral administration was 77% of that obtained after intravenous administration.
Statistical examination was also carried out on the maximum inhibition produced by each route of administration and on the inhibition measured at each time point. The mean maximum inhibition was 98.00 and 75.88 % after intravenous and oral administration respectively and these figures were found to be significantly different ($p = 0.014$). In addition TxB_2 inhibition was significantly greater at all common time points ($p < 0.05$) after intravenous administration than after oral administration, with the exception of 72 hours.

The presence of relatively high concentrations of piroxicam in plasma (up to 0.56 µg/ml) at 72 hours after the administration of a single dose of 0.3 mg/kg by the oral route suggests that daily administration would result in accumulation of the drug. The estimated $t^1/_2\beta$ of around 40 hours, in conjunction with the extent of serum TxB_2 inhibition at 72 hours (up to 64%) suggests that an appropriate dosage interval for

oral administration may be 48 hours. This is supported by reports on the clinical efficacy of this drug (S. Carmichael, personal communication).

In man, during administration of 10, 20 or 30 mg once daily, steady state conditions are achieved after approximately 7 days (Nuotio and Makisara, 1978) and further studies are required to determine the extent of accumulation of drug prior to the attainment of steady state in the dog. The extent to which this species will tolerate repeated dosing also requires to be determined. Alternate day administration of a drug in veterinary therapy is likely to lead to problems of owner compliance. It may be that a dosage regimen designed to produce steady state levels followed by a lower daily maintenance dose may be more beneficial. However, as a single oral dose of 0.3 mg/kg gave a mean TxB_2 inhibition which did not exceed 75 % this strategy may result in a poorer clinical response. Further studies to examine thromboxane inhibition and clinical response after a variety of dosage regimens would be required to determine the optimum strategy for clinical use of piroxicam.

There was no apparent overall change in blood clotting times after administration of piroxicam and gastrointestinal blood loss was not found to be a consistent feature. This, together with other data presented, suggest that piroxicam is a safe and efficacious NSAID for use in dogs and that further investigation of this drug would be justified.

4.7. TABLES AND FIGURES

Time (h)	Route of Administration	
	Intravenous	Oral
Pre	0	0
0.25	1.11 ± 0.05	NS
0.50	0.86 ± 0.04	0.77 ± 0.14
1.00	0.93 ± 0.11	1.21 ± 0.15
2.00	0.98 ± 0.14	1.27 ± 0.14
4.00	1.12 ± 0.15	1.25 ± 0.13
6.00	1.06 ± 0.12	NS
8.00	0.94 ± 0.05	1.00 ± 0.10
12.00	0.78 ± 0.04	0.77 ± 0.08
24.00	0.82 ± 0.08	0.80 ± 0.06
48.00	0.45 ± 0.03	0.44 ± 0.03
72.00	0.30 ± 0.04	0.39 ± 0.06

Table 4.1 Mean (±SEM).concentration of piroxicam in plasma of dogs after administration at a dose rate of 0.3 mg/kg by the intravenous and oral routes .

Parameters		Units	Animal Number 1	2	3	4	5	6	Mean	±	SEM
AUC	(obs)	µg/ml.h	34.63	44.30	49.22	55.48	63.69	37.04	47.39		4.52
+ Δ	(obs)	µg/ml.h	41.92	68.04	49.85	77.82	102.99	48.77	64.90		9.38
AUC	(cal)	µg/ml.h	41.14	67.90	69.30	75.98	74.12	47.63	62.68		5.97
+ Δ	(cal)	µg/ml.h	48.43	91.64	69.57	98.32	113.42	59.36	80.08		10.18
AUMC	(obs)	µg/ml.h^2	911.63	1301.40	1365.78	1473.65	1498.75	1003.69	1259.15		100.41
+ Δ	(obs)	µg/ml.h^2	932.46	3010.75	2978.90	3084.17	3177.18	1823.11	2501.09		374.33
AUMC	(cal)	µg/ml.h^2	1812.62	4890.15	4510.40	4081.42	2708.50	2457.71	3410.13		509.90
+ Δ	(cal)	µg/ml.h^2	1833.45	6599.50	6123.51	5689.94	4386.93	3277.13	4651.74		751.29
MRT	(obs)	hours	26.32	29.38	27.74	26.60	23.53	27.10	26.78		0.79
MRT	(cal)	hours	37.86	72.01	88.02	57.87	38.68	55.21	58.27		7.92
Cp0	(cal)	mg/ml	0.93	0.94	1.08	1.43	0.96	0.92	1.04		0.08
Vdss	(obs)	ml/kg	228.05	198.94	169.13	143.82	110.84	219.47	178.37		18.63
Vdss	(cal)	ml/kg	234.51	235.76	379.56	176.58	102.31	279.01	234.62		38.22
Vd a	(cal)	ml/kg	272.97	235.48	276.04	162.33	205.06	260.42	255.38		18.21
Clb	(obs)	ml/h/kg	8.66	6.77	6.09	5.41	4.71	8.09	6.62		0.62
Clb	(cal)	ml/h/kg	6.19	3.27	4.31	3.05	2.64	5.05	4.04		0.58
$t^1/_{2B}$	(cal)	hours	30.54	49.86	44.36	36.86	53.72	35.36	40.16*		
V_c	(cal)	ml/kg	321.20	319.15	277.78	209.79	312.5	326.89	294.55		18.39
k_{el}	(cal)	hours	0.02	0.01	0.01	0.01	0.01	0.01	0.01		0.002

(obs) = calculated from observed values
(cal) = calculated from computed values

+ Δ = computed values for 1st and last triangle added
* = harmonic mean

Table 4.2 Main pharmacokinetic variables in dogs after administration of piroxicam at a dose rate of 0.3 mg/kg by the intravenous route.

Time (h)	Animal Number 1	2	3	4	5	6	Mean
Pre	0	0	0	0	0	0	0
0.25	93.41	94.08	>99.49	>99.38	96.20	98.50	>96.85
0.50	88.04	90.77	>99.49	99.20	97.52	97.67	>95.45
1.00	89.55	85.86	>99.49	99.38	97.96	93.59	>94.31
2.00	89.55	60.68	96.59	93.52	96.50	83.44	86.72
4.00	86.68	>99.10	97.25	89.44	93.72	86.02	>92.03
6.00	89.27	80.24	92.90	95.30	97.52	89.52	89.79
8.00	89.33	87.86	98.77	94.94	93.43	89.52	92.31
12.00	81.23	81.85	NS	NS	NS	NS	81.54
24.00	76.91	68.00	91.81	83.85	79.42	82.86	80.48
48.00	62.76	54.16	90.36	81.81	72.85	64.23	71.03
72.00	53.60	36.61	50.65	55.10	58.39	46.92	50.21

NS = No sample

Table 4.3 Inhibition (%) of thromboxane B_2 in serum of dogs after administration of piroxicam at a dose rate of 0.3 mg/kg by the intravenous route.

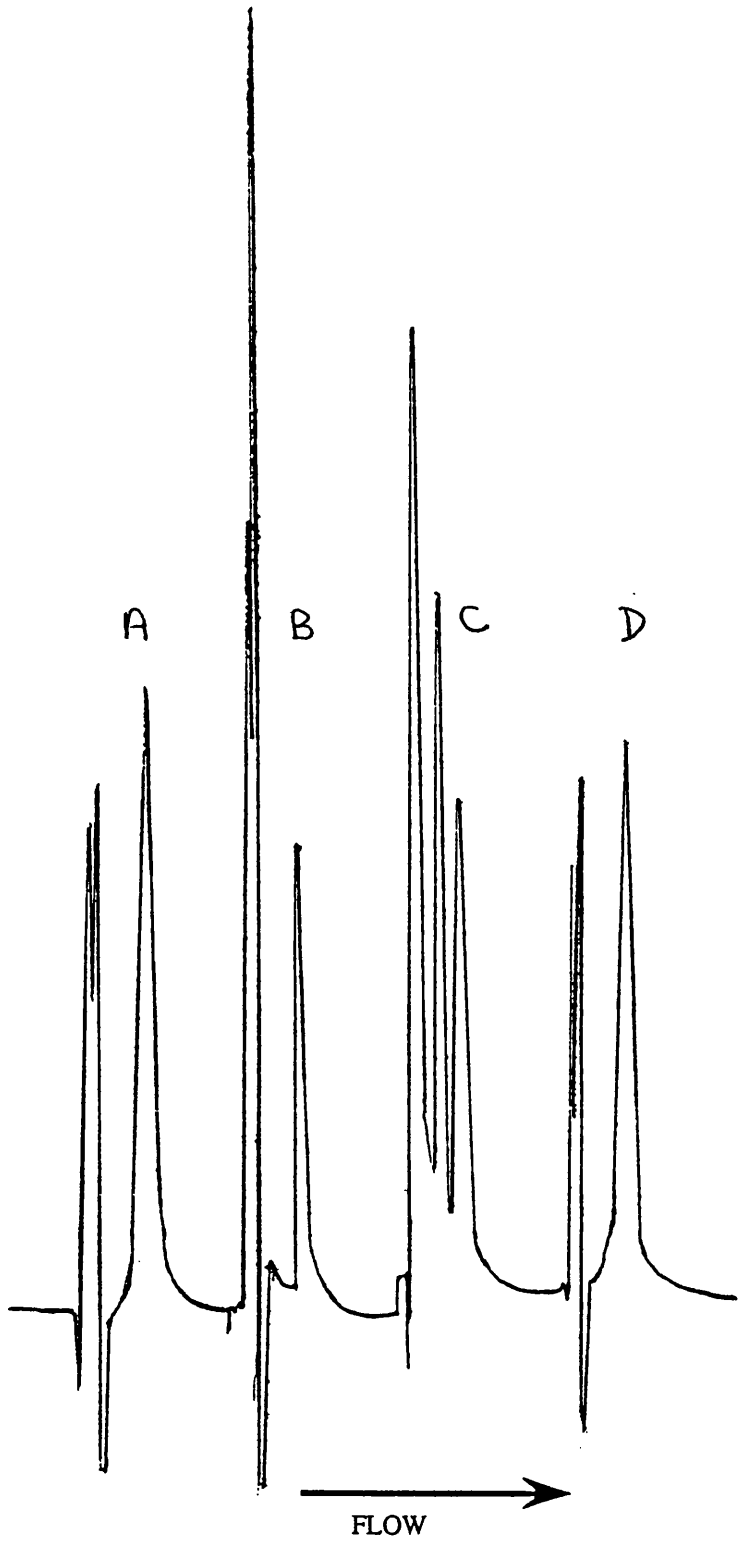
Parameter		Animal Number								
	Units	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	Dog 6	Mean	±	SEM
C_{max}	µg/ml	1.01	1.29	1.27	1.24	1.45	1.86	1.35	±	0.11
t_{max}	hours	4.00	8.00	0.50	2.00	4.00	1&2	3.33	±	1.09
AUC	µg/ml.h	35.76	47.77	40.16	42.03	55.79	59.13	46.77	±	3.75
+ Δ	µg/ml.h	45.71	174.59	60.72	55.83	115.25	152.25	100.72	±	22.33
AUMC	µg/ml.h^2	940.84	1410.10	1158.01	1120.40	1652.40	1750.71	1338.74	±	130.59
+ Δ	µg/ml.h^2	1657.10	23232.18	2717.71	2114.10	5933.70	4537.53	6698.72	±	3370.23
MRT	hours	26.31	29.52	28.83	26.66	29.62	29.61	28.42	±	0.63
MAT	hours	-0.01	0.14	1.09	0.06	6.08	2.83	1.70	±	0.98
F	%	103.26	107.80	81.59	76.24	87.60	159.64	102.69	±	12.44

+ Δ = computed values for 1st and last triangle added

Table 4.4 Main pharmacokinetic variables in dogs after administration of piroxicam at a dose rate of 0.3 mg/kg by the oral route.

Time (h)	Animal Number 1	2	3	4	5	6	Mean	±	SEM
Pre	0	0	0	0	0	0	0		
0.50	54.48	78.67	72.13	75.17	44.95	62.92	64.72	±	5.35
1.00	63.53	76.45	60.37	78.40	79.45	76.53	72.45	±	3.38
2.00	68.00	78.95	67.14	74.29	77.20	66.39	72.00	±	2.25
4.00	60.82	81.35	65.30	72.20	76.54	68.95	70.86	±	3.05
8.00	71.92	82.02	61.78	77.52	78.94	73.93	74.35	±	2.91
12.00	53.26	76.00	64.57	75.62	65.65	61.18	66.05	±	3.56
24.00	49.07	65.70	53.91	63.52	67.68	54.37	59.04	±	3.09
48.00	51.03	63.36	41.00	62.44	66.59	54.42	56.47	±	3.91
72.00	0	39.31	63.67	45.22	39.87	43.80	34.70	±	7.73

Table 4.5 Inhibition (%) of thromboxane B_2 in serum of dogs after administration of piroxicam at at a dose rate of 0.3 mg/kg by the oral route.



A. Standard solution containing 1 µg/ml of drug.

B. Plasma sample estimated to contain 0.99 µg/ml of drug.

C. Blank dog plasma fortified with 1.0 µg/ml drug.

D .Standard solution containing 1 µg/ml of drug.

Figure 4.1 Typical chromatogram of piroxicam in dog plasma

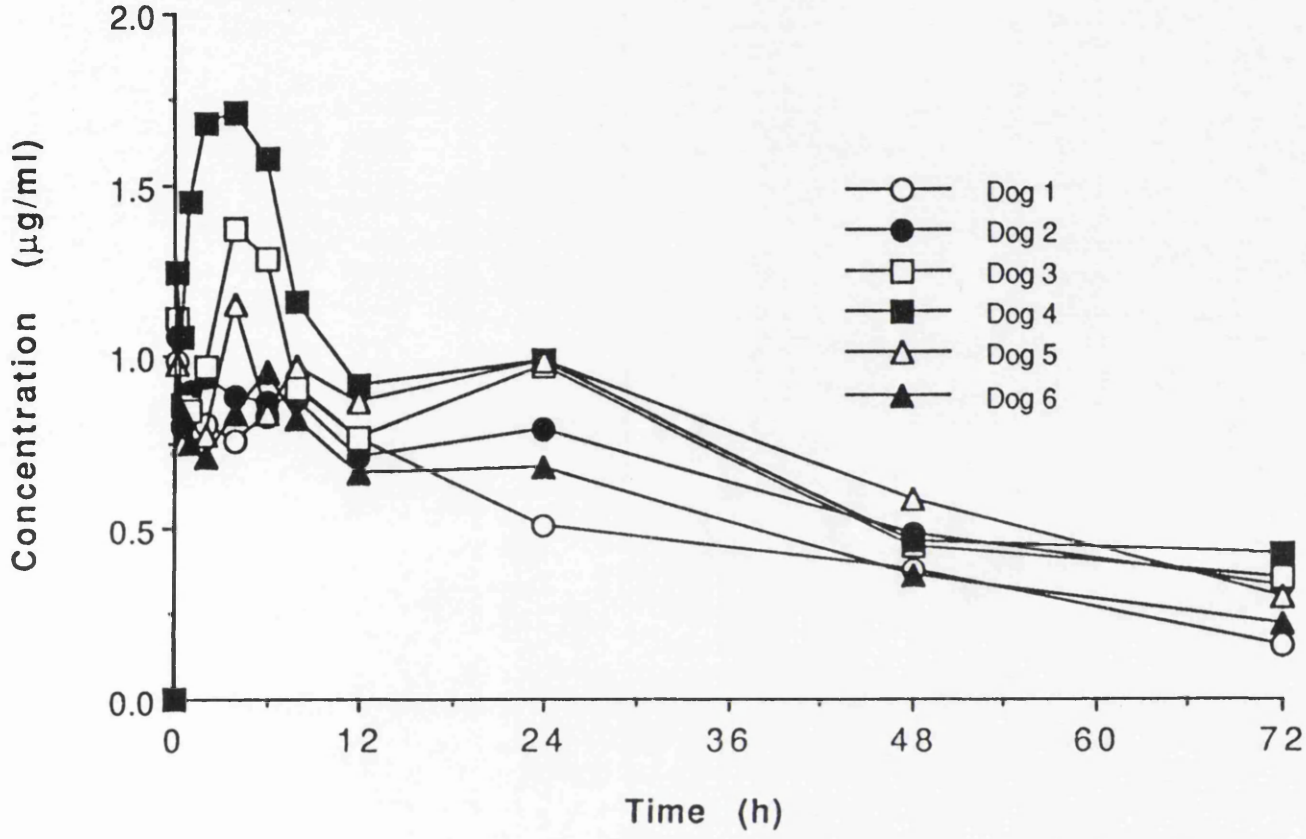


Figure 4.2 Concentration (µg/ml) of piroxicam in plasma of dogs after administration by the intravenous route at a dose rate of 0.3 mg/kg.

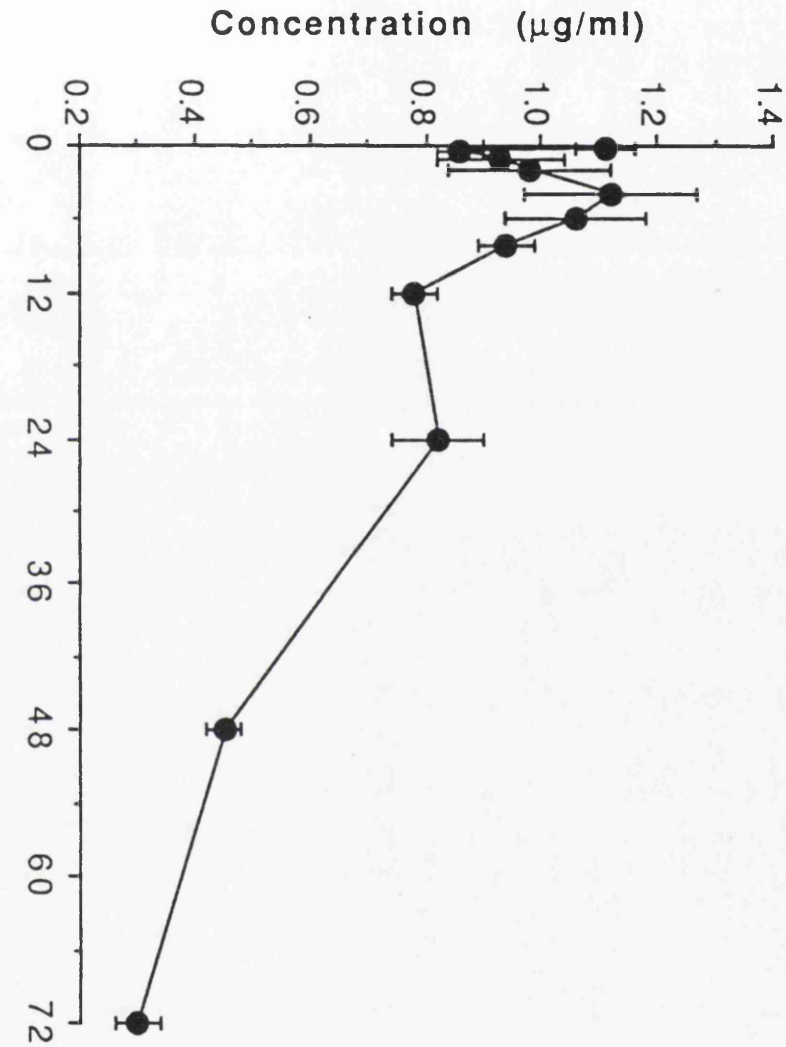


Figure 4.3 Mean (±SEM) concentration (µg/ml) of piroxicam in plasma of dogs after administration by the intravenous route at a dose rate of 0.3 mg/kg .

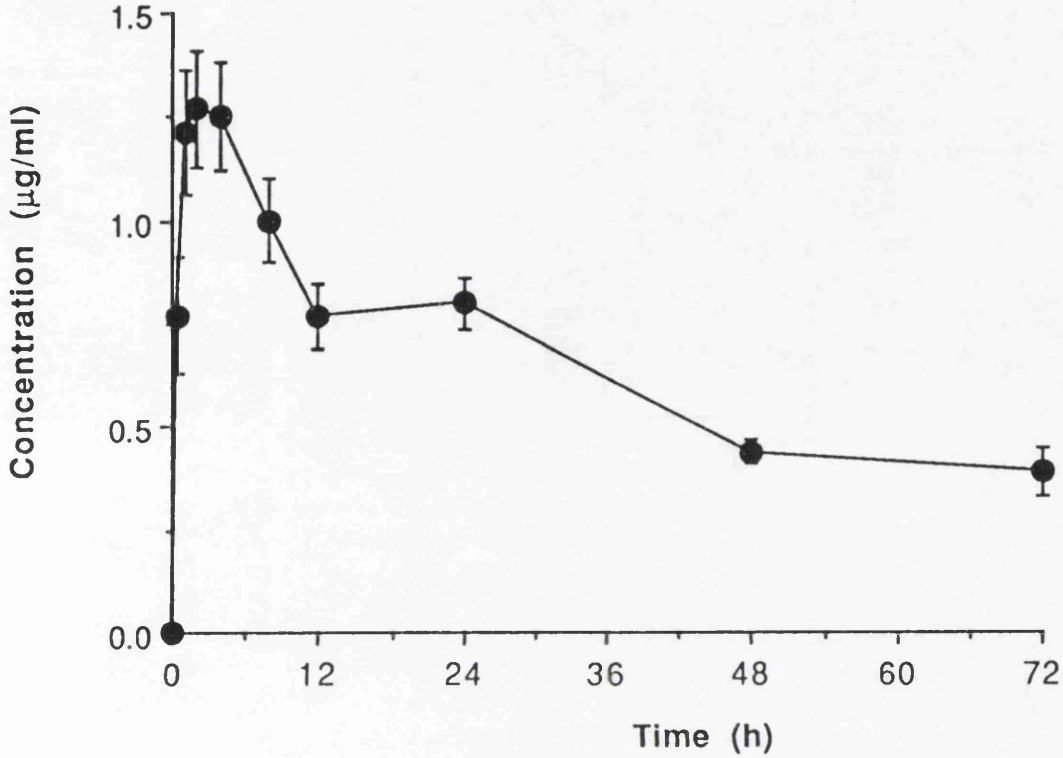


Figure 4.4 Mean (±SEM) concentration (µg/ml) of piroxicam in plasma of dogs after administration by the oral route at a dose rate of 0.3 mg/kg .

Chapter 5

STUDIES WITH CINCHOPHEN

5.1. INTRODUCTION

Cinchophen (2-phenyl quinoline-4-carboxylic acid) was synthesised in 1887 by Dobner and Giesecke. The initial preparation of cinchophen was yellowish in colour and this was thought to be due to the presence of impurities (Hueper, 1948). The pure compound is a white odourless powder which is insoluble in water but somewhat soluble in hot alkali and boiling organic solvents such as alcohol and acetone (Hueper, 1948).
Cinchophen found its first therapeutic application in 1911 for the treatment of gout (Weintraud, 1911 and 1912). This resulted from the discovery of its ability to increase the excretion of uric acid (Nicolairer and Dohrn, 1908). The substance was marketed as Atophan and the recommended human therapeutic dose rate was 22 mg/kg daily. The discovery of its more general anti-inflammatory and analgesic properties (Smith and Hawk, 1915) resulted in a wider range of uses, as did the discovery that it produced anti-pyretic effects when given intravenously or subcutaneously (Fuerst, 1925).

Cinchophen, and the corticosteroid prednisolone, have been available for more than 20 years as a combined preparation for anti-inflammatory treatment of small animals. Despite this there is little literature relating to the clinical efficacy or pharmacokinetics of cinchophen. An oral cinchophen/prednisolone combination preparation has been found to give good improvement in osteoarthritic dogs and its effectiveness was shown to be similar to that produced by phenylbutazone (Pearson, 1989).

Little is known about the precise nature of the metabolism and excretion of cinchophen, however it has been shown (Hueper, 1948) that the urinary metabolites differed in man and dog since 2-o-oxyphenylquinoline-4-carboxylic acid is produced in man but not in the dog. Lichtman (1931) also found that 7-21 % of an administered dose of cinchophen was excreted as oxycinchophen in normal human patients.

Biberfield (1913) reported yellow atrophy of the liver after administration of 5 g of cinchophen twice daily to dogs, and by 1948 there had been 230 documented cases of jaundice associated with its use (Hueper, 1948), however hepatotoxicity appeared to be a feature of prolonged use only.

Although a high incidence of gastrointestinal complaints were noted when cinchophen was used clinically in man (Westfall, 1926), it was not until 1932 that Van Wagoner and Churchill discovered the ulcerogenic action of cinchophen whilst attempting to study its hepatotoxicity. Further studies by these authors (Van Wagoner and Churchill,1932a and b) showed that gastric ulcers were produced in dogs after daily

oral administration of up to 27 times the therapeutic oral dose for man. In 1938, Bollman et al also reported chronic peptic ulceration in man and dogs after administration of cinchophen at a dose rate of 100-200 mg/kg, with food. In addition, duodenal ulcers were observed in this study, but these were found only to be present in conjunction with gastric ulcers. Cinchophen has also been shown to cause gastric ulceration in cats (Hanke, 1934; Schwartz and Simonds, 1935), however neither Shoji (1933) nor Schwartz and Simonds (1935) were able to produce gastric ulceration in rabbits by oral or parenteral administration of cinchophen. Gastric ulceration is not necessarily a feature of a local toxic action of this drug. This was shown by Churchill and Manshardt (1933) who found that gastric ulcers were produced after daily injection of cinchophen into an isolated loop of jejunum. In addition, Hanke (1934) observed gastric ulcers after subcutaneous administration to cats. In an attempt to understand the mechanism of cinchophen induced ulceration, Stalker et al (1937) showed that there was no increase in gastric acidity associated with cinchophen administration, however overall gastric secretion was increased. There is some evidence to suggest that the more refined (white) cinchophen which became available is less ulcerogenic than the original preparation (Simonds, 1938).

Little clear evidence of other side effects exists. Annegers et al (1943), studied human subjects to whom he administered cinchophen at the normal dose rate for up to 39 weeks. No evidence of any changes were seen in the liver, although peptic ulceration was noted.

The occurrence of skin disorders after administration of cinchophen has been recorded (Hueper, 1948), and the frequency of these side effects has been reported as 0.5 % to 20 % of cases studied. This large range in number of skin side effects may have resulted from the fact that cutaneous hypersensitivity in the form of itching and skin rashes has been reported to occur a few hours after initial medication in some cases and only after prolonged periods of medication in others (Hueper, 1948).

The LD_{50} of cinchophen after subcutaneous administration has been shown to be in the region of 1 g/kg for rats, mice and guinea pigs (Barbour and Lozinsky, 1923; Risi, 1932; Hueper, 1948) and a little lower (0.62 g/kg) for dogs (Risi, 1932).

5.2. EXPERIMENTAL OBJECTIVES

The objectives of this work were, to examine the kinetics of cinchophen after administration of a combined preparation containing cinchophen and prednisolone (PLT) at the recommended dose rate of 12.5 mg cinchophen per kilogram of body weight by the intravenous and oral routes. Also to investigate the pharmacodynamics

by quantifying the inhibition of TxB_2 in serum following administration of PLT by each route.

5.3. MATERIALS AND METHODS

5.3.1 ANALYSIS OF CINCHOPHEN

Analysis of cinchophen was carried out by high performance liquid chromatography (H.P.L.C.) using spectrophotometric detection.

5.3.1.1 REAGENTS

1. Cinchophen (C-Vet Ltd.)
2. Diethyl ether, redistilled grade (Rathburn Chemicals)
3. Acetonitrile, redistilled grade (Rathburn Chemicals)
4. Orthophosphoric acid (BDH Ltd)
5. Potassium dihydrogen phosphate 'Analar' (BDH Ltd)
6. 1-Heptane sulphonic acid (Sigma Chemical Co.).
7. Disodium hydrogen orthophosphate 'Analar' (BDH Ltd)
8. Citric acid 'Analar' (BDH Ltd)
9. Citrate/phosphate buffer pH 3.0 was produced by mixing 20.5 ml M/5 Na_2HPO_4 and 79.5 ml M/10 citric acid to give 100 ml of buffer.
10. Water, redistilled in the presence of potassium permanganate prior to use.
11. H.P.L.C. mobile phase comprising 427.5 ml acetonitrile, 5.1 ml phosphoric acid, 1.72 g potassium dihydrogen phosphate and 0.564 g 1-heptane sulphonic acid per litre of water.

5.3.1.2 H.P.L.C. EQUIPMENT

Pump :- Gilson 301

Detector :- Spectraphysics 100

Column :- 100 mm X 8 mm containing 5 μ O.D.S. Hypersil (Shandon Southern).

Wavelength :- 262 nm

Absorbance :- 0.02 or 0.05 AUFS

Flow rate of mobile phase :- 1 ml/min.

Retention time of cinchophen under these conditions was 4.0 minutes.

5.3.1.3 SAMPLE PREPARATION

For samples from the intravenous experiment a 1 ml aliquot of each plasma sample was placed in a 50 ml ground glass tube. To this was added 1.0 ml of citrate / phosphate buffer (pH 3.0), followed by 20 ml diethyl ether. For samples from the oral experiment the volume of sample and buffer were increased to 2 ml. The tube was stoppered and the mixture shaken for 10 minutes by inversion on a slow rotary mixer. Fifteen millilitres of ether was removed from the upper layer and placed in a 50 ml plain glass tube. A further 20 ml diethyl ether was added to the sample mixture prior to shaking for a second period of 10 minutes. Twenty millilitres of the upper ether fraction was removed and combined with the 15 ml previously recovered.
The tube containing the combined ether extracts was placed in a Dri-Block (Techne Ltd) at 50 ^{o}C under a stream of air and allowed to evaporate until approximately 5 ml remained. This remaining diethyl ether was transferred to a 10 ml conical glass tube. The 50 ml glass tube was rinsed three times with approximately 1 ml of diethyl ether which was then added to the conical tube. The ether extract was evaporated to dryness at 50 ^{o}C under a stream of air. A final 1 ml of ether was run down the walls of the conical tube to ensure that all residue present was in the base of the tube. The extract was returned to the Dri-Block and allowed to evaporate to dryness.
Sample tubes were covered with clingfilm and stored overnight at 4 ^{o}C if necessary.

Prior to injection into the HPLC system the residues were reconstituted in methanol. An appropriate known volume (not less than 150 μl) of methanol was added to the bottom of the conical tube which was then tilted and rotated in an ultrasonic bath for 1 minute. A 20 μl injection loop was used for injection, the loop was loaded with 15 μl of sample.

The concentration of cinchophen in each sample was determined by reference to a calibration curve prepared for each analysis. This was prepared by fortifying blank plasma with known amounts of cinchophen to produce the appropriate range of concentrations. A number of fortified plasma aliquots treated in this way were processed in an identical manner to that described for unknown samples. In addition, standard solutions of cinchophen were injected directly into the HPLC. These standard solutions allowed equipment performance to be monitored and were also used to calculate the concentration of cinchophen measured in the fortified samples. From these figures it was possible to calculate the percentage recovery of cinchophen after extraction.

Unknown sample concentrations were also calculated by reference to the standard drug solutions. These concentrations were then adjusted to 100 % to allow for extraction losses by comparison to the recoveries obtained for the fortified samples.

A typical chromatogram is shown in figure 5.1

5.3.1.4 RECOVERY, PRECISION AND LINEARITY

Recovery of drug from plasma samples was approximately 90 %. Typical recovery of cinchophen, inter-assay and inter-assay variations are shown in appendix 1.3.
An assessment of the precision of the assay was made by estimation of the inter-assay coefficient of variation for recovery of cinchophen from fortified plasma (appendix 1.3). The mean inter-assay and intra-assay variations were found to be 4.5 % and 4.8 % respectively.
Linearity of concentration was determined by the use of simple regression for peak height of samples fortified with known concentrations of drug. The correlation coefficient (r) for concentrations ranging from 1 - 50 μg/ml of cinchophen in plasma was 0.997.

The sensitivity of the assay was determined with respect to the background fluctuation recorded on the trace produced from the HPLC system. The minimum peak height which could be reliably recorded was estimated from standard solutions in replicate analyses. This was determined as being at least twice the maximum overall background fluctuation and was set at one unit. A peak height of one unit of cinchophen, under the HPLC conditions described, correspond to 0.03 μg drug per millilitre of plasma.

5.3.1.5 OTHER ANALYSES AND METHODS USED.

Methods for the estimation of serum TxB_2 concentration, platelet numbers in blood, blood clotting time and detection of occult blood in faeces are described in the general material and methods section. Statistics and pharmacokinetic analysis, including equations are also outlined in that section.

5.4. EXPERIMENTS WITH CINCHOPHEN

Three male and three female Beagle dogs aged between 2 and 3 years were used for all experimental work.
Animals were housed in pairs and fed a complete cereal diet once daily, water was available ad libitum.

5.4.1 INTRAVENOUS ADMINISTRATION OF CINCHOPHEN

5.4.1.1 EXPERIMENTAL DESIGN

The six dogs were administered cinchophen at a dose rate of 12.5 mg/kg of cinchophen on a single occasion. Animals were weighed immediately prior to the experiment and the dose rate of cinchophen to be administered calculated according to body weight. Weights ranged from 12.0 - 17.5 kg

5.4.1.2 DRUG ADMINISTRATION

An injectable preparation containing cinchophen (80 mg/ml) and prednisolone (1 mg/ml) (PLT, C-Vet Ltd.) was used. The preparation was administered into the right jugular vein over a 30 second period at a dose rate of 12.5 mg/kg cinchophen, and 0.15 mg/kg prednisolone.
Administration of PLT was carried out one hour prior to feeding.

5.4.1.3 SAMPLING REGIMEN

Blood samples were collected from the left jugular vein into suitable tubes for drug analysis, haematology and serum TxB_2 estimation as described previously. Sampling was carried out prior to and at 0.08, 0.17, 0.33, 0.5, 0.75, 1, 2, 4, 8, 12, 24, 30, 48 and 55 hours after drug administration. No 55 hour sample was collected for measurement of TxB_2 inhibition or platelet numbers.

Samples of blood were also taken into capillary tubes prior to drug administration and at 0.5, 1, 2, 4, 8, 24, 30 and 48 hours. These were used for the estimation of clotting times.
Faecal samples were collected throughout the experiment and an estimate of the presence of occult blood was made.

5.4.2 ORAL ADMINISTRATION OF CINCHOPHEN

5.4.2.1 EXPERIMENTAL DESIGN

The dogs were weighed immediately prior to the experiment and the dose of cinchophen to be administered calculated according to body weight. Weights ranged from 12.0 - 16.0 kg. The six dogs were administered cinchophen at a dose rate of 12.5 mg/kg of cinchophen on a single occasion.

5.4.2.2 DRUG ADMINISTRATION

An oral preparation containing cinchophen (200 mg/tablet) and prednisolone (1 mg/tablet) (PLT, BK Ltd.) was used. Where the dose to be administered did not equal an exact number of tablets, a tablet was shaved with a scalpel blade to reduce the weight to that required. The target dose rate for cinchophen was 12.5 mg/kg and all administered doses were within 0.2 % of this. The resulting dose of prednisolone was 0.06 mg/kg.
Administration of the preparation was carried out one hour prior to feeding.

5.4.2.3 SAMPLING REGIMEN

Blood samples were collected into suitable tubes for drug analysis, haematology and TxB_2 estimation, as described previously.. Sample times were prior to and 0.08, 0.17, 0.33, 0.5, 0.75, 1, 2, 4, 8,12, 24, 30, 48 and 50 hours post drug administration.
In addition, samples of blood were taken into capillary tubes prior to drug administration and at 0.5, 1, 2, 4, 8, 24, 30 and 48 hours for estimation of clotting times.
Faecal samples, collected throughout the experiment, were used to estimate the presence of occult blood.

5.5. RESULTS OF EXPERIMENTS WITH CINCHOPHEN

5.5.1 INTRAVENOUS ADMINISTRATION

5.5.1.1 CINCHOPHEN CONCENTRATIONS AND PHARMACOKINETICS AFTER INTRAVENOUS ADMINISTRATION

The concentration of cinchophen in the plasma of each dog after intravenous administration at a dose rate of 12.5 mg/kg is shown in appendix 7.1. Figure 5.2 shows individual concentrations. Mean (± SEM) concentrations are shown in table 5.1 and figure 5.3. The main pharmacokinetic variables are shown in table 5.2

The mean concentration of cinchophen measured in plasma of dogs at the first sample time (0.08 hours) after drug administration was 120.24 ± 15.09 μg/ml. The inter-animal range in concentration was large 61.58 - 159.01 μg/ml. Decreasing concentrations of cinchophen were then measured in plasma of dogs 1 to 4, as is typical of an intravenous administration. In dogs 5 and 6 this typical pattern was not observed and concentrations were found to be higher than the 0.08 hour value at some sample times during the early part of the sampling period. These later peaks in concentration occured at 0.50 hours for dog 5 (91.97 μg/ml) and 0.75 hours for dog 6 (92.09 μg/ml) and were greater than the concentration measured for these animals in the first sample after drug administration. In addition, dog 5 had a smaller peak in drug concentration at 4 hours (70.36 μg/ml). A likely explanation for the difference in plasma concentration versus drug concentration profiles for these animals is that not all drug was injected intravenously. As a result of this possibility the values for pharmacokinetic parameters calculated for these animals have been excluded from the calculation of pharmacokinetic mean values. Secondary peaks in concentration were also measured in dogs 1, 2 and 4, these were lower than the concentration measured in the first sample and occured at 0.5 (dog 1) and 0.75 hours (dogs 2 and 4) respectively. Drug was present in five animals at the final sample time and the maximum concentration in any animal at this time was 1.04 μg/ml (dog 2). The mean concentration at this time was 0.72 ± 0.20 μg/ml.

The $t^1/_2\beta$ of cinchophen after intravenous administration at a dose rate of 12.5 mg/kg was found to be in the range 7-8 hours for dogs 2-4, however dog 1 had a shorter half life of 4.66 hours. The overall mean $t^1/_2\beta$ in four animals was 6.54 hours.

The range of MRT calculated from observed values for dogs 1-4 was 6.38-10.76 hours. Using these figures to calculate an observed $t^1/_2\beta$, resulted in figures which were not substantially different from the computed values (range 5.14 - 8.68 hours)

Observed AUCs ranged from 694 - 1324 μg/ml.h and the mean observed AUC for cinchophen in dogs 1-4 after intravenous administration at a dose rate of 12.5 mg/kg was 1068 ± 149 μg/ml.h..
The AUMC computed from figures produced by fitting the theoretical curves showed very large inter-animal variation. A similar variation was seen when AUMC was calculated from observed data and in neither case did inclusion of the estimated first and last triangles decrease this variation to an appreciable extent. The mean AUMC for observed data from dogs 1-4 was 10315 (± 2225) $\mu g/ml.h^2$.

Mean body clearance of drug was similar when calculated from observed or computed values (12.59 ± 2.07 ml/kg.h for values from observed data, and 12.77 ± 2.02 ml/kg/h for computed values), once more the inter-animal variation was large. Volume of distribution at steady state was in good agreement whether calculated from observed or computed values and the inter-animal variation was not large. The mean Vdss calculated from observed data was 109.60 ± 6.77 ml/kg and 113.44 ± 5.99 ml/kg for computed values. The volume of the central compartment showed an almost two fold variation between animal 4 and animals 5 and 6. This supports the suggestion that a proportion of the drug was injected peri-venously. Data from animals 1-4 are in the range 74.7 - 92.6 ml/kg.

5.5.1.2 SERUM THROMBOXANE INHIBITION AFTER INTRAVENOUS ADMINISTRATION

Appendix 7.2 contains the serum TxB_2 concentrations for each animal at each time point . Percentage inhibition of TxB_2 is shown in table 5.3.

Inhibition of TxB_2 was between 45 and 84 % in all animals at 0.08 hours after drug administration (mean 69.04 ± 6.19 %). Four animals (dogs 1 to 4) had maximum inhibition in excess of 80 %, however in dogs 5 and 6 the maximum inhibition was 76 and 60 % respectively, and may be further evidence to suggest that a proportion of the drug was injected peri-venously in these animals. The serum TxB_2 inhibition for dogs 3 and 6 was less than 25 % at 0.5 and at 0.75 hours for dog 6, however inhibition was greater at 0.75 hours for dog 3 (82.19

%). At 1 hour after drug administration, mean inhibition had decreased to 43.41 % and mean inhibition remained around 40 - 50 % until 8 hours. With the exception of dogs 5 and 6, inhibition of serum TxB_2 was greater than 50 % at most sampling times up to 4 hours and at 30 hours inhibition was only detected in the sample from one animal (dog 1). The area under the serum TxB_2 inhibition versus time curve was 865 %.h.

5.5.1.3 PLATELET NUMBERS AFTER INTRAVENOUS ADMINISTRATION

Platelet numbers for each dog on each sampling occasion are given in appendix 7.3.

Dog 4 was found to have platelet numbers outwith the normal range quoted for dogs, that is below 200 X 10^9/litre, at most samples times after drug administration. In addition, low platelet numbers were also recorded in dog 2 at 48 hours and dog 6 at 12 hours. Platelet numbers above the normal range were measured in dog 1 prior to and at 8 hours post administration and in dog 2 prior to and at 8 and 12 hours.The cause of these variations is unknown, however, it was not considered likely that these results would affect the overall pattern of TxB_2 production.

5.5.1.4 CLOTTING TIMES AFTER INTRAVENOUS ADMINISTRATION

Clotting times for each animal at each sampling time and mean clotting times are shown in appendix 7.4.

In general, these values remained within the expected range, however it was noted in five animals (dogs 2 - 6), that the clotting times were longer at one hour than the values obtained prior to drug administration. This was found to be a statistically significant difference ($p < 0.05$). No general pattern of increase or decrease was noted thoroughout the experiment and it was therefore considered that this had occured by chance.

5.5.1.5 OCCULT BLOOD IN FAECES AFTER INTRAVENOUS ADMINISTRATION

Gastrointestinal blood loss determined throughout the experiment by Colo-Rectal test is shown in appendix 7.5.

Blood was not detected in any faecal sample collected during the experiment.

5.5.2 ORAL ADMINISTRATION

5.5.2.1 CINCHOPHEN CONCENTRATION AND PHARMACOKINETICS AFTER ORAL ADMINISTRATION

The concentration of cinchophen in the plasma of each dog after oral administration at a dose rate of 12.5 mg/kg is shown in appendix 8.1. Mean (± SEM) concentrations are shown in table 5.1 and figure 5.4
The main kinetic variables are shown in table 5.4

Cinchophen was present in the plasma of only two dogs on the first sampling occasion (0.17 and 0.06 μg/ml, dogs 2 and 4 respectively). The mean concentration of cinchophen measured in plasma of dogs at 0.17 hours post drug administration was 5.63 ± 3.69 μg/ml. As shown by the standard error the inter-animal range in concentration was large at this time (0.07 - 21.21 μg/ml) and this was generally the case at each sampling time. The maximum mean concentration (70.20 ± 8.15 μg/ml) occured at two hours after drug administration and cinchophen was detected in the plasma of all animals at 50 hours (mean concentration 2.05 ± 0.81 μg/ml). Concentrations of drug detected in dogs 1, 5 and 6 at several time points up to one hour were considerably lower than concentrations detected in the other three animals.

The mean observed AUC after administration of cinchophen at a dose rate of 12.5 mg/kg by the oral route was 1010 μg/ml.h. Inter-animal variation in the persistence of cinchophen was determined as the MRT was greater than two fold, the range being 7.62-16.44 hours (mean 12.53 ± 1.48). The AUMC for observed values was in the range 3,500 to 24,500 $\mu g/ml.h^2$ and the mean value was 10944 ± 3637 $\mu g/ml.h^2$. The final blood sample was collected at 50 hours after oral administration of cinchophen, however after intravenous the final sample time was 55 hours. The bioavailability was calculated on concentrations measured up to and including 48 hours and varied by more than a factor of two. The mean value was 64.15 %.

5.5.1.2 SERUM THROMBOXANE CONCENTRATION AFTER ORAL ADMINISTRATION

Serum TxB_2 concentrations for each animal at each time point are shown in appendix 8.2, and percentage inhibition of TxB_2 in table 5.5

There was a very large inter-animal variation in serum TxB_2 inhibition at most sampling times. Inhibition of TxB_2 was measured in only three animals on the first sampling occasion and in all animals there was no inhibition detected on at least one sampling occasion during the first 12 hours. The maximum inhibition was in the range 60 - 70 % for four animals (dogs 2, 3, 4 and 6). Dogs 1 and 5 had lower maximum inhibition values (58.03 and 42.23 % respectively). In five animals maximum inhibition occured between 0.333 and 2 hours, however in one animal (dog 5) the maximum inhibition of TxB_2 occured at 8 hours. The maximum mean inhibition was 50.43 ± 9.29 % and occured at 1 hour, at all other sampling times the mean inhibition was 35 % or less. Inhibtion of TxB_2 was detected in only two animals (dogs 1 and 5) at 50 hours. The area under the serum TxB_2 inhibition versus time curve was 636 %.h.

5.5.1.3 PLATELET NUMBERS AFTER ORAL ADMINISTRATION

Platelet numbers for each dog on each sampling occasion are given in appendix 8.3.

Platelet numbers were found to be outwith the normal range in many samples. This was particularly a feature of dog 1 (which was high) and dog 4 (which was low), however in these animals the platelet numbers in the sample taken prior to drug administration were also outwith the normal range.

5.5.1.4 CLOTTING TIMES AFTER ORAL ADMINISTRATION

Clotting times for each animal at each sampling time and mean clotting times are shown in appendix 8.4.

In general, these values remained within the expected range, however it was noted that the clotting times were shorter ($p < 0.001$) at 24 hour than the values obtained prior to drug administration. No general pattern of increase or decrease was noted thoroughout the experiment and as a result it was concluded that this difference had occured by chance.

5.5.1.5 OCCULT BLOOD IN FAECES AFTER ORAL ADMINISTRATION

Gastrointestinal blood loss determined throughout the experiment by Colo-Rectal test is shown in appendix 8.5.

Blood was not detected in any faecal sample collected during the experiment.

5.6. DISCUSSION

Cinchophen was well tolerated and achieved high concentrations in the plasma of dogs after intravenous administration of a preparation containing cinchophen and prednisolone. It is unfortunate that the relative proportions of cinchophen and prednisolone were different in the intravenous and oral preparations available. As a result of this it is not possible to be entirely sure that any of the similarities and differences between the intravenous and oral administrations were real or were a consequence of the presence of prednisolone. This is particularly true in the case of inhibition of TxB_2, where inhibition was considerably greater after intravenous administration than after oral administration in which the prednisolone content of the preparation administered was less than half of that in the intravenous administration.

For both intravenous and oral routes of administration the plasma concentration versus time profile for cinchophen was characterised, in most animals, by at least one secondary peak. These secondary peaks may have resulted from enterohepatic circulation of drug which has been noted by several workers after administration of NSAIDs (Dupont et al, 1982; Hobbs and Twomey 1979). In the case of animals 5 and 6 after intravenous administration this may have been contributed to by the continuing absorbtion of drug from around the injection site as it seems likely that some drug had been administered peri-venously. Mathematical modeling of plasma concentration versus time curves revealed that best fit theoretical curves for intravenous data were produced by 2 exponents for animals 1-4,. It was not possible to describe the data obtained from dogs 5 and 6 by anything other than a curve using a single exponent, and as a result the pharmacokinetic variables have been calculated accordingly. It is assumed that this discrepancy may have resulted from the incomplete intravenous administration discussed earlier. The plasma concentration versus time profile for dog 6 after intravenous administration was not typical of an intravenous administration. The concentration of drug in plasma increased between the first sample time at 0.08 hours and 0.33 hours, the maximum concentration occured at 0.75 hours. This pattern suggests that some drug had been deposited peri-venously, although there was no indication of this at the time of drug administration, that any drug had been deposited peri-venously. Drug was detected in the plasma of all but one animal on the last sampling occasion after intravenous administration (55 hours) and in all animals on the last sampling occasion after oral administration (50 hours).

Mathematical fitting of best theoretical curves for the concentration data generated by the oral administration resulted in a very poor correlation with observed data, only two animals had correlations of greater than 0.9. As a result pharmacokinetic variables were

calculated from observed data. A wide inter-animal variation in the concentration of cinchophen was noted in each experiment and the t_{max} after oral administration varied from 0.75 - 4 hours.

Preparations containing cinchophen and prednisolone have been marketed in the UK for more than 20 years, yet there is a paucity of information regarding their pharmacokinetics and suitable therapeutic plasma concentrations. It has been established (Hart, 1983) that prednisolone is well absorbed after oral administration. In addition, Graham et al, (1977) have shown that concurrent administration of aspirin and corticosteroid decrease salicylate levels due to an increase in salicylate metabolism. It is therefore possible that the presence of the prednisolone component and the fact that it was administered at different dose rates may have affected the kinetics of cinchophen. The $t^1/_2\beta$ of cinchophen was found to be 6.84 hours after administration of the intravenous product. Calculation of an approximate $t^1/_2\beta$ for cinchophen from the MRT value after oral administration showed that the $t^1/_2\beta$ was 10.06 hours and was therefore somewhat longer than after intravenous administration. Cinchophen achieved larger AUCs when combined with the higher dose rate of prednisolone indicating that it was unlikely that any increase in the rate of metabolism had occured.

Cinchophen was found to have widely variable bioavailability (range 54-120 %) after oral administration, although in general it was well absorbed. In two animals (dogs 2 and 3) the bioavailability was greater than 100 % (119 and 116 % respectively). This may have resulted from good absorption coupled with intra-animal variation and experimental errors. In addition, it is possible that this is an anomaly as a result of the need to calculate the bioavailability over a restricted time period, however the bioavailability for dog 2 was in excess of 100 % whether or not it was calculated using AUCs measured after intravenous administration which had been adjusted to include the theoretical first and last triangles of the plasma concentration time curves.

Mean absorption times varied widely between animals and one animal (dog 4) had a negative value (range -1.55 - 5.68). It is not possible to explain this negative value, which theoretically implies that oral absorbance was more rapid than the injection of an intravenous bolus of drug. It may be that the rapid absorption rate was complicated by the occurrence of inter-animal variation between intravenous and oral experiments.

The inability to produce accurate theoretical curves for the data produced after the oral administration of cinchophen and the presence of drug in plasma at the final sampling times means that comparison of MRT values cannot be straightforward and it is not possible to accurately calculate the last triangle of the AUC for the oral administration. Comparison of MRT values based on observed data indicate that for each animal the

MRT is considerably longer after oral administration. Inclusion of the AUCs of the calculated first and last triangles for the intravenous data makes little difference to the overall MRT values, since the AUC of these triangles is small in comparison to the total AUC. The concentration of drug in the plasma at the last sample time was similar after intravenous and oral administration. It is therefore likely that the contribution to the total AUC by the inclusion of the last triangle for the oral AUC would make little difference to the overall MRT value.

Maximum inhibition of serum TxB_2 was generally in the range 75-90 % after intravenous administration of cinchophen, however for one animal (dog 6) the maximum inhibition was only 60 %. This lower level of inhibition is more typical of the inhibition measured after oral administration where maximum values were generally in the range 40-60 %. In most animals inhibition of 50 % or greater was maintained for 4-8 hours after intravenous administration, however one animal (dog 6) had an inhibition greater than 50 % at only one sample time (0.333 hours).

Comparison of the AUC of TxB_2 inhibition versus time curve after intravenous and oral administration of cinchophen showed that the AUC after oral administration was 73 % of that obtained after intravenous administration. Statistical examination was also carried out on the maximum inhibition produced by each route and the inhibition measured at each time point. The mean maximum inhibition was 78.17 and 59.91 % after intravenous and oral administration respectively and these figures were found to be significantly different ($p = 0.02$). In addition, TxB_2 inhibition was significantly higher at all time points until 1 hour ($p < 0.05$) after intravenous administration than after oral administration. After both intravenous and oral administration of cinchophen the graph of inhibition of TxB_2 was characterised by peaks and troughs. This pattern was somewhat similar to that seen in the plasma concentration versus time graph although the time of appearance of peaks and troughs was not synchronised. This feature was very striking in the case of the data for inhibition after oral administration where in many cases the inhibition fell to zero prior to rising again. The occurrence of much smaller peaks and troughs in the plasma concentration versus time curves, which may have been caused by enterohepatic circulation, may explain this. However, this seems unlikely as high concentrations of cinchophen were required to produce substantial inhibition in TxB_2 production, and it seems unlikely that the small increases in drug concentration which were detected could produce relatively large increases in TxB_2 inhibition unless the dose response curve is very steep. The presence of prednisolone in the administered preparations is a complication. Although the recognised mode of action of corticosteroids does not include inhibition of cyclooxygenase, it is possible that drug interference at the level of phospholipase may

have resulted in TxB_2 inhibition. However, Seppala et al (1985) have demonstrated that a single 20 mg dose of prednisolone does not significantly reduce the concentration of PGE_2, 6-keto-PGF_{1alpha} or TxB_2 in synovial fluid of arthritic patients. In this study, fluctuations in inhibition were greatest after oral administration and the dose rate of prednisolone administered orally was less than 50 % of that administered intravenously. This factor suggests that the erratic levels of inhibition of serum TxB_2 were not as a result of the prednisolone component.

Platelet numbers were found to be outwith the expected range (both high and low) on a number of occasions after both intravenous and oral administration of drug. In no animal did this appear to be a feature associated with drug administration. Also, the variation in platelet numbers does not mirror the unusual pattern of serum TxB_2 inhibition, indicating that it is unlikely that poor inhibition was an anomaly caused by low production of TxB_2 resulting from a decreased number of platelets.

It is interesting that the blood clotting times recorded at one hour after intravenous administration of cinchophen were significantly longer than those measured prior to drug administration. Some NSAIDs have been shown to prolong bleeding time (Meilke et al, 1969, Hirsch et al, 1973). This is thought to be linked to inhibition of cyclooxygenase and the resulting reduction in the production of platelet TxA_2 (Buchanan, et al, 1983). Clotting times measured at 24 hours after the oral administration of cinchophen were found to be significantly shorter than those recorded prior to drug administration. In light of this, and the fact that significant alteration in clotting times was not a consistent feature in either experiment, it seems unlikely that cinchophen had any clinically significant effect on blood clotting times after a single administration at the dose rate studied.

The continued use of this drug combination over a period of 20 years indicates that it is relatively safe and the observations of this study support this.
Baggot (1982) has suggested that the dosage interval for most analgesics used in veterinary medicine should be twice the half life and since the half life for cinchophen was found to be approximately 6.5 hours, a twice daily dosage interval would be appropriate. This half life also indicates that drug accumulation during prolonged therapy is unlikely. Examination of the serum TxB_2 inhibition data demonstrates that this dosage strategy is unlikely to maintain inhibition of greater than 50 %. The presence of prednisolone in the preparation means that it is not possible to predict the efficacy of treatment on the basis of serum TxB_2 inhibition alone. A clinical trial using the oral preparation given twice daily at a dose rate of between 25 and 44 mg/kg daily over a period of 14 days showed it to have similar efficacy to phenylbutazone in the

treatment of osteoarthritis. In this study dogs treated with the combined preparation showed almost twice the incidence of adverse reactions than was noted in the group treated with phenylbutazone (Pearson, 1989).

Further studies using each drug independently would be required to establish the contribution of each component to the efficacy of the preparation and to confirm the kinetics and serum TxB_2 inhibition of cinchophen in the absence of prednisolone. It is apparent, however, that cinchophen is a relatively poor and reversible inhibitor of serum TxB_2 inhibition. In light of the fluctuation in platelet numbers and the occasional significant change in blood clotting times it would also be of interest to examine these parameters for each drug individually.

5.7. TABLES AND FIGURES

Time	Route of Administration	
(h)	Intravenous	Oral
Pre	0	0
0.08	120.24 ± 15.09	0.04 ± 0.03
0.17	116.16 ± 12.53	5.63 ± 3.69
0.33	112.39 ± 9.90	23.08 ± 14.29
0.50	107.35 ± 8.24	40.97 ± 17.93
0.75	104.44 ± 7.84	45.45 ± 18.64
1.00	99.93 ± 8.13	54.85 ± 15.46
2.00	84.00 ± 5.15	70.20 ± 8.15
4.00	67.72 ± 4.96	57.96 ± 7.07
8.00	45.67 ± 4.88	39.40 ± 6.02
12.00	33.48 ± 4.41	29.20 ± 4.89
24.00	11.26 ± 2.37	13.14 ± 3.40
30.00	7.50 ± 1.70	10.34 ± 3.42
48.00	1.34 ± 0.34	2.85 ± 1.03
50.00	NS	2.05 ± 0.81
55.00	0.72 ± 0.20	NS

Table 5.1 Mean (±SEM) concentration (μg/ml) of cinchophen in plasma of dogs after administration at a dose rate of 12.5 mg/kg by the intravenous (in combination with 0.15 mg/kg prednisolone) and oral (in combination with 0.06 mg/kg prednisolone) routes.

Parameters		Units	Animal Number 1	2	3	4	5	6	Mean§	±	SEM
AUC	(obs)	µg/ml.h	694.27	1324.10	962.23	1290.47	1114.29	1442.74	1067.76	±	148.87
+ Δ	(obs)	µg/ml.h	705.48	1351.47	978.52	1309.46	1132.95	1467.80	1086.23	±	151.86
AUC	(cal)	µg/ml.h	681.95	1323.55	938.02	1239.37	1081.56	1251.01	1045.72	±	146.80
+ Δ	(cal)	µg/ml.h	693.16	1347.84	954.31	1258.36	1100.22	1276.01	1063.39	±	149.40
AUC 0-48	(obs)	µg/ml.h	699.60	1323.34	962.83	1291.05	1109.50	1446.48	1069.20	±	147.68
AUMC	(obs)	µg/ml.h²	4432.99	14548.07	9477.96	12801.75	12226.39	18657.27	10315.19	±	2224.89
+ Δ	(obs)	µg/ml.h²	4433.92	15210.03	9762.75	13102.67	12842.95	19696.93	10627.34	±	2349.37
AUMC	(cal)	µg/ml.h²	4461.84	15143.63	9559.56	12467.44	12879.06	17390.98	8258.12	±	3326.78
+ Δ	(cal)	µg/ml.h²	4462.77	15805.59	9844.35	12768.35	13495.62	18430.64	10720.26	±	2414.86
MRT	(obs)	hours	6.38	10.76	9.98	10.01	10.97	12.93	9.28	±	0.98
MRT	(cal)	hours	6.54	11.44	10.19	10.06	11.91	13.90	9.55	±	1.05
Cp0	(cal)	µg/ml	135.00	151.67	135.64	167.34	90.93	89.99	147.41	±	7.68
Vdss	(obs)	ml/kg	111.36	104.09	127.96	95.52	125.07	114.28	109.73	±	6.88
Vdss	(cal)	ml/kg	115.10	108.75	129.11	100.79	139.36	141.49	113.44	±	5.99
Vd a	(cal)	ml/kg	123.30	109.10	143.30	105.72	133.57	138.90	120.35	±	8.54
Clb	(obs)	ml/h/kg	18.30	9.40	12.99	9.69	11.22	8.66	12.59	±	2.07
Clb	(cal)	ml/h/kg	18.30	9.40	13.30	10.09	11.56	9.99	12.77	±	2.02
$t^{1}/_{2B}$	(cal)	hours	4.66	8.01	7.45	7.26	8.25	9.63	6.84*	±	--
V_c	(cal)	ml/kg	92.60	82.40	92.20	74.70	137.47	138.90	85.47	±	4.30
k_{el}	(cal)	hours	0.20	0.11	0.14	0.13	0.08	0.07	0.14	±	0.02
k12	(cal)	hours	0.40	0.67	0.30	0.25	-	-	0.33	±	0.14
k21	(cal)	hours	1.35	2.16	0.63	0.69	-	-	1.21	±	0.36

(obs) = calculated from observed values
(cal) = calculated from computed values
+ Δ = computed values for 1st and last triangle added
* = harmonic mean § mean calculated for dogs 1-4

Table 5.2 Main pharmacokinetic variables in dogs after administration of cinchophen (in combination with 0.15 mg/kg prednisolone) at a dose rate of 12.5 mg/kg by the intravenous route.

Time	Animal Number								
(h)	1	2	3	4	5	6	Mean	±	SEM
Pre	0	0	0	0	0	0	0	±	-
0.08	84.00	78.65	55.91	77.27	73.35	45.04	69.04	±	6.19
0.17	88.39	75.89	82.26	83.12	64.03	34.02	71.28	±	8.20
0.33	74.50	81.42	74.18	66.07	62.38	57.91	69.41	±	3.59
0.50	80.46	75.80	18.41	68.51	75.94	23.77	57.15	±	11.53
0.75	75.10	72.60	82.19	45.13	68.75	10.47	59.04	±	10.99
1.00	75.77	60.59	54.42	9.74	35.61	24.32	43.41	±	10.04
2.00	56.02	58.82	61.15	54.22	48.82	40.46	53.25	±	3.08
4.00	51.67	51.51	56.59	57.63	25.47	0	40.48	±	9.42
8.00	57.23	13.83	64.67	62.82	28.89	60.52	47.99	±	8.70
12.00	36.01	0.95	19.91	17.86	0	0	12.45	±	6.01
24.00	48.13	0	NS	46.59	27.83	0	24.51	±	10.62
30.00	23.83	0	0	0	0	0	5.96	±	5.96
48.00	NS	NS	NS	NS	NS	0	0	±	-

Table 5.3 Inhibition(%) of thromboxane B_2 in serum of dogs after administration of cinchophen (in combination with 0.15 mg/kg prednisolone) at a dose rate of 12.5 mg/kg by the intravenous route.

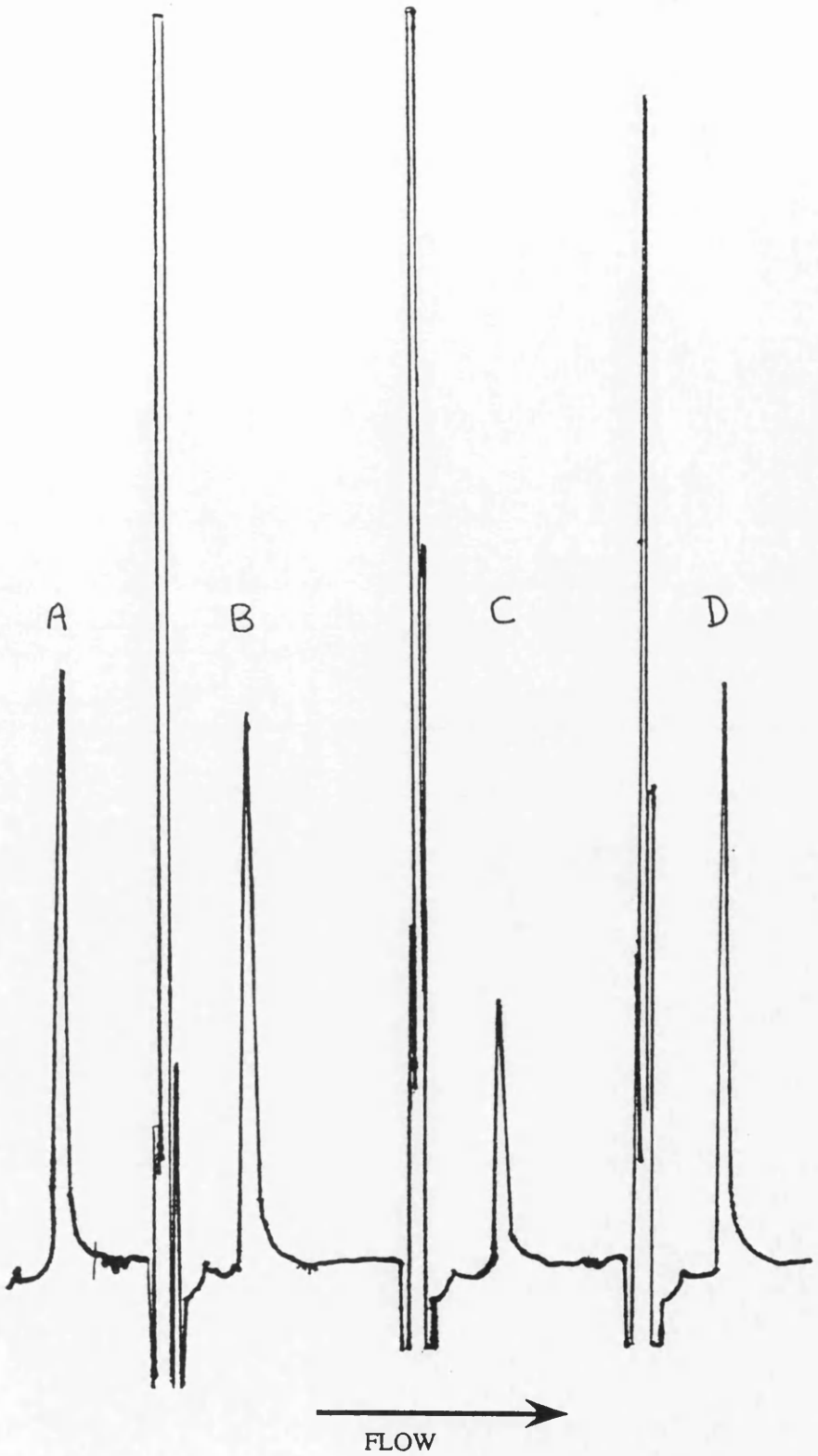
Parameters	Units	Animal Number 1	2	3	4	5	6	Mean	SEM
C_{max}	µg/ml	68.78	94.60	99.56	80.29	61.47	78.53	80.54	5.96
t_{max}	hours	2.00	0.75	0.75	2.00	2.00	4.00	1.92	0.49
AUC	µg/ml.h	473.52	1591.67	1119.68	686.68	968.64	1217.25	1009.57	161.97
AUC 0-48	µg/ml.h	472.48	1578.89	1115.48	686.04	964.87	1210.28	1004.68	160.24
AUMC	µg/ml.h^2	3476.85	24447.48	14148.79	5703.95	14130.87	16490.70	10944.77	3637.98
MRT	hours	7.62	16.44	12.12	8.46	15.08	14.46	12.36	1.48
MAT	hours	1.34	5.68	3.14	-1.55	4.11	1.53	2.15	1.52
F	%	67.53	119.31	115.85	53.91	ND	ND	64.15	14.65

ND = not determined

Table 5.4 Main pharmacokinetic variables in dogs after administration of cinchophen (in combination with 0.06 mg/kg prednisolone) at a dose rate of 12.5 mg/kg by the oral route.

Time	Animal Number								
(h)	1	2	3	4	5	6	Mean	±	SEM
Pre	0	0	0	0	0	0	0	±	-
0.08	31.86	0	0	27.23	0	12.72	11.97	±	5.94
0.17	20.21	0	4.58	0	21.19	7.65	19.36	±	9.31
0.33	5.96	23.08	5.09	62.53	12.34	0	14.26	±	5.93
0.50	5.76	31.84	41.68	39.08	0	11.74	22.52	±	7.80
0.75	0	64.39	70.71	44.12	0.37	26.87	27.06	±	13.51
1.00	50.00	63.89	66.81	0	5.72	61.57	50.43	±	9.29
2.00	58.03	54.06	55.35	54.57	14.28	30.67	35.40	±	9.97
4.00	34.54	16.54	0	0	37.32	21.46	18.30	±	6.60
8.00	44.65	8.05	0	0	42.23	0	15.82	±	8.83
12.00	6.56	0	0	0	38.07	17.43	13.20	±	5.90
24.00	3.48	18.30	2.63	17.17	0	1.84	9.54	±	5.07
30.00	25.84	0	0	30.97	0	24.15	14.99	±	7.04
48.00	12.25	0	0	39.59	11.97	0	4.04	±	2.55
50.00	12.18	0	0	0	10.56	0	4.55	±	2.80

Table 5.5 Inhibition(%) of thromboxane B_2 in serum of dogs after administration of cinchophen (in combination with 0.06 mg/kg prednisolone) at a dose rate of 12.5 mg/kg by the oral route.



A. Standard solution containing 5 µg/ml of drug.

B. Blank dog plasma fortified with 20 µg/ml drug.

C. Plasma sample estimated to contain 10.17 µg/ml of drug.

D .Standard solution containing 5 µg/ml of drug.

Figure 5.1 Typical chromatogram of cinchophen in dog plasma

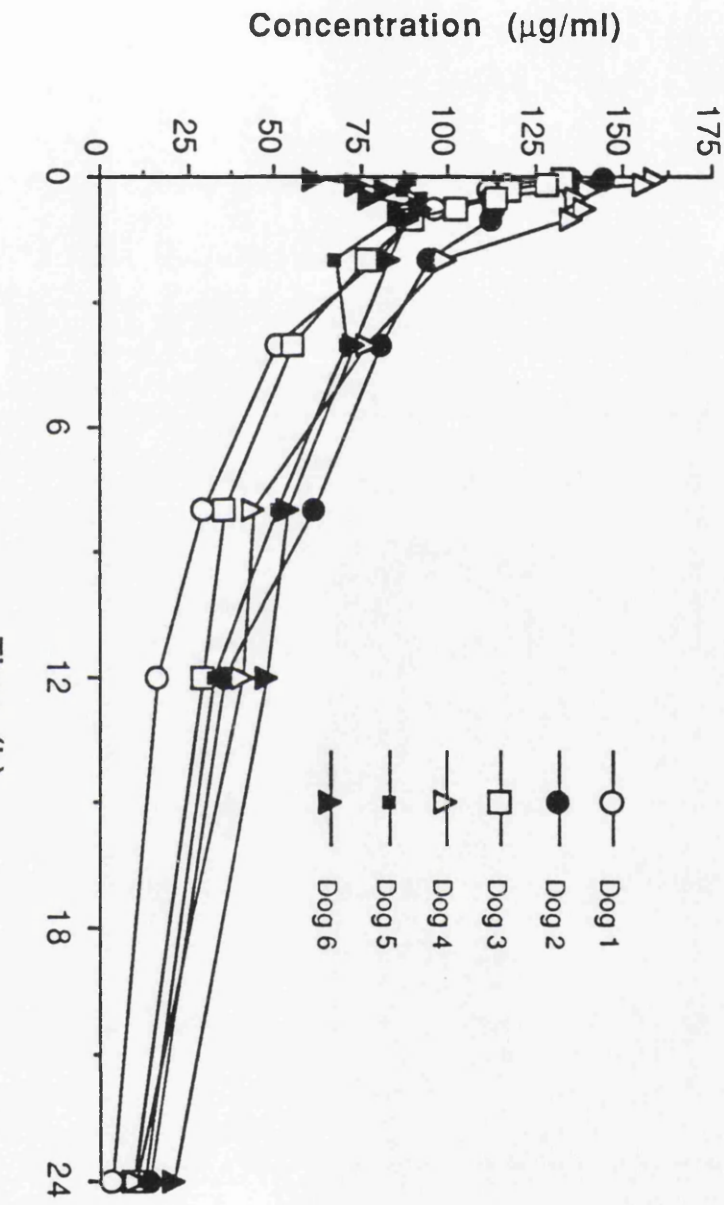


Figure 5.2 Concentration (µg/ml) of cinchophen in plasma of dogs after administration by the intravenous route at a dose rate of 12.5 mg/kg .

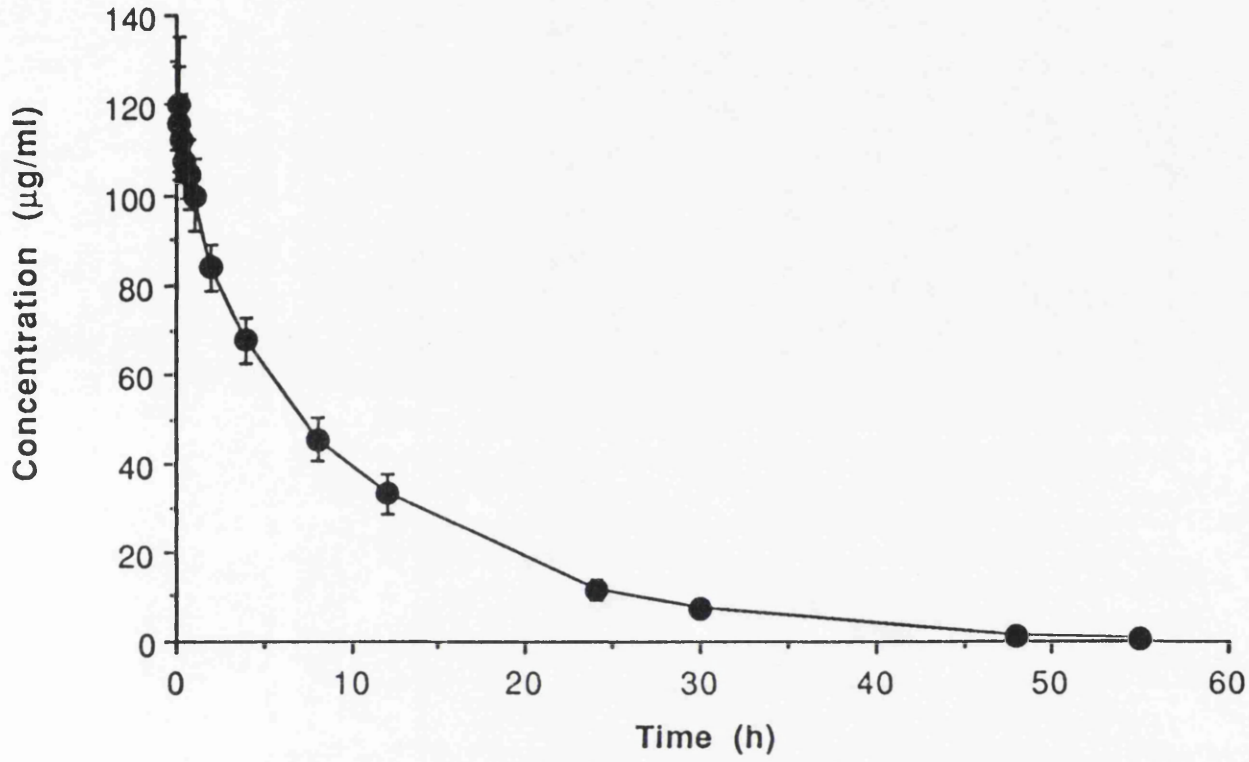


Figure 5.3 Mean (±SEM) concentration (µg/ml) of cinchophen in plasma of dogs after administration by the intravenous route at a dose rate of 12.5 mg/kg .

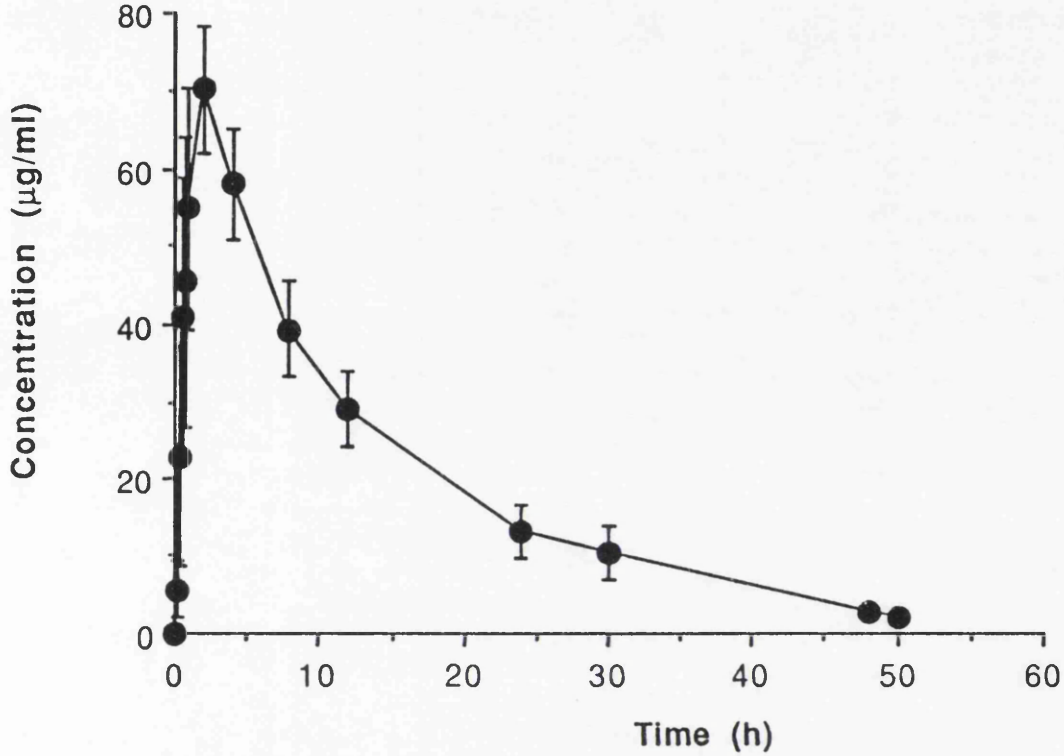


Figure 5.4 Mean (±SEM) concentration (µg/ml) of cinchophen in plasma of dogs after administration by the oral route at a dose rate of 12.5 mg/kg .

Chapter 6

STUDIES WITH TOLFENAMIC ACID

6.1. INTRODUCTION

Tolfenamic acid (N-(2-methyl-3-chlorophenyl) anthranilic acid) is a member of the fenamic acid group of NSAIDs. It is structurally similar to other substituted anthranilic acids, particularly mefenamic acid and flufenamic acid, which have analgesic and antiinflammatory properties (Barnardo et al, 1966). Individual members of this group differ only by the presence or absence of methyl and chloro groups in the basic structure. Tolfenamic acid, manufactured by GEA Ltd (Denmark), is marketed for human use under the trade name Clotam. The normal human therapeutic dosage regimen is 100-200 mg three times daily. It is also available for use in small animals (dogs and cats) in France where is is marketed as Tolfedine by Vetoquinol Ltd (France).

In general, fenamic acids have a pKa of 3-4 (tolfenamic acid pKa 4.3) and are thus almost completely ionised at physiological pH, however they are very lipophilic for this class of drug and as a result, are likely to be present in reasonable concentrations in lipid fractions of the body (Scherrer, 1985).

Numerous clinical trials have been carried out comparing tolfenamic acid to other NSAIDs. Kajander et al (1972) compared tolfenamic acid at dose rates of 300 and 600 mg daily with acetylsalicylic acid at 300 mg per day for four days, and found the higher dose rate of tolfenamic acid to give significantly better analgesia. Yamashita et al (1981a) confirmed the analgesic superiority of tolfenamic acid when compaired to phenylbutazone and also found it to be two to six times as potent as aspirin and equipotent to mefenamic acid and diclofenac. Tolfenamic acid has also been demonstrated to have good analgesic efficacy in the treatment of dysmenorrhea (Kauppila and Ylikorkala, 1977) and migraine (Hakkarainen et al, 1979). In patients with rheumatoid arthritis, 600 mg of tolfenamic acid was more effective than 300 mg phenylbutazone using subjective and objective evaluation (Rejholec et al, 1979b). A comparative study of tolfenamic acid, aspirin and a placebo in the treatment of osteoarthritis of the knee showed no significant difference between drug or placebo when range of movement and other objective perameters were measured (Zachariae and Sylvest, 1972). The analgesic efficacy (as measured by patient preference) was, however, equal to aspirin.

A $t^1/_2\beta$ of 2.5 hours after intravenous and oral administration has been established in human subjects (Pentikainen et al, 1981) and this was also shown to be independant of dose rate over the range examined (100-800 mg). This is in the same range as $t^1/_2\beta$ values obtained for other chemically similar NSAIDs such as flufenamic acid ($t^1/_2\beta$ 3.0 hours) and mefenamic acid ($t^1/_2\beta$ 1.8 hours) (Von Dell et al, 1977; Adamska-Dyniewska et al, 1979). A slightly lower $t^1/_2\beta$ of 1.7 hours was established for

tolfenamic acid when administered to patients with cirrhosis of the liver (Stenderup et al, 1985).

After oral administration, tolfenamic acid was found to be rapidly absorbed. Maximum concentrations in plasma occurred by 2 hours, and bioavailability was reported to be 60 % (Pentikainen et al, 1981). In the same study, maximum plasma concentration and AUC were also found to be linearly correlated with dose. Pentikainen et al (1981) have suggested that the comparatively low bioavailablility may be due to a combination of incomplete gastric absorbtion and first pass metabolism.

Tolfenamic acid is highly bound to plasma proteins in vivo (99.7 %) and the extent of binding has been shown to be independent of plasma concentration (Pentikainen et al, 1981). This figure also correlated well with the value of 99.7 % determined in vitro for protein binding. High protein binding is typical of many NSAIDs and this figure equals or exceeds the degree of binding established for fenoprofen, ibuprofen, tolmetin and naproxen (Brogden et al, 1979). Pentikainen et al (1981), have established that tolfenamic acid does not saturate binding sites in the normal therapeutic concentration range.

In line with other NSAIDs it has been established that tolfenamic acid inhibits cyclooxygenase activity in vivo and in vitro (Linden et al, 1976; Vapaatalo et al, 1986). In vitro, it has been found to be as effective as indomethacin and to be effective at lower concentrations than acetylsalicylic acid in inhibiting the synthesis of PGE_2 in rabbit kidney medulla microsomal fractions. In addition, tolfenamic acid is similar to indomethacin and acetylsalicylic acid as a competitive inhibitor of PG synthesis (Linden et al, 1975 and 1976). Alanko and co-workers (1989, 1990) measured a 31 % decrease in PGE_2 produced by PMNs, and a 70 % decrease in TxB_2 production by platelets after administration of tolfenamic acid to human subjects. It has been established that plasma drug concentrations produced by the administration of three 100mg doses of tolfenamic acid per day exceed the concentrations required to produce a 50 % inhibition of PG synthesis in an experimental model using rabbit kidney medulla microsomes (Linden et al, 1976).

An interesting feature of tolfenamic acid is its additional ability to inhibit the synthesis of LTs in man at clinically achievable concentrations (Moilanen et al, 1988; Alanko et al, 1989). Oral administration of tolfenamic acid has been shown to reduce the production of LTB_4 in human PMN by 26 % and of LTC_4 by 33 % (Alanko, 1990) and this inhibition has shown a positive correlation with drug concentration. More recently, Kankaanranta et al (1993) demonstrated that tolfenamic acid was approximately ten times less effective (on a molar basis) than flunixin in inhibiting

serum TxB_2 production. However it was capable of inhibiting LTB_4 synthesis and PMN migration at clinically achievable concentrations. Inhibition of LT synthesis has also been shown for flufenamic acid and meclofenamic acid (Fruchtmann, 1984; Boctor, 1986). Compounds capable of inhibiting the production of both PGs and LTs (so called dual inhibitors), have been shown to be more effective antiinflammatory agents than classic NSAIDs (Moncada, 1986). A further distinctive property of fenamates is the influence of co-factors on the ability of these drugs to inhibit cyclooxygenase. Many of the cofactors (such as phenols and indoles) are substances which may be present naturally at the site of activity. Enhancement of activity by as much as 100 fold has been reported (Egan et al, 1978), and it has been sugested (Scherrer, 1985) that the local presence of such natural co-factors may result in an action of fenamates which is tissue selective.

Fenamates differ from other NSAIDs since they may block prostaglandin receptors. This was initially reported by Collier and Sweatman (1968), and later confirmed by Zor et al (1976). When tested on two in vitro smooth muscle preparations, tolfenamic acid was 20 times more effective than mefenamic acid in the inhibition of PGF_{2alpha} contraction of rat uterus, and equal to mefenamic acid in the inhibition of PGF_{2alpha} contraction of guinea pig trachea (Vapaatalo et al, 1977). Concentrations required to cause this effect are 30 times higher than those required to cause inhibition of prostaglandin synthesis, however they are still within the range which may be achieved during clinical use.
Bennett et al (1980a , 1980b), investigating the action of various PGs in gut preparations, found that sodium meclofenamate antagonised the stimulation of gastrosecretion induced by PGF_{2alpha} but not the inhibitory effect of PGE_2. The ability of fenamates to antagonise PG induced contractions varied with species and tissue, and these workers suggested that the apparent anomolies were a result of the existence of several types of PG receptors.

Yamashita et al (1981b) have shown tolfenamic acid to be superior to mefenamic acid, diclofenac and indomethacin in inhibiting the in vitro migration of leukocytes and to be almost as potent as diclofenac and indomethacin in the inhibition of platelet aggregation. This is in line with previous findings for fenamic NSAIDs (O'Brien, 1986).

Studies by Pentikainen et al (1981) indicated elimination of tolfenamic acid in human subjects is predominantly by extrarenal mechanisms. This is similar to the chemically related compounds mefenamic and flufenamic acid which are removed by hepatic metabolism (Glazko, 1967). There is good correlation between elimination rate

constants or plasma clearances of tolfenamic acid and liver function in patients with cirrhosis of the liver (Stenderup et al, 1985). It has been well documented that other fenamates are extensively metabolised to hydroxylated derivatives with only a small percentage of initial dose being excreted in the urine as unaltered drug or glucuronide (Glazko, 1967). Pentikainen et al (1981, 1982) have shown that less than 10 % of an intravenous dose is detected in urine as unchanged drug or glucuronide when given to healthy patients. These workers have also identified two major metabolites of tolfenamic acid in urine and have established that these are both hydroxylated derivatives. Studies conducted by Kuninaka et al (1981) on humans, rats and rabbits indicated that the major metabolites present in urine were glucuronides and these metabolites have been found to be less potent antiinflammatory agents than the parent compound .

In general, clinical use of tolfenamic acid has been found to produce few side effects (Kajander et al, 1972; Zachariae and Sylvest, 1972; Axelsson et al, 1977), even after administration for several months (Rejholec et al, 1979b). Gastrointestinal irritation has been noted in some studies (Sorensen and Christiansen, 1977; Rejholec et al, 1979a) although not all (Axelsson et al, 1977) and administration of 10 times the human dose to dwarf pigs over a period of six weeks resulted in no adverse effects on gastric mucosa (Axelsson et al, 1977). Slight dysuria, particularly in male patients was noted in some studies (Sorensen and Christiansen, 1977; Rejholec et al, 1979a). Tolfenamic acid has been shown to have a better therapeutic index than mefenamic acid, indomethacin, diclofenac and phenylbutazone (Rejholec et al, 1979a). Axelsson (1977) reported that tolfenamic acid produced significantly fewer cases of gastritis than acetylsalicylic acid, and studies carried out in Japan suggest that tolfenamic acid may be up to five times less ulcerogenic than mefenamic acid when administered to rats (Yamashita et al, 1981a).

6.2 EXPERIMENTAL OBJECTIVES

The objectives of this work were to examine the kinetics of tolfenamic acid after administration at the recommended dose rate of 4.0mg/kg body weight by the intravenous and subcutaneous routes in dogs, to investigate the pharmacokinetics and to quantify the inhibition of TxB_2 in serum following administration of tolfenamic acid by each route.

6.3 MATERIALS AND METHODS

6.3.1 ANALYSIS OF TOLFENAMIC ACID

Analysis of tolfenamic acid was carried out by high performance liquid chromatography (H.P.L.C.) using spectrophotometric detection.

6.3.1.1 REAGENTS

1. Tolfenamic acid (Sigma Chemical Co.)

2. Diethyl ether, redistilled grade (Rathburn Chemicals)

3. Methanol 'Analar' (BDH Ltd), redistilled prior to use

4. Disodium hydrogen orthophosphate 'Analar' (BDH Ltd)

5. Citric acid 'Analar' (BDH Ltd)

6. Citrate/phosphate buffer pH 3.0 was produced by mixing the following solutions :-

   20.5 ml M/5 Na_2HPO_4 + 79.5 ml M/10 citric acid,to give 100 ml of buffer.

7. Perchloric acid 70 % 'Analar' (BDH Ltd)

8. Water, redistilled in the presence of potassium permanganate prior to use.

7. H.P.L.C. mobile phase comprised 80 : 20, methanol : water containing 50 µl of 1 in 55 aqueous solution of perchloric acid per 100 ml mobile phase.

6.3.1.2 H.P.L.C. EQUIPMENT

Pump :- Gilson 301

Detector :- Spectraphysics 100

Column :- 100 mm X 8 mm containing 5 µ O.D.S. Hypersil (Shandon Southern).

Wavelength :- 342 nm

Absorbance :- 0.02 AUFS

Flow rate of mobile phase :- 1.5 ml/min.

The retention time of tolfenamic acid under these conditions was 3.5 minutes.

6.3.1.3 SAMPLE PREPARATION

A 2 ml aliquot of each plasma sample was placed in a 50 ml ground glass tube. To this was added 2 ml of citrate / phosphate buffer (pH 3.0), followed by 20 ml diethyl ether. The tube was stoppered and the mixture shaken for 10 minutes by inversion on a slow rotary mixer. Fifteen millilitres of ether was removed from the upper layer and placed in a 50 ml glass test tube. A further 20 ml diethyl ether was added to the sample mixture and shaken for a second period of 10 minutes after which 20 ml of the upper ether fraction was removed and combined with the 15 ml previously recovered.
The tube containing the combined ether extracts was placed in a Dri-Block (Techne Ltd) at 50 °C under a stream of air and allowed to evaporate until approximately 5 ml remained. The remaining diethyl ether was transferred to a 10 ml conical glass tube. The 50 ml glass tube was rinsed three times with approximately 1 ml of diethyl ether which was then added to the conical tube. The ether extract was evaporated to dryness at 50 °C under a stream of air. A final 1 ml of ether was run down the walls of the conical tube to ensure that all residue present was in the base of the tube. The extract was returned to the Dri-Block and allowed to evaporate to dryness. Sample tubes were covered with clingfilm and stored overnight at 4 °C if necessary. Prior to injection onto the HPLC system the residues were reconstituted in methanol. An appropriate known volume (not less than 150 µl) of methanol was added to the bottom of the conical tube which was then tilted and rotated in an ultrasonic bath for 1 minute A 20 µl injection loop was used for injection, the loop was loaded with 15 µl of sample.

The concentration of tolfenamic acid in each sample was determined by reference to a calibration curve prepared for each analysis. This was prepared by fortifying blank plasma with known amounts of tolfenamic acid to produce the appropriate range of concentrations. A number of fortified plasma aliquots treated in this way were processed in an identical manner to that described for unknown samples. In addition, standard solutions of tolfenamic acid were injected directly onto the HPLC. These standard solutions allowed equipment performance to be monitored and were also used to calculate the concentration

of tolfenamic acid measured in the fortified samples. From these figures it was possible to calculate the percentage recovery of tolfenamic acid after extraction.

Unknown sample concentrations were calculated by reference to the standard drug solutions. These concentrations were then adjusted to 100 % to allow for extraction losses by comparison to the recoveries obtained for the fortified samples.

A typical chromatogram is shown in figure 6.1

6.3.1.4 RECOVERY, PRECISION, LINEARITY AND SENSITIVITY

Recovery of drug from plasma samples was approximately 70 %. Typical recovery of tolfenamic acid, inter-assay and intra-assay variation are shown in appendix 1.4.
Calculation of the inter-assay coefficient of variation for recovery of tolfenamic acid from fortified plasma allowed an assessment of the precision of the assay to be made (appendix 1.4). The mean inter-assay variation was found to be 5.2 % and the mean intra-assay variation was 5.5 %.
Simple regression was used to determine the linearity of concentration with respect to peak height for fortified samples. The correlation coefficient (r) for concentrations ranging from 0.1 - 5.0 μg/ml of tolfenamic acid in plasma was 0.999.

The sensitivity of the assay was determined with respect to the background fluctuation recorded on the trace produced from the HPLC system. The minimum peak height which could be reliably recorded was estimated from standard solutions in replicate analyses. This was determined as at least twice the maximum overall background fluctuation and was set at one unit. A peak height of one unit of tolfenamic acid, under the HPLC conditions described, corresponded to 0.05 μg drug per millilitre of plasma.

6.3.1.5 OTHER ANALYSES AND METHODS USED

Estimation of serum TxB_2 concentration, estimation of platelet numbers in blood, estimation of occult blood in faeces, pharmacokinetic analysis, statistical analysis
are described in the general Materials and Methods section.

6.4 EXPERIMENTS WITH TOLFENAMIC ACID

Three male and three female Beagle dogs aged between 2.0 and 2.5 years were used for all experimental work. The animals were housed in pairs and fed a complete cereal diet once daily, water was available ad libitum.

6.4.1 INTRAVENOUS ADMINISTRATION OF TOLFENAMIC ACID

6.4.1.1 EXPERIMENTAL DESIGN

Tolfenamic acid, at a dose rate of 4.0 mg/kg, was administered to the six dogs on a single occasion.The dogs were weighed immediately prior to the experiment and the dose of tolfenamic acid to be administered calculated according to body weight. Weights ranged from 13.1 - 16.9 kg.

6.4.1.2 DRUG ADMINISTRATION

A 40 mg/ml injectable solution (Tolfedine, Vetoquinol) was administered into the right cephalic vein over a 30 second period.
Administration of tolfenamic acid was carried out one hour prior to feeding.

6.4.1.3 SAMPLING REGIMEN

Blood was collected by jugular venipuncture into suitable tubes for drug analysis, haematology and serum TxB_2 estimation as described previously. Sampling was carried out sequentially prior to and at 0.08, 0.17, 0.33, 0.50, 0.75, 1, 2, 4, 8,12, 24, 48 and 72, 96 and 168 hours after drug administration. The first sample time for serum TxB_2 inhibition was 0.5 hours and no sample was taken at 0.75 hours. Addition samples of blood were taken into capillary tubes prior to drug administration and at 0.5, 1, 2, 4, 8,12, 24, 48, 72, 96 and 168 hours after drug administration. These were used for the estimation of blood clotting time.
Faecal samples were collected throughout the experiment and an estimate of the presence of occult blood was made as described in the general material and methods section.

6.4.2 SUBCUTANEOUS ADMINISTRATION OF TOLFENAMIC ACID

6.4.2.1 EXPERIMENTAL DESIGN

The six dogs were administered tolfenamic acid at a dose rate of 4.0 mg/kg on a single occasion. The dogs were weighed immediately prior to the experiment

and the dose of tolfenamic acid administered calculated according to body weight. Weights ranged from 12.3 - 16.3 kg.

6.4.2.2 DRUG ADMINISTRATION

A 40 mg/ml injectable solution of tolfenamic acid (Tolfedine, Vetoquinol) was used. The drug was administered under the loose skin at the back of the neck. Administration of tolfenamic acid was carried out one hour prior to feeding.

6.4.2.3 SAMPLING REGIMEN

Samples were taken for drug analysis, haematology and serum TxB_2 estimation as described previously. Sampling was carried out prior to and at 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48, 72, 96 and 168 hours after drug administration. Samples of blood also taken into capillary tubes prior to drug administration and at 0.5, 1, 2, 4, 8, 24, 48, 72, 96 and 168 hours after drug administration for estimation of clotting time.

An estimate of the presence of occult blood was made from faecal samples which were collected throughout the experiment.

6.5 RESULTS OF EXPERIMENTS WITH TOLFENAMIC ACID

6.5.1 INTRAVENOUS ADMINISTRATION

6.5.1.1 TOLFENAMIC ACID CONCENTRATION AND PHARMACOKINETICS AFTER INTRAVENOUS ADMINISTRATION

The concentration of tolfenamic acid in the plasma of each dog after intravenous administration of tolfenamic acid at a dose rate of 4.0 mg/kg is shown in appendix 9.1. Figure 6.2 shows individual concentrations. Mean (± SEM) concentrations are shown in table 6.1 and figure 6.3. The main pharmacokinetic variables are shown in table 6.2.

The mean concentration of tolfenamic acid measured in plasma of dogs at 0.08 hours after drug administration was 25.41 ± 4.50 μg/ml and a large inter-animal range in concentration was noted (14.50 - 44.13 μg/ml). Concentration of drug declined rapidly and in two dogs (dog 1 and 2) no drug could be detected at 2 hours. Tolfenamic acid was detected in plasma from two dogs at 48 hours (dogs 3 and 4), and at 96 hours was only detected in plasma from dog 4. Mean concentration of tolfenamic acid in plasma was 0.28 ± 0.06 μg/ml at 12 hours and 0.07 ± 0.03 μg/ml at 24 hours.

Two animals showed plasma concentration versus time profiles containing several peaks and troughs. Drug could not be detected in plasma from dogs 1 and 2 at 2 hours post administration, however, at 4 hours concentrations of 0.20 μg/ml and 0.32 μg/ml (respectively) were measured. Drug was then detected in samples from these animals until 12 hours (dog 2) and 24 hours (dog 1).
A large inter-animal variation in $t^1/_2$ ß was observed over the range 3.91 to 16.38 hours. The inter-animal variation of persistence of tolfenamic acid in plasma is reflected in the large variation in MRT values calculated from observed data (range 1.32 to 19.27 hours). In addition, observed AUCs ranged from 9.70 - 35.05 μg/ml.h. The mean AUC after administration of tolfenamic acid at a dose rate of 4.0 mg/kg by the intravenous route was 18.10 μg/ml.h.

The area under the first moment curve also varied widely between animals. The mean AUMC was 193 (± 111) μg/ml.h^2, range 17.34 - 727.41 μg/ml.h^2.
Mean body clearance of drug was 230 ± 41 ml/h/kg, range 106 ml/h/kg (dog4) to 351 ml/h/kg (dog 1) and Vd_{ss} was 1247 (± 293) ml/kg, range 396 (dog 5) to 2058 ml/kg (dog 1) .

6.5.1.2 SERUM THROMBOXANE INHIBITION AFTER INTRAVENOUS ADMINISTRATION

Serum TxB_2 concentrations for each animal at each time point are shown in appendix 9.2, and percentage inhibition of TxB_2 in table 6.3.

Inhibition of TxB_2 was between 84 and 96 % in all animals at 0.5 hours after drug administration (mean 91.30 %). Inhibition exceeded 70 % in only three animals (dogs 2, 3 and 6) at one hour and serum TxB_2 was near to control concentrations during the period 2 to 4 hours in several animals. However, all animals showed a secondary peak in inhibition at some point during the sampling times from 4 hours onwards. The area under the serum TxB_2 inhibition versus time curve was 1857 %.h.

6.5.1.3 PLATELET NUMBERS AFTER INTRAVENOUS ADMINISTRATION

Platelet numbers for each dog on each sampling occasion are given in appendix 9.3. Dogs 2, 4, and 5 were found to have platelet numbers outwith the range quoted for dogs prior to drug administration, that is below 200 X 10^9/litre. Low counts were recorded for dog 4 at each sampling time until eight hours,

from which time platelet counts were within the normal range until 72 hours. Blood samples taken from dog 6 at 2 hours and dog 2 at 8 hours were also found to be outwith the normal range. No time related pattern of increase or decrease was noted throughout the experiment.

6.5.1.4 CLOTTING TIMES AFTER INTRAVENOUS ADMINISTRATION

Clotting times for each animal at each sampling time and mean clotting times are shown in appendix 9.4. These values remained within the expected range at all sample times with the exception of the two hour sample from dog 1 and 3, the 4 hour sample from dog 4 and the 96 hour sample from dog 3 which were slightly lower than expected. No pattern of increase or decrease was noted thoroughout the experiment.

6.5.1.5 OCCULT BLOOD IN FAECES AFTER INTRAVENOUS ADMINISTRATION

Colo-Rectal tests were used throughout the experiment to determine gastro-intestinal blood loss (appendix 9.5). Blood was not detected in the faeces samples with the exception of the single sample from pen 3 (dogs 5 and 6) at 72 hours in which a trace of blood was detected.

6.5.1 SUBCUTANEOUS ADMINISTRATION

6.5.1.1 TOLFENAMIC ACID CONCENTRATION AND PHARMACOKINETICS AFTER SUBCUTANEOUS ADMINISTRATION

The concentration of tolfenamic acid in the plasma of each dog after subcutaneous administration at a dose rate of 4.0 mg/kg is shown in appendix 10.1 and figure 6.4. Mean (± SEM) concentrations are shown in table 6.1 and figure 6.4. Figure 6.5 shows individual concentrations and the main kinetic variables are shown in table 6.4.

Plasma concentrations after subcutaneous administration of tolfenamic acid were determined for only five of the six dogs to which drug had been administered. This was due to loss of samples in storage.

The mean concentration of tolfenamic acid measured in plasma of dogs at 0.25 hours after subcutaneous administration of drug was 1.11 ± 0.35 μg/ml. As shown by the standard error the inter-animal range in concentration was large at 0.25 hours and from 8 hours onwards. The maximum mean concentration (4.05 ± 0.65 μg/ml) occured at 2 hours after drug administration and tolfenamic acid was detected in the plasma of all animals at 12 hours (mean concentration 1.17 ± 0.28 μg/ml). Tolfenamic acid could be detected in the plasma of two dogs (dogs 1 and 6) at 48 hours. Examination of the drug concentration versus time profile shows that for all but one dog (dog 5), after an initial decline, concentrations rose again. The profile for dog 1 showed two such secondary peaks and the concentration of drug detected rose again from below the limit of detection at 24 hours to 0.06 μg/ml at 48 hours, before falling below the limit of detection once more at 72 hours.

As in the intravenous experiment, the inter-animal variation in persistence of tolfenamic acid in plasma is reflected in the large variation in MRT values calculated from observed data (3.89 - 11.06 hours). In addition, observed AUCs ranged from 12.53 - 41.47 μg/ml.h. The mean observed AUC after administration of tolfenamic acid at a dose rate of 4.0 mg/kg by the subcutaneous route was 29.62 μg/ml.h. The area under the first moment curve ranged from 49 to 429 $\mu g/ml.h^2$ (mean 246 $\mu g/ml.h^2$).

6.5.1.2 SERUM THROMBOXANE CONCENTRATION AFTER SUBCUTANEOUS ADMINISTRATION

Serum TxB_2 concentrations for each animal at each time point are shown in appendix 10.2, and percentage inhibition of TxB_2 in table 6.5

Inhibition of TxB_2 ranged from 32 - 99 % at 0.25 hours and from 54 -90 % at 0.5 hours after drug administration. Maximum inhibition was greater than 80 % in each animal and the maximum mean inhibition of 80 % occured at 2 hours. Twelve hours after drug administration inhibition of TxB_2 had declined to 50 % or less in four animals (dogs 2, 3, 5 and 6), and at 24 hours concentrations were near pr- drug administration values in dogs 2 and 5. The area under the serum TxB_2 inhibition versus time curve was 2785 %.h.

6.5.1.3 PLATELET NUMBERS AFTER SUBCUTANEOUS ADMINISTRATION

Platelet numbers for each dog on each sampling occasion are given in appendix 10.3. In general, throughout the experiment these remained within the range considered to be normal for dogs. However, decreased platelet numbers were detected in samples collected at 48 and 96 hours from dog 4 and at one and 96 hour from dog 6.

6.5.1.4 CLOTTING TIMES AFTER SUBCUTANEOUS ADMINISTRATION

Clotting times for each animal at each sampling time and mean clotting times are shown in appendix 10.4. These values remained within the expected range at all sample times with the exception of the 1 hour sample from dog 1 and the 96 hour sample from dog 4, which were lower than expected .

6.5.1.5 OCCULT BLOOD IN FAECES AFTER SUBCUTANEOUS ADMINISTRATION

Gastro-intestinal blood loss determined throughout the experiment by Colo-Rectal test is shown in appendix 10.5. No blood was detected with the exception of faeces from pen 2 (dogs 3 and 4) and pen 3 (dogs 5 and 6) at 48 hours. These samples showed a trace and definite reaction respectively.

6.6 DISCUSSION

Tolfenamic acid was well tolerated after intravenous and subcutaneous administration to dogs.

The general pattern of plasma drug concentration versus time after intravenous and subcutaneous administration indicated that enterohepatic circulation had occured, to some extent, in most dogs after drug administration. This is in keeping with findings for other NSAIDs (Hobbs and Twomey, 1979; Dupont et al, 1982)

Concentrations of tolfenamic acid in plasma of dogs after intravenous administration were found to have a wide inter-animal variation and to decline rapidly. This was most notable in animals 1 and 2 in which no drug was detected at two hours. Inter-animal variations in drug concentration were particularly apparent at 0.25 hours and from eight hours onwards after subcutaneous administration. A pattern of troughs and secondary

peaks in concentration similar to that noted in the intravenous experiment was observed in most animals after intravenous and subcutaneous administration.

Large inter-animal variation in observed AUC (9.70 - 35.05 μg/ml.h intravenous and 12.53 - 41.47 μg/ml.h subcutaneous) and MRT (1.32 - 19.27 hours intravenous and 3.89 - 11.06 hours subcutaneous) was observed after both intravenous and subcutaneous administration of drug. It is possible that the plasma concentration profile may have played a part in the wide variation of $t^1/_2\beta$ and enterohepatic cycling may occur to varying degrees in different animals. The presence of this phenomenon makes it extremely difficult to accurately determine pharmacokinetic variables for this drug. Mathematical modelling of the concentration versus time data obtained after intravenous administration of tolfenamic acid was best described by two exponents. This was found to be a reasonably accurate description of drug disposition, a correlation of greater than 0.95 was obtained between observed and estimated values for a biexponential curve in all animals. For comparative purposes, pharmacokinetic values calculated directly from observed data were also computed.

Mathematical modelling of the concentration versus time curves obtained after subcutaneous administration demonstrated that the data was best described by two exponents. Goodness of fit of mathematically derived model curves was generally poor. The correlation (r^2) of observed to estimated drug concentrations was found to be lower than 0.90 for all but one animal (dog 6). As a result the kinetic information obtained from directly observed data is likely to be a more accurate indication of the disposition of the drug and therefore observed data was used to calculate kinetic parameters. Comparison of computed $t^1/_2\beta$ with half lifes estimated from observed MRT data indicated that all computed $t^1/_2\beta$ values were an overestimate, in some cases by more than 50%.

It is apparent that the mean AUC obtained after subcutaneous administration was considerably greater than after intravenous administration (29.62 vs 18.10 μg/ml.h). Expression of these figures as bioavailability (F) indicates that in all but one animal (dog 5) the bioavailability was greater than 100 %. This result may be due to the errors in estimating values for AUCs, particularly for that section of the AUC from the time of injection to the first sample. Drug concentrations detected at the earlier sampling times do, however, indicate that tolfenamic acid may be well absorbed after subcutaneous administration.

Mean absorption times (MAT) were in good agreement (2.57 -3.82) hours for dogs 1, 2 and 5. Dog 6 had a longer MAT of 8.14 hours and dog 4 had a negative MAT, indicating that the MRT was longer after intravenous than after subcutaneous

administration. This is theoretically only possible if some of the drug administered during an intravenous infusion does not enter the vein and is deposited subcutaneously or intramuscularly. The occurence of a comparatively small degree of enterohepatic circulation of a drug can result in a large increase in the calculated AUMC. If enterohepatic circulation were more apparent after subcutaneous than after intravenous administration this may result in a small increase in AUC, however the increase in AUMC could outweigh this resulting in a value for the AUMC which is smaller after intravenous than after subcutaneous administration of the drug.

Comparison of bioavailability and MRT indicates that there is no advantage to be derived by intravenous administration.

The inhibition of serum TxB_2 after intravenous administration was initially higher than after subcutaneous administration but was of shorter duration. Mean inhibition of TxB_2 0.5 hours after intravenous administration of tolfenamic acid was greater than 90 %. The pattern of inhibition versus time reflected the apparently erratic plasma drug concentrations with a lower mean inhibition measured at 2 hours (inhibition 23.36 % at drug concentration 0.41 ± 0.20 μg/ml tolfenamic acid) than at four hours (inhibition 31.78 % at drug concentration 0.62± 0.30 μg./ml tolfenamic acid). At one hour the mean inhibition had decreased to 54.47 % although some individual animals had inhibition of greater than 50% at time points thereafter.The maximum inhibition of TxB_2 after subcutaneous administration of tolfenamic acid (99.78 %) was detected in dog 3 at 0.25 hours, however the general level of inhibition during the period up to two hours was in the range 70 - 90 % and this was reflected in the mean values. The pattern of increasing and decreasing inhibition related to drug concentrations at these time points is apparent in samples from some animals (eg dog 1 at 24 and 48 hours, dogs 2 and 4 at 8 and 12 hours) but is not reflected in mean figures due to inter-animal variation.

Calculation of the area under the serum thromboxane inhibition versus time curve confirmed the wide inter-animal variation in inhibition achieved after drug administration. The mean AUC obtained was greater after subcutaneous administration, however this difference was not found to be statistically significant.

Statistical examination was also carried out on the maximum inhibition produced by each route of administration and on inhibition measured at each time point. The mean maximum inhibition was found to be 91.30 and 88.12 % after intravenous and subcutaneous administration respectively. There was no significant difference between these observations. Mean TxB_2 inhibition was found to be significantly higher after subcutaneous administration at 2 and 8 hours post drug administration.

A number of low platelet counts were seen at various time points during the intravenous and subcutaneous experiments and in three animals (dogs 2, 4, and 5) prior to intravenous administration of the drug. The pattern of these did not suggest that they were in any way associated with the administration of tolfenamic acid and were not a consistent feature of drug administration. It is therefore considered unlikely that reduced concentrations of TxB_2 in serum were due to low platelet numbers.

The blood clotting times after administration of tolfenamic acid indicated that there was no apparent change in clotting times which could be associated with drug administration.
Whilst blood was occasionally detected in faecal samples collected throughout the course of each experiment this was never a consistent feature and appeared not to present likely complications associated with the administration of tolfenamic acid at this dose rate.

These results suggest that subcutaneous administration would be an appropriate route for the administration of tolfenamic acid. Intravenous administration appears to confer no advantage in terms of plasma concentration nor TxB_2 inhibition and there is some evidence that TxB_2 inhibition may be superior after subcutaneous administration. The difference in $t^1/_2\beta$ measured for dogs in this study (5.9 hours), when compaired with that reported in man (2.5 hours) (Pentikainen et al, 1981), underlines the need for examination of individual drugs in each species. Greater inhibition of TxB_2 was noted in this study than that reported in man (Pentikanen et al, 1981; Alanko et al, 1989), where tolfenamic acid has been shown to be a superior analgesic than phenylbutazone. The 12 hour persistence of this inhibition and the relatively short $t^1/_2\beta$, suggest that a twice daily oral dosage regimen may be appropriate to maintain good levels of TxB_2 inhibition in the dog. However, further studies are required to confirm the time taken to achieve steady state and confirm the safety of drug concentrations achieved.

It is of interest to note that Espinasse et al, (1992) found good inhibition of PGE_2 production, by lower dose rates of tolfenamic acid, in experimental models of inflammation. These workers administered tolfenamic acid intramuscularly at a dose rate of 2 mg/kg to cattle. Good inhibition of PGE_2 in tissue cage exudates was measured at 24 hours (88 %) and inhibition was greater than 50 % at 48 hours. This indicates that as good or better levels of inhibition of PGs may be expected in the dog, and that 4 mg/kg would be an appropriate dose for further testing in clinical trials or inflammatory models. Recent studies in dogs using tissue cages as the inflammatory model confirm this observation (McKellar, Lees and Gettinby, personal communication).

The comparatively large volume of distribution which was measured after the intravenous administration of tolfenamic acid to dogs is in contrast to that reported in humans (Vdss 0.16 litres/kg) by Pentikainen et al (1981). A small volume of distribution is generally indicative of extensive binding of drug to plasma proteins and is a common feature of NSAIDs, having been reported in several studies (Pentikainen, et al, 1981; Snow et al, 1981ab Rainsford, 1985). It is, however, of interest to consider the findings of Pentikainen et al and Snow et al as these workers also examined the extent of binding of drug to plasma protein. Tolfenamic acid was shown to be 99.7 % bound to human plasma protein and Snow et al found meclofenamic acid to be more than 99 % bound to plasma protein in the horse and to have a volume of distribution of 0.13 litres/kg. The fact that the volume of distribution in this study was almost ten times higher than these reported figures suggests that the plasma protein binding of tolfenamic acid in the dog is considerably lower than that in man, and indeed lower than normal for NSAIDs. A lower percentage of drug bound could result in a higher circulating concentration of unbound (potentially pharmacologically active) drug. If this were the case then it might be expected that high levels of inhibition of serum TxB_2 would have been observed. In fact levels of inhibition generally did not exceed 90 % and it must therefore be assumed that the degree of inhibition of serum TxB_2 is not dependant on unbound drug, or that in the case of tolfenamic acid the drug leaves the circulation and is bound within the tissues. The possibility exists that lower plasma protein binding of tolfenamic acid would result in a lower accumulation of this drug in inflammatory exudates, as suggested by Higgins et al (1986). However if tissue binding had occurred, then it is possible that physical or chemical changes resulting from tissue damage or the inflammatory process could free drug which had been bound, and result in increased localised concentrations of free drug. One possible factor which may be involved in such a process is the action of tissue destructive free radicals.

Further studies to examine the concentrations of drug, degree of protein binding and level of serum TxB_2 and tissuePGE_2 inhibition in experimental models of inflammations are required to provide a greater insight into the action of tolfenamic acid in the dog.

6.7 TABLES AND FIGURES

Time	Route of Administration	
(h)	Intravenous	Subcutaneous
Pre	0	0
0.08	25.41 ± 4.50	NS
0.17	18.18 ± 3.28	NS
0.25	NS	1.11 ± 0.35
0.33	9.92 ± 1.78	NS
0.50	4.98 ± 1.32	2.16 ± 0.32
0.75	3.47 ± 0.66	NS
1.00	1.61 ± 0.31	4.04 ± 0.50
2.00	0.41 ± 0.20	4.05 ± 0.65
4.00	0.62 ± 0.30	1.60 ± 0.38
8.00	0.50 ± 0.14	0.72 ± 0.20
12.00	0.28 ± 0.06	1.17 ± 0.28
24.00	0.07 ± 0.03	0.11 ± 0.07
48.00	0.04 ± 0.02	0.03 ± 0.02
72.00	0.01 ± 0.01	0
96.00	0.02 ± 0.01	0.02 ± 0.02
168.00	0	0

NS = No sample

Table 6.1 Mean (±SEM) concentration (μg/ml) of tolfenamic acid in plasma of dogs after administration at a dose rate of 4.0 mg/kg by the intravenous and oral routes.

Parameters		Units	Animal Number 1	2	3	4	5	6	Mean	± SEM
AUC	(obs)	µg/ml.h	9.70	10.54	23.76	35.05	10.28	19.25	18.10	± 4.12
+ Δ	(obs)	µg/ml.h	11.40	113.31	26.22	37.76	13.33	23.48	20.92	± 4.18
AUC	(cal)	µg/ml.h	13.50	15.53	27.03	42.80	13.82	26.88	23.26	± 4.67
+ Δ	(cal)	µg/ml.h	16.98	18.30	29.49	44.17	16.87	31.12	26.16	± 4.44
AUMC	(obs)	µg/ml.h^2	66.71	51.96	224.66	727.41	17.34	68.28	192.73	± 110.88
+ Δ	(obs)	µg/ml.h^2	66.85	52.19	224.87	727.64	17.60	68.63	192.96	± 110.88
AUMC	(cal)	µg/ml.h^2	68.08	91.70	284.00	897.58	23.73	78.56	240.61	± 136.47
+ Δ	(cal)	µg/ml.h^2	68.37	91.93	284.20	897.69	23.73	78.91	240.81	± 136.46
MRT	(obs)	hours	5.87	3.92	8.58	19.27	1.32	2.92	6.98	± 2.66
MRT	(cal)	hours	5.04	5.90	10.51	20.97	1.72	2.92	7.84	± 2.90
Cp0	(cal)	µg/ml	26.00	42.82	34.69	18.52	42.98	58.00	37.17	± 5.71
Vdss	(obs)	ml/kg	2057.56	1178.36	1308.35	2041.32	396.19	497.94	1246.66	± 293.34
Vdss	(cal)	ml/kg	1493.61	1520.21	1555.16	1960.02	496.88	434.78	1243.41	± 255.68
Vd a	(cal)	ml/kg	1982.48	2502.49	2529.89	2209.45	1669.86	851.66	1957.64	± 257.83
Clb	(obs)	ml/h/kg	350.88	300.52	152.55	105.93	300.07	170.36	230.05	± 40.60
Clb	(cal)	ml/h/kg	296.25	257.51	148.00	93.46	289.39	148.78	205.57	± 35.14
$t^1/_{2\beta}$	(cal)	hours	4.64	6.73	11.85	16.38	4.00	3.91	5.91*	--
V_c	(cal)	ml/kg	153.87	93.41	115.31	215.84	93.06	68.97	123.41	± 21.85
k_{el}	(cal)	hours	1.93	2.76	1.28	0.43	3.11	2.16	1.94	± 0.40
k_{12}	(cal)	hours	4.87	3.85	1.82	2.73	1.01	1.74	2.67	± 0.59
k_{21}	(cal)	hours	0.56	0.25	0.14	0.34	0.23	0.33	0.31	± 0.06

(obs) = calculated from observed values
(cal) = calculated from computed values
+ Δ = computed values for 1st and last triangle added
* = harmonic mean

Table 6.2 Main pharmacokinetic variables in dogs after administration of tolfenamic acid at a dose rate of 4.0 mg/kg by the intravenous route.

Time	Animal Number								
(h)	1	2	3	4	5	6	Mean	±	SEM
Pre	0	0	0	0	0	0	0		-
0.50	85.50	95.25	94.60	84.63	91.25	96.56	91.30	±	2.10
1.00	12.83	76.27	73.37	66.67	26.17	71.53	54.47	±	11.26
2.00	0	46.40	0	31.07	35.36	27.36	23.36	±	7.83
4.00	7.62	50.25	70.73	55.83	0	6.28	31.78	±	12.49
8.00	8.03	47.74	37.92	42.40	0	20.52	26.10	±	7.98
12.00	20.70	50.25	50.41	13.11	7.80	16.26	26.42	±	7.75
24.00	0	48.35	21.14	22.33	15.25	14.45	20.25	±	6.49
48.00	34.69	24.62	17.00	0	29.38	18.14	20.63	±	4.95
72.00	0	17.42	0	0	36.05	26.73	13.37	±	6.44
96.00	12.42	14.52	15.08	0	39.78	45.57	21.21	±	7.16
168.00	0	0	0	0	0	17.66	2.94	±	2.94

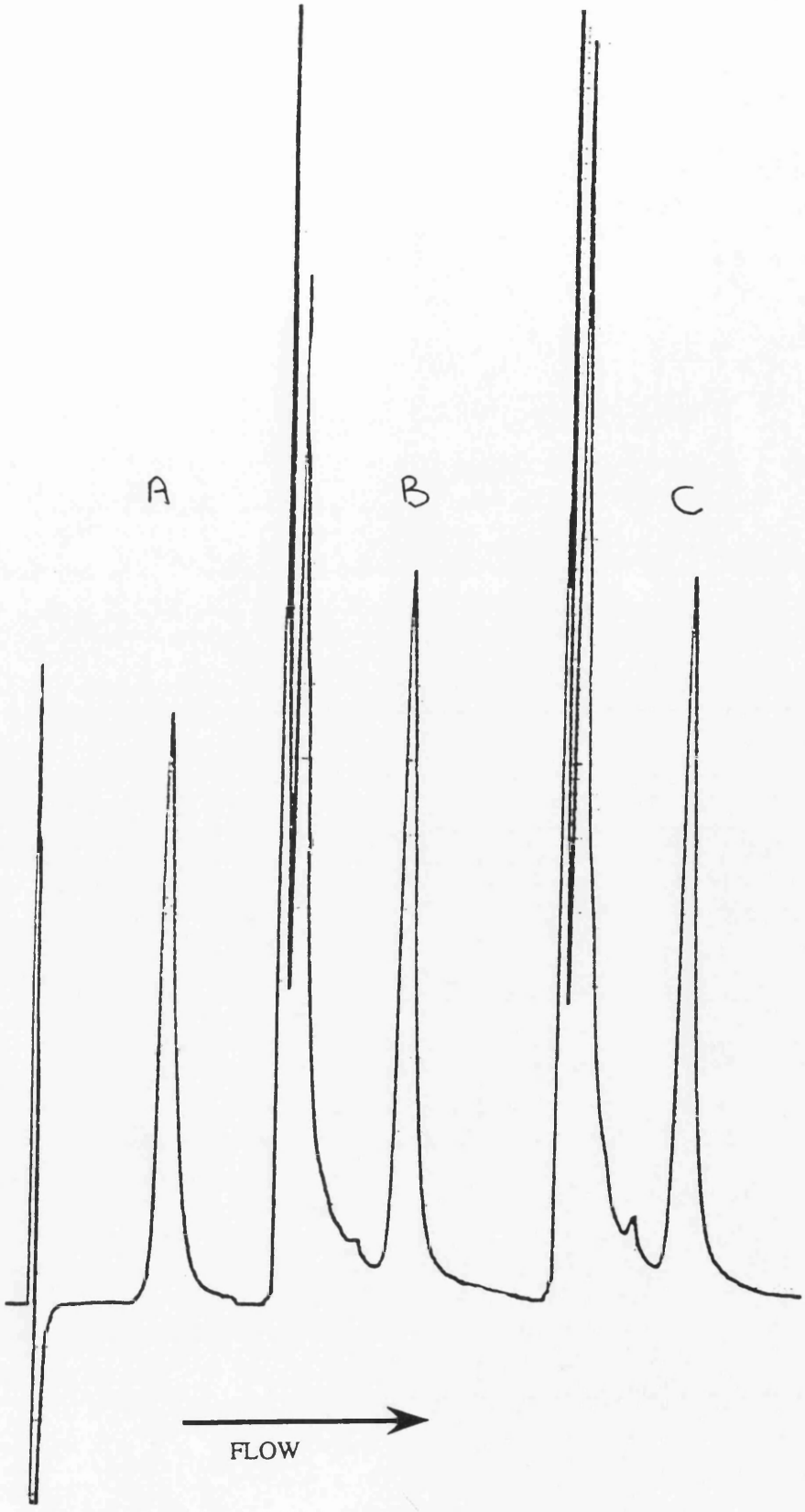
Table 6.3 Inhibition (%) of thromboxane B_2 in serum of dogs after administration of tolfenamic acid at a dose rate of 4.0 mg/kg by the intravenous route.

Parameter	Units	Animal Number 1	2	4	5	6	Mean	±SEM
C_{max}	µg/ml	4.14	4.04	5.76	2.63	5.40	4.39	± 0.55
t_{max}	hours	1.00	1.00	2.00	1.00	2.00	1.40	± 0.24
AUC	µg/ml.h	30.56	24.84	41.47	12.53	38.69	29.62	± 5.18
+ Δ	µg/ml.h	30.72	25.10	41.48	12.73	38.77	29.76	± 5.14
AUMC	$\mu g/ml.h^2$	280.03	194.12	279.06	49.51	428.97	246.34	± 61.95
+ Δ	$\mu g/ml.h^2$	280.07	194.18	279.06	49.56	428.99	246.37	± 61.95
MRT	hours	9.12	7.74	6.73	3.89	11.06	7.71	± 1.20
MAT	hours	3.28	3.82	-12.54	2.57	8.14	1.05	± 3.53
F	%	269.47	188.58	109.85	95.50	165.12	165.70	± 3.10

Table 6.4 Main pharmacokinetic variables in dogs after administration of tolfenamic acid at a dose rate of 4.0 mg/kg by the subcutaneous route.

Time	Animal Number								
(h)	1	2	3	4	5	6	Mean	±	SEM
Pre	0	0	0	0	0	0	0		-
0.25	74.12	68.61	99.78	91.11	62.68	32.30	71.43	±	9.68
0.50	87.30	82.79	90.29	87.92	66.40	54.58	78.21	±	4.98
1.00	89.35	81.15	84.39	85.37	77.51	57.23	79.17	±	4.68
2.00	83.33	75.31	78.49	79.42	82.90	82.79	80.37	±	1.30
4.00	71.04	39.85	63.22	50.97	52.17	56.48	55.62	±	4.39
8.00	60.41	42.46	45.88	78.74	21.02	51.07	49.93	±	7.85
12.00	59.33	48.49	50.73	79.31	0	28.27	44.35	±	11.15
24.00	20.83	9.21	30.60	45.30	8.43	14.80	21.53	±	5.82
48.00	35.81	21.92	0	21.67	29.14	16.52	20.84	±	4.99
72.00	29.17	0	0	21.67	29.14	16.52	16.08	±	5.45
96.00	49.51	0	0	8.83	0	14.45	12.13	±	7.86
168.00	21.13	15.00	0	2.45	24.86	8.87	12.05	±	4.09

Table 6.5 Inhibition (%) of thromboxane B_2 in serum of dogs after administration of tolfenamic acid at a dose rate of 4.0 mg/kg by the oral route.



A. Blank dog plasma fortified with 1.0 µg/ml drug.

B. Plasma sample estimated to contain 0.96 µg/ml of drug.

C. Standard solution containing 5 µg/ml of drug.

Figure 6.1 Typical chromatogram of tolfenamic acid in dog plasma.

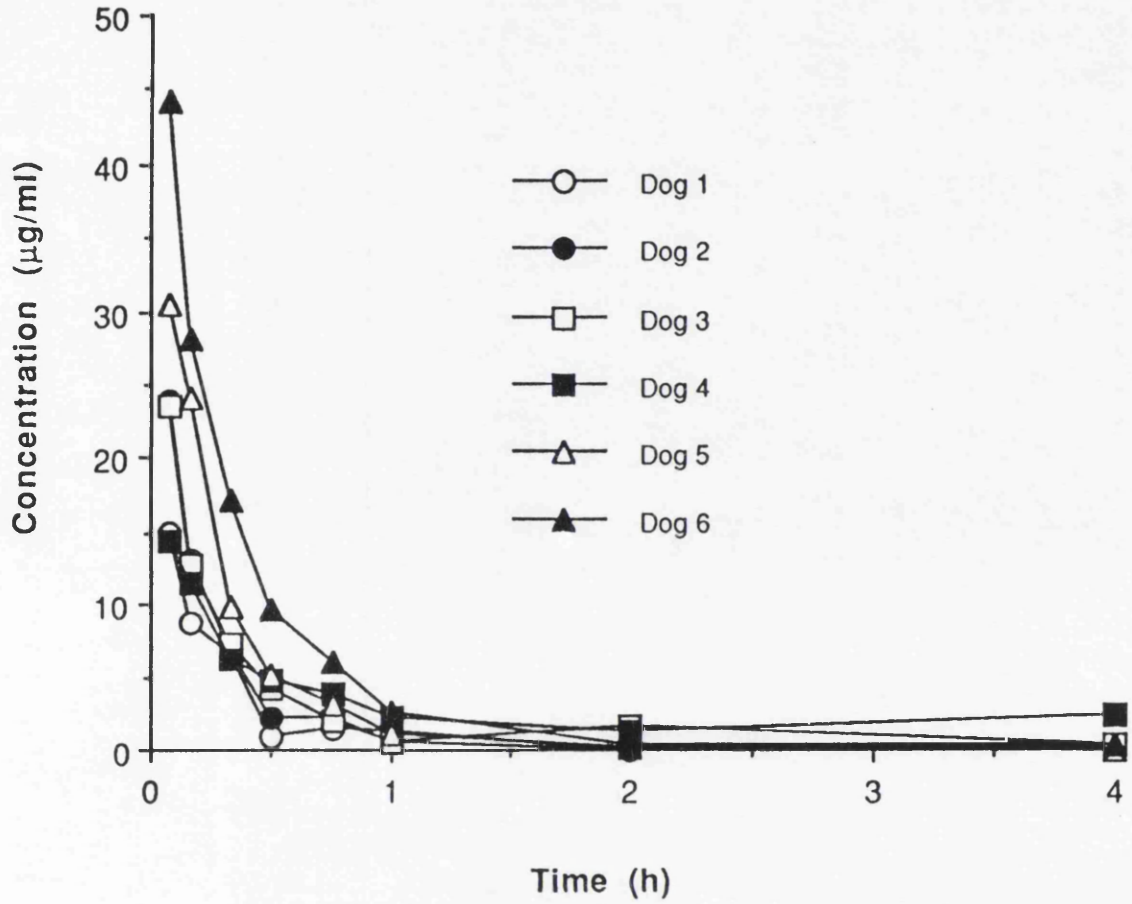


Figure 6.2 Concentration (µg/ml) of tolfenamic acid in plasma of dogs after administration by the intravenous route at a dose rate of 4.0 mg/kg .

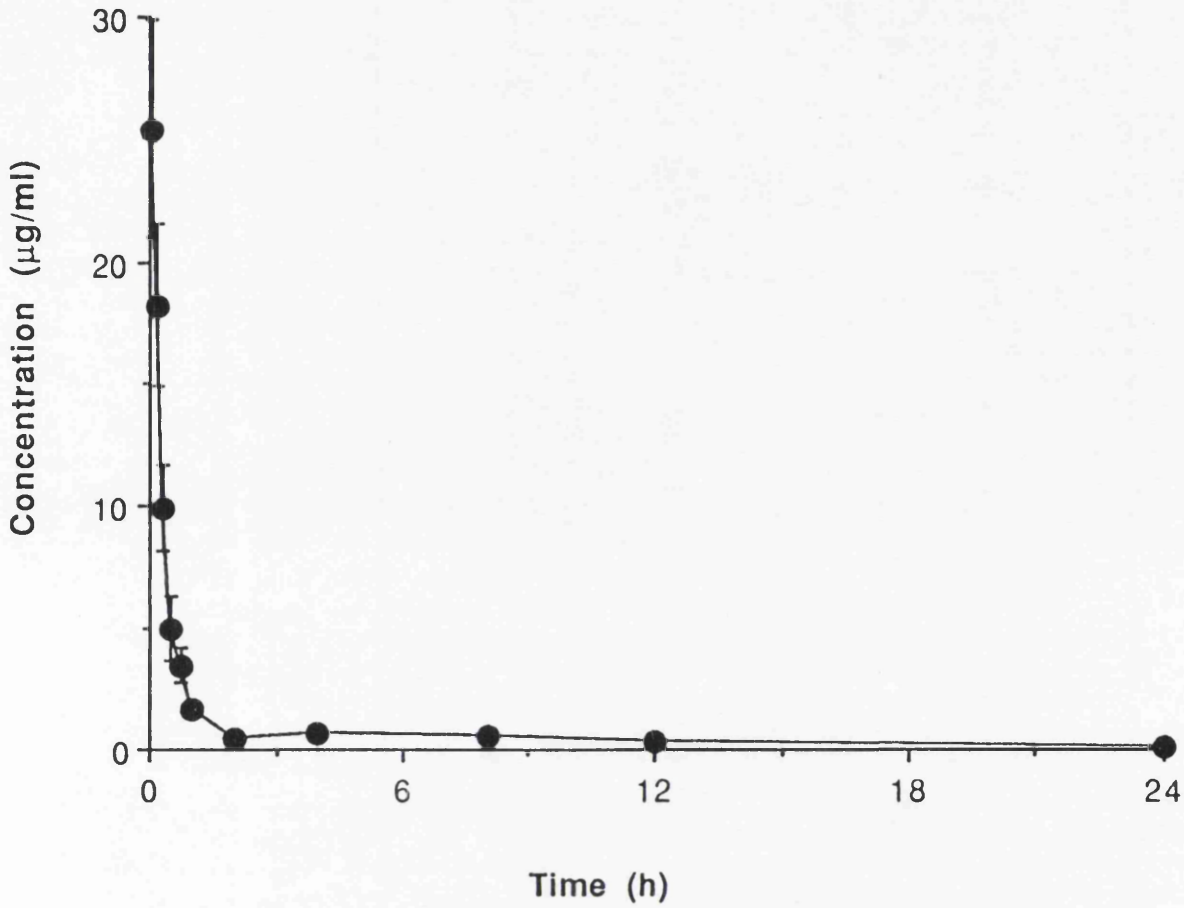


Figure 6.3 Mean (±SEM) concentration (µg/ml) of tolfenamic acid in plasma of dogs after administration by the intravenous route at a dose rate of 4.0 mg/kg body weight.

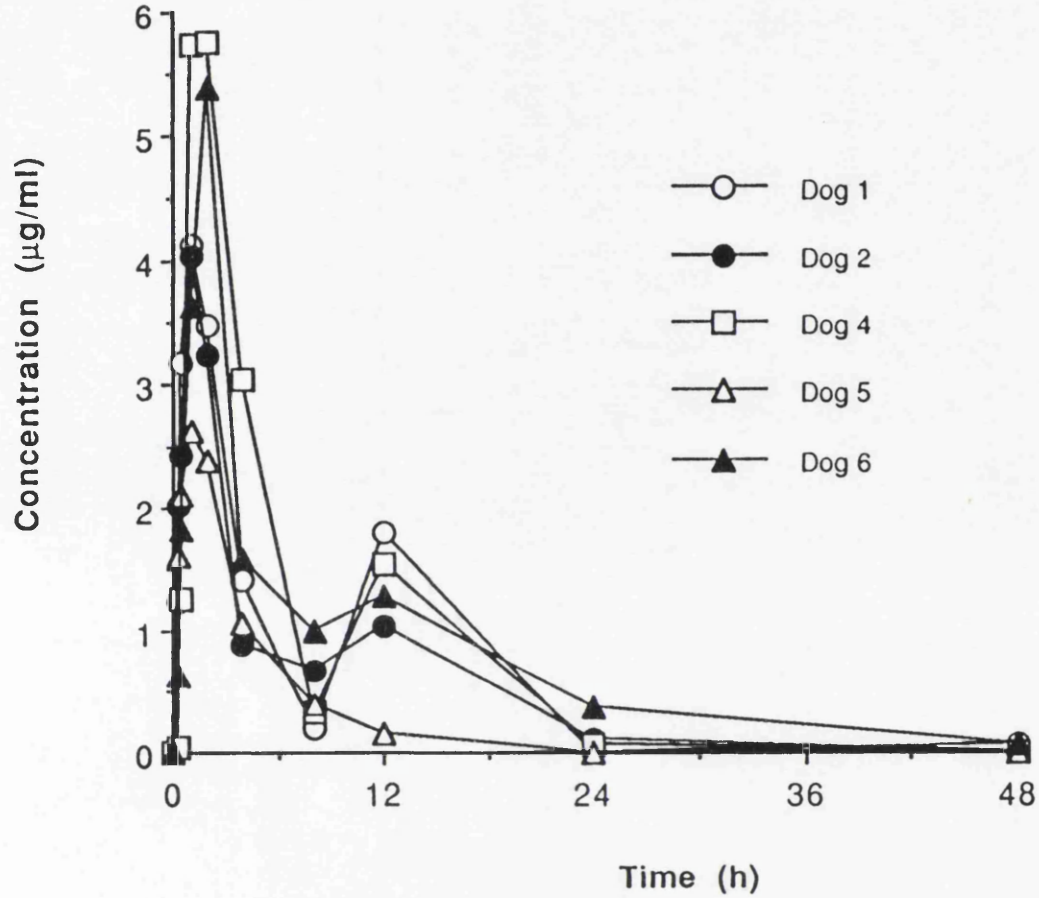


Figure 6.4 Concentration (µg/ml) of tolfenamic acid in plasma of dogs after administration by the subcutaneous route at a dose rate of 4.0 mg/kg body weight.

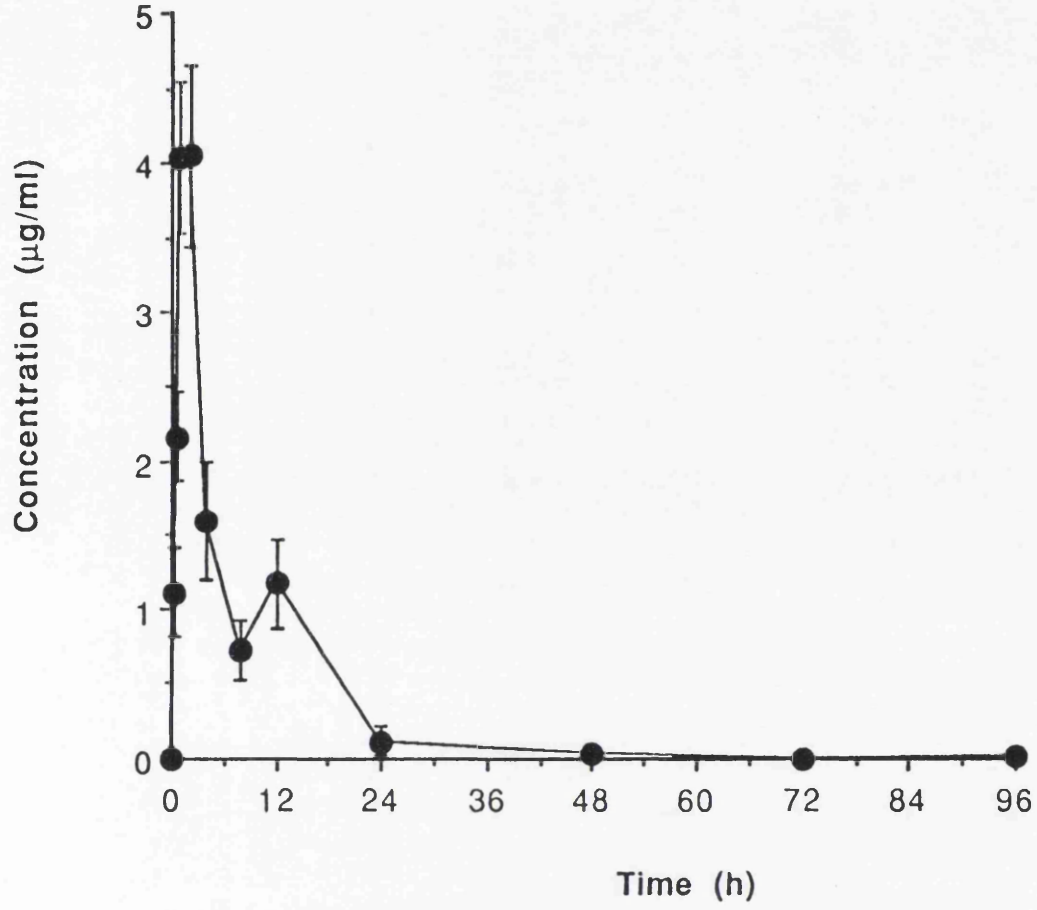


Figure 6.5 Mean (±SEM) concentration (µg/ml) of tolfenamic acid in plasma of dogs after administration by the subcutaneous route at a dose rate of 4.0 mg/kg body weight.

Chapter 7
PROTEIN BINDING OF FLUNIXIN MEGLUMINE AND MECLOFENAMIC ACID

7.1. INTRODUCTION

Serum albumin is the most abundant protein in plasma (Doolittle, 1979) and performs several important physiological roles. It is responsible for the maintenance of the osmotic pressure of blood, the transport of fatty acids and the sequestration and transport of bilirubin. It is a simple protein, comprised of a single polypeptide chain containing 585 amino acids and has a molecular weight of 66500 (Behrens et al, 1975: Meloun et al, 1975). Albumin is unique amongst the major plasma proteins in having no carbohydrate component (Peters, 1975). The albumin molecule is hydrophilic and is highly soluble in aqueous media, this results from the presence of more than 200 positive and negative charges which are distributed over its surface (Peters, 1975). Serum albumin has been shown to have a repeating loop structure which is comprised of two large and one small loop in each of three separate areas, these are formed by the presence of disulphide bonds (Brown and Shockley, 1982).

The liver is the primary site of albumin production (Miller et al, 1951). Here, synthesis occurs on ribosomes attached to endoplasmic reticulum. Albumin is secreted from ribosomes to the rough endoplasmic membrane and subsequently through the smooth endoplasmic reticulum to the golgi complex, after which it passes through the cell membrane (Peters et al, 1971; Redman and Cherian, 1972; Jamieson and Ashton, 1973). The average time for this process is about 30 minutes.

The normal half life of circulating albumin is about 19 days in man (Schultze and Heremans, 1966; Peters, 1970) and the horse (Kaneko, 1989) and approximately 8 days in the dog (Kaneko, 1989) and it appears to be directly correlated to body size. About 10 % of plasma albumin is generally lost in the gut (Jeffries et al, 1962). A small proportion is not reabsorbed after glomerular filtration and is lost in urine. It is thought that the balance of catabolism results from the small, but continual, uptake into cells by pinocytosis of microscopic droplets of circulating plasma (Cushman, 1970).
In man only 40% of albumin is in the circulation at any given time. Five percent of circulating albumin passes from the blood into interstitial fluid each hour, and returns during the subsequent 24-28 hours via the thoracic duct (Peters, 1975). There is albumin present in almost every body fluid and in most body secretions (Schultze and Heremans, 1966).

Serum albumin plays an important role in the binding of drugs within the body. It is the serum protein primarily involved in the binding of drugs, although many basic drugs are known to bind to acid glycoproteins and lipoproteins (Riviere, 1988). Drug protein binding (in blood and tissue proteins) is an important factor for regulation of drug distribution within the body, and it has been widely accepted that only unbound

drug is able to exert any pharmacological action. Monitoring of drug concentration in plasma has become standard practice and for most drugs clinical response is more closely correlated with plasma drug concentrations than with dose administered (Booker and Darcy, 1973; Borga, 1980; Levy, 1980; Kilpatrick et al, 1984). The monitoring of plasma concentrations is of particular importance for drugs with narrow therapeutic indices, drugs with concentration dependant kinetics, or in patients for whom the risk of toxicity is abnormally high (eg with impaired renal function). Most analytical procedures used for therapeutic monitoring measure total drug concentration in plasma or serum and, unless information on the binding of drug is available, these results may be misleading.

In general, drugs are reversibly bound to proteins and this may involve a combination of a variety of bonds including van der Waals and electrostatic forces (Perrin, 1985). Initial studies on human serum albumin suggested that the conformational flexibility of the molecule results in the existence of a number of relatively non-selective sites and this was proposed as an explanation for its ability to bind a wide variety of substances with different chemical structures (Nilsen, 1976; Muller and Wollert, 1979). Although the situation has not yet been fully elucidated, it is now clear that a relatively small number of specific binding sites exist on the molecule (Sudlow et al, 1976; Fehske et al, 1981). A number of these sites have been localised, and two of the most important sites have been named I and II. Sudlow et al (1976) were able to characterise these sites according to the properties of the drugs which they generally bind. Site I binds hydrophilic drugs, generally aromatic acids which are ionised at physiological pH. Site II attracts more hydrophobic compounds, these are also generally aromatic acids but tend to be larger heterocyclic molecules. Table 7.1 shows some drugs which have been shown to bind to sites I and II.

NSAIDs have been shown to be extensively bound to plasma protein (Glazko et al, 1978; Brune and Lanz, 1985) and this has resulted in considerable interest in the binding of this group of drugs. Table 7.2 shows the extent of protein binding in man for some common NSAIDs.

Although plasma binding of drugs may differ considerably between species (Sturman and Smith, 1967; Borga et al, 1968), intra-species protein binding is thought to vary little in healthy individuals, and in man it is rare to see a variability in free drug concentration of greater than two fold (Routledge, 1985). There are, however, some circumstances under which the free fraction of drug in blood is known to vary. Physical factors including temperature and pH are important (Chignell, 1977), although there is limited variation in these parameters in vivo. The variability of the

concentration of free drug is also related to changes in albumin concentration, and the effect of this will be most profound in cases where protein binding is high (Routledge, 1985). In cases of chronic renal failure the extent of protein binding has been found to be lower than that predicted from the albumin concentration of the patient (Boobis, 1977), and protein binding of salicylates was found to be significantly decreased in children with Kawasaki disease (a disease of unknown origin, most common in children, characterised by high fever, conjunctivitis, skin rash and swelling of glands) compared with normal controls (Koren et al, 1991). It has been suggested that this may be due to structural changes in the protein which reduce its ability to bind ligands, or to the production of unidentified endogenous inhibitors of drug binding (Sjoholm et al, 1976; Boobis, 1977).

A further factor which affects the binding of drugs to albumin is the presence of other drugs. It is known that drugs are able to compete for binding sites on albumin, and some of the most active displacing substances are acidic compounds such as NSAIDs (Sellers and Koch-Weser, 1971). There are some circumstances under which these displacement reactions are of great importance. When a drug with high protein affinity is given as a bolus injection displacement may be almost instantaneous, and the concentration of free displaced drug may be significantly increased. Under normal conditions the rates of displacement and re-equilibration of a drug within the body preclude excessive elevations of free (pharmacologically active) concentrations of drug in plasma. However, if the re-distribution of free drug is delayed or does not occur uniformly, or if the safe therapeutic range is narrow this may result in toxic effects. Also, since plasma drug monitoring generally assumes that the ratio of free and bound drug remains constant, it is possible for total plasma concentrations to be misinterpreted if the overall concentration of drug in plasma has decreased, due to a reduction in only the bound component (D'Arcy and McElnay, 1985).

In addition to displacement interactions it is also thought that the presence of one drug may alter the ability of protein to bind another drug. Acetylsalicylic acid has been shown to acetylate serum albumin and it is thought that this results in the modification of the binding sites (Erill and Calvo, 1985). Binding of acetrizoate was enhanced after treatment with acetylsalicylic acid (Farr et al, 1966; Hawkins et al, 1968), however the binding decreased when treatment was replaced by sodium salicylate (Hench and Farr, 1967). Other binding changes which have been shown to occur after treatment with acetylsalicylic acid include increased binding of phenylbutazone and decreased binding of flufenamic acid (Chignell and Starkweather, 1971). For some chiral drugs the enantiomers exhibit different protein binding. Binding of the R(-) enantiomer of ibuprofen and flurbiprofen is lower than that of the S(+) enantiomer, and binding was

found to be influenced by the presence of the optical antipode (Evans et al, 1989; Knadler et al, 1989).

Although albumin is the most abundant of the serum proteins, blood plasma contains numerous other proteins, and many of these proteins also bind drugs. Globulins are known to bind sex hormones and may also have a binding capacity for pharmacological agents (Routledge, 1985). Another important group of blood proteins are the lipoproteins. These are macromolecular complexes which transport plasma lipids in the circulation and are generally grouped, according to density, into four main classes named chylomicrons. A wide variety of hydrophobic drug groups have been shown to bind to lipoproteins, including non-ionisable neutral compounds (eg Probucol) (Urien, et al, 1984), acidic mucopolysaccharides (eg heparin) (Mahley et al, 1979), acidic drugs (eg diclofenac) (Chamouard et al, 1985), steroids (Counsell, and Pohland, 1982), basic compounds (eg tetracycline) (Powis, 1974) and peptides (eg cyclosporin A) (Lemaire and Tillement, 1982). A large variety of drugs including acepromazine, propranolol and warfarin, are also known to bind to alpha$_1$-acid glycoprotein. Current evidence suggests that these, and other drugs, compete for a single binding site on this plasma protein (Muller, et al, 1985)

Approximately 45% of the volume of circulating blood is composed of blood cells and of this 95% comprises erythrocytes. The remaining 5% contains blood leucocytes and thrombocytes and this small proportion is generally ignored when binding to blood cells is measured. The terms erythrocyte and blood cells are therefore used synonymously in this area of research. The size of an erythrocyte is many hundreds of times larger than an albumin molecule and the major protein constituent of erythrocytes (haemoglobin) is approximately 8 times more abundant in blood than albumin on a molar basis, although the molecular weight of 64500 is similar to that of albumin. Phenothiazines and pentobarbital are amongst the drugs known to bind to haemoglobin (Wind et al, 1973; Ehrnebo, 1980).There is some evidence to suggest that binding is linearly related to the concentration of drug, and that there is competition between plasma proteins and blood cells for the binding of free drug available in whole blood (Ehrnebo, 1985). The equilibration rate of drug and blood cells is highly variable and 5 minutes and 90 minutes have been reported in vitro for diphenylhydantoin and chlorthalidone (a more hydrophobic drug), respectively (Beermann et al, 1975; Ehrnebo and Odar-Cederlof, 1977). It has also been demonstrated that drug binding may be reversible and competitive with other drugs (Ehrnebo and Odar-Cederlof, 1977).

Drug binding is also known to occur in tissues (D'Arcy and McElnay 1982; McElnay and D'Arcy, 1983). It was thought that tissue binding was principally the result of drug binding to the extravascular fraction of albumin (Jusko and Gretch, 1976). However, more recently drugs have been shown to bind to a variety of structures in tissues (Kurz and Fichtl, 1983).

Fichtl and Schuhmann (1985) studied the relationship between tissue and plasma binding of 44 drugs in the rabbit and rat. These workers reported a poor intra-species correlation in binding between plasma and tissue for a given drug. However, they found good correlation in binding to different tissue types, and this correlation was found to extend to a number of unrelated drugs. It was also found that there was a close correlation for binding of drugs to muscle of rat, rabbit and man and it has been suggested that tissue binding may be governed by similar mechanisms in different species. In this respect tissue binding in one species may serve as a model for others. Examination of published data for 29 drugs in different species led Fichtl and Schuhmann (1985) to conclude that inter-species differences in the distribution volume of drugs principally result from inter-species differences in binding of drugs in plasma and not to binding in tissue.

Binding has a marked effect on the distribution, metabolism and excretion of drugs (Routledge, 1985). Protein binding plays a major role in glomerular filtration and may be of some importance in tubular secretion (Balat, 1985). Only free drug is filtered into the Bowman's capsule and tubular secretion may depend on the rate of dissociation of bound drug. Studies on the plasma protein binding of propranolol in man, dog, rat and monkey have shown the volume of distribution to be inversely related to plasma protein binding (Evans et al, 1973).

Drug retention in plasma is regulated by a number of factors which include the ability to pass through vascular membranes, and lipid solubility. In addition, drug is retained in plasma when the capacity of plasma to bind the drug is greater than that of the tissues. The resultant trapping of drug in plasma and restricted diffusion into tissues is seen as high concentrations of drug in blood and a low volume of distribution. This predominantly occurs for acidic drugs which are highly ionised at the physiological pH of plasma. Tissue retention of drug occurs when the binding capacity in plasma is low relative to that in the tissues. In this situation, which is most common for basic drugs, the plasma concentration is often lower than that in tissues and the volume of distribution of the drug is large (Houin, 1985).

Current interpretation of drug behaviour in the body is largely based on the widely held belief that only the unbound fraction of a drug can reach its target site and be pharmacologically active. However it has been suggested that the major biologically active fraction of a drug is that bound to protein, and that drug bound to protein is available for transport to tissues (Pardridge, 1985). Some drugs have been shown to leave the blood stream many times faster than the plasma proteins (Dewey, 1959; Pardridge et al, 1985), indicating that transport of protein bound drugs into tissues involves enhanced dissociation of drug from plasma protein. One possible explanation for this is that the enhanced dissociation from plasma protein may be the result of interaction with, as yet, unidentified endogenous substances within the microcirculation (Pardridge and Landaw, 1984).

7.2 EXPERIMENTAL OBJECTIVES

The objectives of this work were - to examine the protein binding of two common NSAIDs, flunixin and meclofenamic acid, in the serum of dogs, goats and horses.

7.3 MATERIALS AND METHODS

7.3.1 DIALYSIS EXPERIMENTS WITH MECLOFENAMIC ACID AND FLUNIXIN

7.3.1.1 ANIMALS

All animals used throughout the experiment were housed indoors. Animals used in the study appeared to be clinically normal and had no recent history of any clinical abnormality.

7.3.1.2 SERUM COLLECTION

One hundred millilitres of blood were collected into unheparinised glass Vacutainers (Becton Dickinson Ltd) from each of three dogs, goats and horses. The blood was allowed to clot for 24 hours at room temperature and the clot gently eased from the side of the tube by means of a glass rod prior to removal. The serum fraction was separated from the resultant liquor by centrifugation at 4°C and 3000g for 15 minutes. Serum from each animal was pooled to give one sample for each species and this was stored frozen in glass universal bottles at -20 °C until use. Prior to use the serum was defrosted overnight and allowed to equilibrate to room temperature.

7.3.1.3 DRUG PREPARATION

On the day of use a 300 μg/ml solution of each drug (flunixin meglumine and meclofenamic acid) was prepared using 0.85 % aqueous sodium chloride as solvent. The solution was sonicated in an ultrasonic bath for 5 minutes to ensure complete dissolution and was then stored at room temperature until use.

7.3.1.4 FORTIFICATION OF SERUM WITH DRUG

Thirty millilitres of serum of a single species were placed in a glass beaker and stirred constantly at a slow speed with a magnetic stirrer. To this was added, dropwise over a period of one minute, 0.5 ml of the appropriate 300 μg/ml drug solution, to produce a final concentration of 5 μg/ml of drug in serum. The serum was then left to mix at room temperature for 30 minutes.

7.3.1.5 PREPARATION OF DIALYSIS TUBING

Seamless cellulose visking tubing (25 mm diameter) which was permeable to water and had a molecular cut off in the range 12,000 - 14,000 (BDH Ltd) was cut into a number of 12 inch lengths and pre-wetted in distilled water. To ensure removal of impurities the lengths were boiled twice for 1 minute in a solution containing 5 mM EDTA and 200 mM sodium bicarbonate (prepared using distilled water). The tubing was thoroughly rinsed with distilled water after each boiling. The lengths of visking tubing were prepared on the day prior to use and stored at 4 °C overnight, in a covered beaker containing distilled water. On the day of use the tubing was transferred to a beaker containing fresh distilled water and allowed to equilibrate to room temperature prior to use.

7.3.1.6 DIALYSIS

Fifteen millilitres of fortified serum were transferred into dialysis tubing which had been prepared as described above. After securing, this was placed in a large dialysis tank containing 25 litres of 0.85 % sodium chloride. The tank was maintained undisturbed at room temperature (18 °C) for 18 hours.
The balance of the fortified serum was transferred to a universal bottle and stored at -20 °C until drug analysis was performed.
After dialysing for 18 hours the equilibrated serum was removed from the dialysis tubing and transferred to a universal bottle for storage in a similar manner to the undialysed aliquot.

Preparation and dialysis for flunixin was carried out for all three species on a single occasion. A similar procedure was performed for meclofenamic acid on a separate occasion.

7.3.2 ANALYTICAL METHODS

7.3.2.1 ANALYSIS OF FLUNIXIN

Analysis of flunixin was carried out by H.P.L.C. using spectrophotometric detection according to the method described for experiments with flunixin in chapter 3.

The concentration of flunixin in each sample was determined by reference to aliquot of the sample of serum which had been fortified with drug prior to dialysis. These samples were processed in an identical manner to that described for the dialysed samples. In addition, standard solutions of flunixin were injected directly onto the H.P.L.C.. These standard solutions allowed equipment performance to be monitored. Before and after injection of each sample an injection was made of standard drug solution of known concentration. The concentration (20 μg/ml) was chosen to produce a peak height on the final trace which was similar to that expected from the samples.

For each sample the quantity of drug detected was expressed as a ratio of peak height of sample : peak height of standard. The ratios calculated for dialysed and undialysed samples were compared and the difference expressed as a percentage. This allowed the calculation of the degree of protein binding.

No calculation of actual percentage extraction of drug from serum was made nor were extraction losses assessed as these were assumed to be constant between the analysis of dialysed serum samples and the samples of undialysed fortified bulk serum to which they were compared.

7.3.2.2 ANALYSIS OF MECLOFENAMIC ACID

Analysis of meclofenamic acid was carried out by high performance liquid chromatography (H.P.L.C.) using spectrophotometric detection.

7.3.2.3 REAGENTS

1. Meclofenamic acid, sodium salt (Sigma Chemical Co.)

2. Chloroform, H.P.L.C. grade (Rathburn Chemicals Ltd)

3. Methanol 'Analar' (B.D.H. Ltd), redistilled prior to use

4. Disodium hydrogen orthophosphate 'Analar' (B.D.H. Ltd)

5. Citric acid 'Analar' (B.D.H. Ltd)

6. Citrate/phosphate buffer pH 3.0 was produced by mixing the following solutions :-

20.5 ml M/5 Na_2HPO_4 + M/10 citric acid

7. Water, redistilled in the presence of potassium permanganate prior to use.

8. Perchloric acid, 70 % 'Analar' (B.D.H. Ltd)

9. H.P.L.C. mobile phase comprising 80 : 20, methanol : water containing 50 µl of 1 in 55 aqueous solution of perchloric acid per 100 ml mobile phase.

7.3.2.4 H.P.L.C. EQUIPMENT

Pump:-	Gilson 301
Detector:-	Spectraphysics 100
Column:- (Shandon	100mm X 8mm containing 5µ O.D.S. Hypersil Southern)
Wavelength:-	287nm
Absorbance:-	0.05 AUFS
Flow Rate:-	1.5 ml/min.

The retention time of meclofenamic acid under these conditions was 5 minutes.

7.3.2.4 SAMPLE PREPARATION

A 1 ml aliquot of each serum sample was placed in a 50 ml ground glass tube. To this was added 0.3 ml of citrate/phosphate buffer (pH 3.0), followed by 6 ml chloroform. The tube was stoppered and the mixture shaken for 10 minutes by inversion on a slow rotary mixer. Four millilitres of the chloroform were recovered and placed in a 10 ml conical glass tube. These tubes were placed in a Dri-Block (Techne Ltd) at 50 °C under a stream of air and allowed to evaporate to dryness. A final 1 ml of chloroform was run down the walls of the conical tube to ensure that all residue present was in the base of the tube. The

tube was returned to the Dri-Block and allowed to evaporate to dryness. Samples were covered with clingfilm and stored overnight at 4 °C if necessary. Prior to injection onto the H.P.L.C. system the residues were reconstituted in methanol. An appropriate volume (not less than 150 µl) of methanol was added to the bottom of the conical tube which was then tilted and rotated in an ultrasonic bath for 1 minute. A 20 µl injection loop was used for injection, the loop was loaded with 15 µl of sample.

The procedure for comparison of dialysed and undialysed samples, and the calculation of percentage binding was as described for flunixin. A typical chromatogram is shown in figure 7.1.

7.4 RESULTS OF DIALYSIS EXPERIMENTS WITH FLUNIXIN AND MECLOFENAMIC ACID

The ratio of peak height of drug in sample : peak height of drug in standard for each species are shown in appendix 11.1 for flunixin and 11.2 for meclofenamic acid. The percentage binding for each drug in each species is shown in table 7.3.
As can be seen from the standard error (appendix 11.1 and 11.2) the inter sample variation within each species was small. Serum protein binding of flunixin was 92.0, 87.0 and 86.6 % in dog, goat and horse, respectively. The inter-species variation in binding was not large. Protein binding of meclofenamic acid in serum showed larger inter-species variation.The extent of binding was 93.8, 84.2 and 100.0 % for dog, goat and horse respectively.

7.5 DISCUSSION

The binding study was restricted to only two drugs because of the insolubility of other NSAIDs in suitable aqueous solvents and the unsuitability of non aqueous solvents for use in protein binding studies. Drugs which were considered for use, and which had limited solubility in saline were piroxicam, tolfenamic acid, carprofen, and phenylbutazone. Each of these appeared to dissolve in saline to produce a concentration of 1 µg/ml. However, analytical methods sensitive enough to accurately determine the degree of dissolution at this concentration were not available. Also, the addition of a drug solution of this concentration to serum would have resulted in a final concentration below the limit of analytical detection and outwith the in vivo circulating concentrations generally targeted for these drugs.

The concentration of drug used to fortify serum in this study was targeted to be within the range of blood concentrations achieved during normal clinical use. As a result of

the high degree of binding normally reported for NSAIDs (Brune and Lanz, 1985), coupled with the comparatively low concentration of drug studied, it was anticipated that the total concentration of unbound drug in serum would be small. The reliable limit of detection of available methods for the quantification of drug did not permit the measurement of unbound drug, consequently estimation of the extent of binding was based on the concentration of bound drug detected following dialysis.

Estimation of the concentration of unbound drug would have been possible by increasing the total concentration of drug used to fortify the serum by a factor of 5 or 10. It would then have been possible to employ a system of ultrafiltration using centrifugation to collect the unbound fraction. The concentration of drug in this fraction could then have been determined by the analytical methods employed in this study to quantify the bound fraction. Such an increase in the concentration of drug with which the serum was fortified would only have been justified if additional studies had been carried out to establish that the binding of drug to serum protein was linear throughout the concentration range in each species. Such studies were not performed for the drugs studied as they were examined at a single concentration targeted to be within the normal therapeutic range.

The results of this study are in agreement with the general findings that most NSAIDs are highly protein bound (Brune and Lanz, 1985). Binding estimations have generally been carried out in human serum or plasma. In this study, the binding of flunixin to serum proteins was very similar between horse and goat (86.6 and 87.0 % respectively), however a higher degree of binding was measured for flunixin in the dog (92 %). In all cases the extent of binding was lower than that reported for mares by Soma et al (1988). Using a micropartition centrifugation method, these workers determined the binding of flunixin to serum proteins to be greater than 99 % in samples which contained known concentrations of flunixin, and also in serum which had been fortified with flunixin.

Blood protein binding of meclofenamic acid has been reported to be 99.8 % in man (Glazko et al, 1978). A similar degree of binding (>99 %) was reported in horses (Snow et al, 1981b), however this figure was determined from samples containing a high total drug concentration. These figures are similar to the results obtained for horse serum in this study, where binding was determined to be 100 %. The extent of binding for meclofenamic acid in serum from dogs was determined as 93.8 %, the variation in these figures, coupled with the considerably lower estimate of binding in the goat (84.2 %) indicates that blood protein binding of this drug may vary considerably between species. The present results confirm the findings of Sturman and Smith (1967) and

Borga et al (1968) and indicate that significant variations in binding of some NSAIDs may occur between species. It is interesting to note that in this study the variation does not appear to show any correlation with respect to species and drug. For flunixin the binding is similar between horse and goat, whereas for meclofenamic acid binding is similar between horse and dog. It would appear, therefore, that it is necessary to establish the degree of blood protein binding separately for each drug and each species.

Fichtl and Schuhman (1985) were unable to demonstrate a correlation between plasma and tissue binding of drug. In a study relating to 29 drugs these workers concluded that there was a close correlation between tissue binding in different species and accordingly hypothesised that the inter-species difference in distribution volume of these drugs was due to species differences in drug binding in plasma. If this proves to be generally correct it may be possible to estimate plasma protein binding in a target species from a knowledge of the plasma and tissue binding in any species together with a knowledge of the volume of distribution of the drug in the target species. In addition, as the concentration of NSAID at target tissues may be more important than the concentration in blood, it may be possible to determine the concentration of a drug in the tissues of all species by investigation of a single model species.

Examination of figures, from the present study, for protein binding show maximum inter species differences of 5 % and 16 % respectively for flunixin and meclofenamic acid. However if we consider that the 'active' portion of any drug dose is generally thought to be the unbound drug then these differences are seen to be more extreme. Table 7.4 shows the concentration of unbound drug which would exist in each species when the total blood concentration of drug is 5 μg/ml. From these figures it is apparent that, in percentage terms, these differences are large. For example there would be 162 % more unbound flunixin and 255 % more meclofenamic acid circulating in the blood of goats than dogs. These very large differences may go some way towards explaining the many anomalies which are seen in NSAID use. It is interesting that estimations in these experiments indicated that all meclofenamic acid in the serum of horse is bound to protein. This may simply reflect inaccuracies in the technique. It is apparent, however, that the percentage of this drug which exists in the unbound state in the horse will be very low. As this is undoubtedly an efficacious drug, this raises questions as to its ability to be active in the bound state or at extremely low concentrations of free drug.

It may be that some anti-inflammatory and analgesic therapies are characterised by accumulation (or decreased rate of removal) of drug at the site of action (Higgins et al, 1986). In inflammatory exudates, where the protein concentration is initially relatively low, this accumulation may be partly founded on free drug available, and large inter-

species variations in binding could result in significantly different degrees of efficacy. Higgins et al (1986) have shown that in experimentally produced equine inflammation the concentration of albumin in inflammatory exudate increases during the course of the inflammatory process. Dissociation of bound drug at the site of inflammation as a result of equilibration would continue to ensure that the concentration of unbound drug remains higher in species with a lower binding ratio.

Pardridge (1985) has suggested that there may be some process by which protein bound drugs undergo enhanced dissociation, thereby enabling free access to tissues. This possibility does of course require that the accepted theory of access of many drugs to their target sites be re-evaluated. The existence of such a mechanism may explain the ability of meclofenamic acid to act as an effective NSAID in horses, when it is apparently totally bound to serum protein. It is interesting to note that the concentration of flunixin in experimentally produced inflammatory exudate decreases more slowly than in plasma during the period 3 to 12 hours after drug administration (Higgins et al,1986). However, during this time it was also noted that the total protein concentration in exudate was increasing and it is therefore not clear if the maintained drug concentrations result from this protein accumulation or some other mechanism.

Studies on the extent, nature and relevance of protein binding of drugs require to be continued until the significance and the interaction of the many unexplained factors have been clarified. These studies are of particular importance for NSAIDs which are highly bound to blood protein and are widely used in both veterinary and human medicine.

7.6 TABLES AND FIGURES

Site I	Site II
Warfarin	Flufenamic acid
Phenylbutazone	Ibuprofen
Oxyphenbutazone	Naproxen

(Data from Sudlow et al, 1976 & Birkett and Wanwimolruk, 1985)

Table 7.1 Some drug ligands known to bind at sites I and II on the serum albumin molecule.

Drug	Protein Binding (%)
Acetylsalicylic Acid	>70
Carprofen	99.9
Ibuprofen	99
Ketoprofen	99
Indomethacin	99
Piroxicam	99
Meclofenamic Acid	99.8

(Data from Brune and Lanz, 1985 and Glazko et al, 1978)

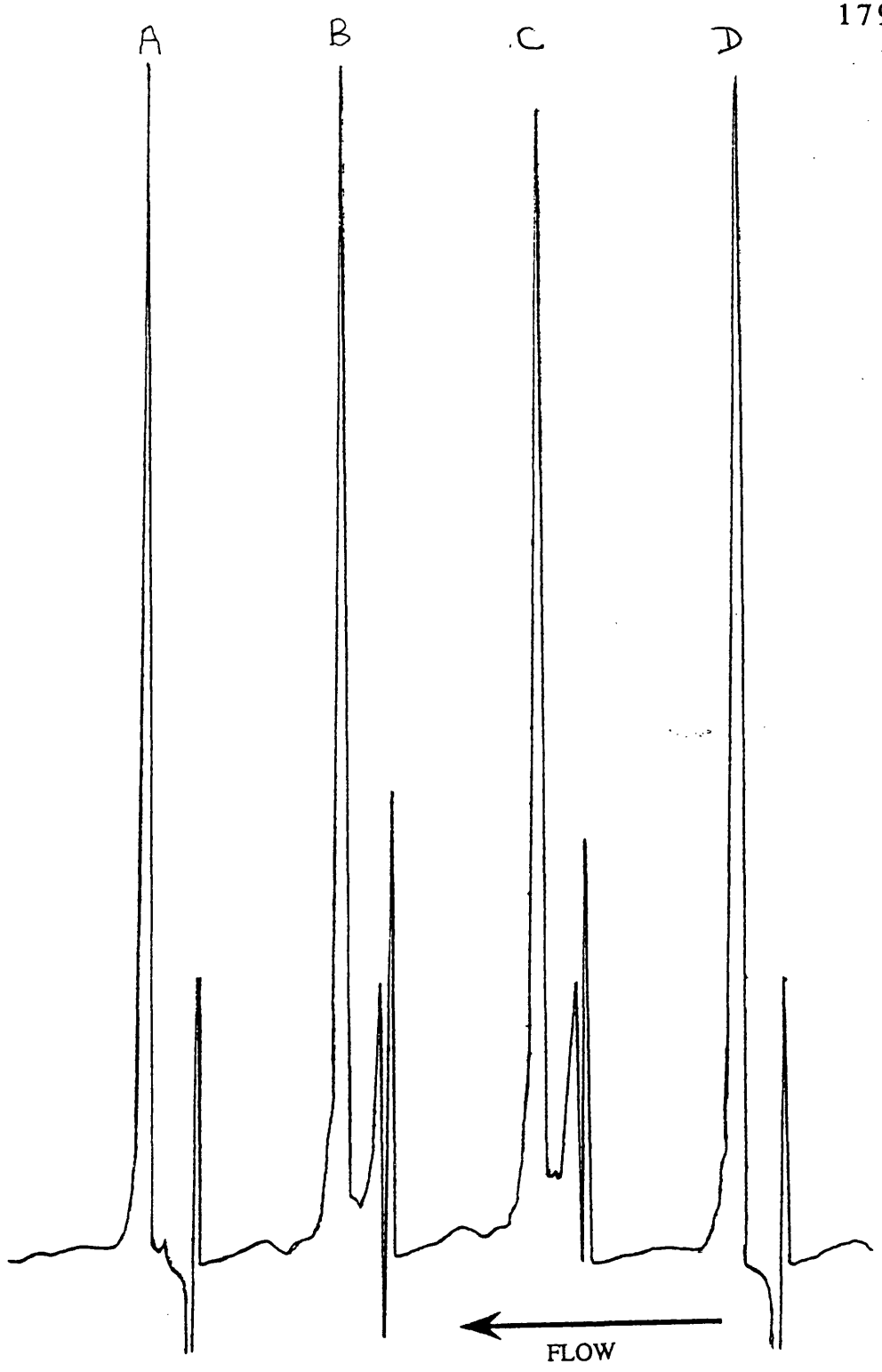
Table 7.2 The extent of plasma protein binding in man for some common NSAIDs.

Drug	Species		
	Dog	Goat	Horse
Flunixin	92.0	87.0	86.6
Meclofenamic Acid	93.8	84.2	100

Table 7.3 Binding (%) of flunixin and meclofenamic acid to serum protein of three animal species.

Drug	Species		
	Dog	Goat	Horse
Flunixin	0.40 (0.81)	0.65 (1.32)	0.67 (1.36)
Meclofenamic Acid	0.31 (0.97)	0.79 (2.47)	0

Table 7.4 Concentration (μg/ml) of unbound flunixin and meclofenamic acid in blood of dogs, goats and horses when the total blood concentration of drug is 5 μg/ml. Figures in parenthesis show micromolar concentrations (μM).



A. Standard solution containing 20µg/ml of drug

B. Blank dog serum which has been fortified with 5µg/ml drug

C. Blank dog sserum which has been dialysed after fortification with 5µg/ml drug

D. Standard solution containing 20µg/ml drug

Figure 7.1 Typical chromatogram of meclofenamic acid in dog plasma.

Chapter 8

STUDIES ON THE INHIBITION OF IN VITRO PRODUCTION OF SERUM THROMBOXANE BY NON-STEROIDAL ANTI-INFLAMMATORY DRUGS.

8.1 INTRODUCTION

Chemical or mechanical injury to the body tissues results in the release of eicosanoids. These are not stored in the body, but are produced de novo on the occasion of each stimulation (Piper and Vane, 1971). The formation of these important mediators of the inflammatory process, which include PGs, Txs, PGI_2 and LTs, is a result of the activity of cyclooxygenase or lipoxygenase enzymes which cause oxygenation of arachidonic acid (Samuelsson et al, 1978; Samuelsson et al, 1980). Within the cyclooxygenase pathway dioxygenase enzyme acts initially on arachidonic acid to produce PGG_2, from which PGH_2 and prostaglandins A, B, C, D, E, and F are formed (Ramwell et al, 1980), PGI_2 and the thromboxanes are formed from PGH_2. Thromboxanes are not true prostaglandins as they do not have the cyclopentane ring typical of the group. However, they are the only members of the so called endoperoxides formed by the cyclooxygenase pathway which are known to have biological activity (Ramwell et al, 1980).

Some of the general properties of eicosanoids have been discussed earlier and little doubt remains that they exhibit a range of activities which are significant in the inflammatory process. The discovery of the pro-inflammatory role of eicosanoids resulted in the search for compounds which could inhibit their action. Early work by Ferreira et al (1971) and Vane (1971) identified this to be the mechanism of action of aspirin. Later studies also suggested that this inhibitory process was the basis of many of the undesirable side-effects which result from the usage of anti-inflammatory drugs (Vane, 1976).

A variety of techniques have been developed to test and compare existing and novel NSAIDs. Some of these techniques are based on the ability of the drug to provide analgesia under experimental conditions, for example in phenylquinone-induced writhing in mice (Roszkowski et al, 1971). Others require the production of experimental inflammation in which the response to the introduction of a pro-inflammatory substance, such as carrageenan, is studied. Such tests are often performed in small laboratory animals, for example carrageenan is used in the production of rat paw oedema (Vinegar et al, 1969). Carrageenan may also be used to produce skin blisters, or in conjunction with implanted tissue cages for the collection of inflammatory exudate in some domestic animal species (Higgins et al, 1984a). In addition, NSAIDs have been studied in clinical trials where their true clinical efficacy could be assessed. In each of these cases there are ethical and practical difficulties. The use of animals for experimental purposes has become increasingly unacceptable to society and this is particularly true where there is a requirement to inflict

discomfort or pain. Although tests such as the production of oedema can be carried out in small animals, the implantation of devices to collect inflammatory exudate is generally only practical in larger animals, and as a result economic factors also become important. Experimentally induced inflammation has been used, in various species, to study the active factors present in inflammatory exudate (Willis, 1969; Velo et al, 1973; Higgs et al, 1983; Higgins et al, 1984b). These studies indicate that eicosanoid content of carrageenan-induced inflammation shows inter-species similarities. Prostaglandin E_2 concentration has been shown to become elevated in experimentally produced inflammatory exudates from rats, horses and ponies (Willis, 1969; Higgs at al, 1983; Higgins et al, 1984b; Higgins and Lees 1984b), and smaller increases in TxB_2 and 6-keto-prostaglandin F_{1alpha} concentration have also been demonstrated in rats and ponies (Higgs et al, 1983; Higgins and Lees, 1984b). The discovery of these changes in the concentration of eicosanoids in experimentally produced exudate led to their use as monitors of the action of NSAIDs. As the increase in concentration of these substances is inhibited by the administration of NSAIDs, quantitative and qualitative changes in exudate eicosanoid composition are used to evaluate and compare the potential efficacy of NSAIDs

The use of clinical studies is, of course, important in determining the efficacy of proven NSAIDs in specific situations. However, there is little moral justification for initiating clinical trials to test a compound until some positive indication of its efficacy has been established.

In vitro testing of NSAIDs has been carried out using a variety of species and tissues (Flower et al, 1972; Flower and Vane 1972; Gryglewski, 1974), and has often been based on the inhibition of PGE_2 synthesis. This technique employs the collection of fresh tissue from clinically normal animals which are known to be free from interfering substances and, therefore, generally uses purpose killed experimental animals. From these tissues (eg dog spleen, guinea pig lung and bovine seminal vesicles) homogenates containing microsomes are prepared and these are incubated under controlled conditions in the presence or absence of inhibitors of eicosanoid synthesis. Supernatants from these incubations are then compared for prostaglandin -like activity in a bioassay system (eg using rat fundic strips).

The search for a test, to assess NSAIDs, which was inexpensive and which conformed to current moral guidelines, resulted in the development of an ex vivo TxB_2 assay. Lees and co-workers (1987a) employed this method to assess the inhibition of production of thromboxane in clotting blood by measuring differences in the concentration of the relatively stable endoperoxide TxB_2. Using this technique it is possible to compare the

concentration of TxB_2 generated in animals to which NSAID has been administered with control animals, or to compare TxB_2 concentration in the same animal prior to and after NSAID administration.

Thromboxane A_2 is a product of the arachidonic cascade and therefore its generation can be reduced by cyclooxygenase inhibitors (Lees et al, 1987). The half life of eicosanoids has been shown to be short, PGI_2 has a half life of about 4 minutes (Salmon and Flower, 1979). An even shorter in vitro half life for TxA_2 of 32 seconds (Salmon and Flower, 1979) has been determined, and as a result measurement of this compound is quite impractical. Thromboxane A_2 is hydrolysed to TxB_2 (Hamberg et al, 1975) which is relatively stable, having a half life of 20-30 minutes (McCann et al, 1981). As a result, the measurement of TxB_2 by radioimmunoassay is accepted as an indicator of the production of TxA_2 (Fitzpatrick et al, 1977) and the accuracy of this technique has been verified by gas liquid chromatography-mass spectrometry (Granstrom et al, 1976; Fitzpatrick et al, 1977).

The principal effects of TxA_2 are its potent induction of platelet aggregation and its action as a vasoconstrictor (Hamberg et al, 1975; Svensson et al, 1975). The primary source of TxA_2 within the body is blood platelets (Hamberg et al, 1975) although migrating leucocytes are also known to be a source (Higgs et al, 1983). In addition, it is thought that TxA_2 may be produced by migrating macrophages (Humes et al, 1977). The major endogenous antagonist of TxA_2 induced platelet aggregation is PGI_2 (Gorman, 1982). This substance not only inhibits platelet aggregation but also promotes vasodilation and is produced in the cells of the blood vessel walls.

The technique initially used to measure TxB_2 ex vivo has been adapted to develop a technique which may be used to monitor the effects of NSAIDs in vitro. Since the major source of thromboxanes in the body is blood platelets, it is possible to study inhibition of thromboxane production in blood which has been removed from the body and maintained under controlled conditions. Normal concentrations of TxB_2 in human blood are in the range 7-300 pg/ml (Fitzgerald et al, 1983). Blood platelets generate increased quantities of TxB_2 during the clotting process, and this increase is time related (Lees et al, 1987). By ensuring rigourous control of the conditions under which this clotting occurs it is possible to use this as the basis of a test system. The addition of a cyclooxygenase inhibitor to samples of clotting blood can be used to create a dose response curve for the inhibition.

8.2 EXPERIMENTAL OBJECTIVES

To utilise an in vitro test system for measurement of TxB_2.to determine and compare the inhibition of cyclooxygenase by two NSAIDs (flunixin and meclofenamic acid) in three different species, namely dogs, goats and horses.

8.3 MATERIALS AND METHODS

8.3.1 REAGENTS

1. Sodium chloride 'Analar' (BDH Ltd)

2. Water, redistilled in the presence of potassium permanganate prior to use.

3. Disodium hydrogen orthophosphate 'Analar' (BDH Ltd)

4. Sodium dihydrogen orthophosphate 'Analar' (BDH Ltd)

5. Ethanol 'Analar' (BDH Ltd)

6. Flunixin meglumine (Schering Inc., USA)

7. Meclofenamic acid, sodium salt (Sigma Chemical Co. Ltd)

8. 0.85 % Sodium chloride

9. Phosphate buffer pH 7.0 was produced by mixing the following solutions:-

   195 ml of 28.392 g/litre disodium hydrogen orthophosphate + 305 ml of 31.202 g/litre sodium dihydrogen orthophosphate + 8.5 g sodium chloride per litre of water.

10. Phosphate buffer pH 7.5 was produced by mixing the following solutions :-

    80 ml of 28.392 g/litre disodium hydrogen orthophosphate + 420 ml of 31.202 g/litre sodium dihydrogen orthophosphate + 8.5 g sodium chloride per litre of water.

11. Phosphate buffer pH 8.0 was produced by mixing the following solutions:-

26.5 ml of 28.392 g/litre disodium hydrogen orthophosphate + 473.5 ml of 31.202 g/litre sodium dihydrogen orthophosphate + 8.5g sodium chloride per litre of water.

8.3.2 EXPERIMENTS TO MEASURE THE INHIBITION OF TxB_2 USING THE IN VITRO DRUG TEST SYSTEM

8.3.2.1 ANIMALS

Blood from three species : dogs, goats and horses, was used in these experiments. In all cases three or more individual animals of each species were studied. All animals within a species were of one breed, that is Beagle dogs, Saanen goats and Thoroughbred horses. All dogs and goats were aged between 1 and 3 years. Horses were 19, 17, 8 and 11 years old (horse 1, 2, 3 and 4 respectively). Animals were maintained indoors for a period of at least one week prior to experimental work and appeared to be clinically normal, none had a recent history of any clinical abnormality.

8.3.2.2 DESIGN OF THE TEST SYSTEM

One millilitre of blood was added to a fixed volume of 0.85 % NaCl containing the desired concentration of drug. This was achieved by the initial production of solutions containing 400, 40, 4. 0.4 and 0.04 µg/ml of flunixin meglumine or meclofenamic acid in 0.85 % NaCl. An appropriate volume of the drug solution was then added to a glass test tube. The final volume was made up to 0.5 ml by the addition of 0.85 % NaCl, and the solution thoroughly mixed by vortexing. Final concentrations of drug which were tested were in the range 0.0005 - 10.0 µg/ml for flunixin and 0.0005 - 133.33 µg/ml for meclofenamic acid. These concentrations fall within the range likely to be achieved in plasma after administration at the recommended dose rates (Snow et al, 1981b; Hardie et al, 1985a; Lees et al, 1987; Soma et al, 1992). Tubes containing each concentration of drug were produced in duplicate and layed out so that duplicate concentrations were placed distant from each other in the assay design. In addition, tubes containing blank 0.85 % NaCl were included as every tenth tube. This arrangement was employed so that the mean of the results obtained for each concentration and blank would minimise any differences in TxB_2 concentration which may have occured due to the time taken to pipette each blood sample.

The necessity to work to a tight time schedule for a large number of samples required that the inhibition experiments were performed on blood from only one animal on any given day.

8.3.2.3 SAMPLES FOR THE ESTIMATION OF THROMBOXANE IN SERUM BY RADIOIMMUNOASSAY

All necessary equipment was set up in an area immediately adjacent to the animal. Blood was drawn into a 20 ml disposable syringe (Plastipack, Beckton Dickenson Ltd.) from the jugular vein, via a 20 g one inch needle (Becton Dickenson Ltd.). If, for any reason, the entire 20 ml collection could not be completed without delay the collection was abandoned and begun again using a fresh needle and syringe. After collection of 20 ml of blood, the needle was removed from the syringe and the blood gently ejected into a 50 ml glass beaker which was suspended in a water bath at 37 °C. One millilitre aliquots of this blood were then pipetted, using an automatic pipette, into glass test tubes which were also suspended in the 37 °C water bath. Each tube contained a test solution. The disposable tip on the automatic pipette was not changed between each pipetting. The content of each tube was immediately mixed by gentle shaking, the time was noted and the tubes covered with aluminium foil to prevent accidental contamination. This process was repeated until the required number of 1 ml aliquots had been dispensed. Each set of tubes was incubated in the water bath at 37 °C for 90 minutes, after which they were centrifuged and serum harvested, as described in the general material and methods section.

8.3.3 EXAMINATION OF THE EFFECT OF A RANGE OF SOLUTIONS ON THE INHIBITION OF TxB_2

As a result of the low aqueous solubility of piroxicam it had been necessary to employ a solution in ethanol for the study of this drug in which intravenous administration was required. It was not clear if the presence of ethanol in the solution administered had influenced the inhibition of thromboxane which was subsequently measured. Many other NSAIDs are poorly soluble in water and consequently the possibility of employing other solvents for use in in vitro studies had been considered. A study was therefore carried out to establish the effect on thromboxane generation of a range of solutions. The test system as described above was utilised, however, in each tube 0.85 % NaCl was replaced by the solution for which the inhibitory properties were to be

tested. Solutions tested in this way were pH 7.0, 7.5 and 8.0 phosphate buffered saline, and a range of concentrations of ethanol (0.01 - 10 %) prepared in 0.85 % NaCl. The inhibition of serum TxB_2 which occured in the presence of these solutions was measured in comparison with that which occured in the presence of 0.85 % NaCl. A significant ($p<0.05$) inhibition of serum TxB_2 was measured in the presence of all test solutions with the exception of 0.01, 0.1 and 1.0 % ethanol when compared with 0.85 % NaCl (table 8.1).

8.3.4 CURVE FITTING AND ESTIMATION OF IC_{50}

Non-linear curve fitting was carried out by means of the computer package Sigmaplot (Jandel Scientific, Germany). This package uses the Marquardt-Levenberg algorithm in an iterative process. Least squares are calculated and used to minimise the sum of squares of differences between equation values and experimental values. The IC_{50} is then calculated from the best fit curve.

8.4. RESULTS

8.4.1 IN VITRO EXPERIMENTS WITH FLUNIXIN

The concentration of TxB_2 generated from serum of dogs, goats and horses in vitro in response to inhibition by flunixin is shown in appendices 12.1-12.3. From this data it was possible to calculate the difference between TxB_2 generated in the presence of a range of concentrations of flunixin with that generated in control samples. The results are expressed as a percentage with respect to control values (tables 8.2 - 8.4). Mean (± SEM) inhibition of TxB_2 production is shown in table 8.5 and figure 8.1.

8.4.1.2 DOGS

The mean inhibition of TxB_2 in serum of dogs at the minimum concentration of flunixin tested (0.0005 µg/ml) was 9.57 ± 4.94 %. The inter-animal range in inhibition was large at this, and many of the other concentrations tested, and at this concentration there was no inhibition of TxB_2 production in one animal (dog 4). Inhibition of approximately 50 % was measured at flunixin concentrations of 0.05 and 0.075 µg/ml. At each of these concentrations inhibition did not exceed 50 % of control in all animals. In one animal (dog 3), inhibition was 48.81 % at 0.05 µg/ml and in dogs 2 and 3 inhibition was 34.01 and 49.87 % respectively at 0.075 µg/ml. A concentration of 0.1 µg/ml flunixin produced inhibition of TxB_2 greater than 50

% in all dogs, and at this concentration the mean inhibition was 63.87 ± 4.18 %. Production of TxB_2 was reduced by 90 % or more in all animals at concentrations of 1.0 µg/ml of flunixin, and the mean inhibition at this concentration was 94.48 ± 1.40 %. At all higher concentrations tested, the inhibition of TxB_2 production exceeded 90 % in all animals, and at the maximum concentration tested (10 µg/ml), inhibition exceeded 95 % in all animals (mean 97.71 ± 0.51 %). The maximum mean inhibition was 98.11 %, this was produced by a solution of 5.0 µg/ml flunixin.

8.4.1.3 GOATS

The mean inhibition of TxB_2 in serum from goats at the minimum concentration of flunixin tested (0.0005 µg/ml) was 8.52 ± 3.64 %. Although the inter-animal range in inhibition was large, some degree of inhibition of TxB_2 production was detected in all goats at this drug concentration. Inhibition in excess of 50 % were measured at flunixin concentrations of 0.02 µg/ml and above (mean inhibition 64.74 ± 5.92 %). Production of TxB_2 was reduced by more than 90 % (94.82 %) in one animal (goat 3) at a drug concentration of 0.05 µg/ml, and inhibition in excess of 90 % was measured in blood from all animals at drug concentrations of 0.10 µg/ml and above (mean inhibition at 0.10 µg/ml was 95.98 ± 1.43 %). At the maximum drug concentration tested (10 µg/ml), inhibition of serum TxB_2 exceeded 98 % in all animals (mean 99.42 ± 0.36 %).The maximum mean inhibition was 99.63 %, this was produced by a solution of 5.0 µg/ml flunixin.

8.4.1.4 HORSES

The mean inhibition of TxB_2 in serum from horses at the minimum concentration of flunixin tested (0.0005 µg/ml) was 11.41 ± 5.78 %, however in one animal (horse 3) no inhibition of TxB_2 production was detected. The mean inhibition exceeded 50 % at a flunixin concentration of 0.025 µg/ml (60.28 ± 8.64 %), however in one animal (horse 3) the inhibition of TxB_2 at this drug concentration was only 38.56 %. A concentration of 0.05 µg/ml flunixin produced inhibition of TxB_2 greater than 60 % in all horses at which concentration the mean serum TxB_2 inhibition was 76.26 ± 7.10 %. Production of TxB_2 was reduced by 90 % or more in all animals at concentrations of 0.75 µg/ml of flunixin, and the mean inhibition at this concentration was 97.55 ± 0.42 %. At all higher concentrations tested the inhibition of TxB_2 production exceeded 90 % in all animals and at the maximum concentration

tested (10 μg/ml) inhibition exceeded 97 % in all animals (mean 98.57 ± 0.57 %).The maximum mean inhibition was 99.66 %, this was produced by a solution of 2.5 μg/ml flunixin.

8.4.2 IN VITRO EXPERIMENTS WITH MECLOFENAMIC ACID

The concentration of TxB_2 generated from serum of dogs, goats and horses in vitro in response to inhibition by meclofenamic acid is shown in appendix 12.4-12.6. From these data it was possible to calculate the difference between TxB_2 generated in the presence of a range of concentrations of meclofenamic acid with that generated in control samples. The results are expressed as a percentage with respect to control values (tables 8.6 - 8.8). Mean (± SEM) inhibition of TxB_2 production is shown in table 8.9 and figure 8.2

8.4.2.1 DOGS

The mean inhibition of TxB_2 in serum of dogs at the minimum concentration of meclofenamic acid tested (0.0005 μg/ml) was 22.46 ± 3.96 %. A mean inhibition greater than 50 % (61.98 %) was measured at 0.2 μg/ml meclofenamic acid, however this result is misleading as it is based on values from only two animals (dogs 1 and 2) for which inhibitions of 94.06 and 29.90 %, respectively, were measured. Inhibition did not exceed 50 % in all animals sampled until 2.5 μg/ml (mean 81.22 ± 0.72 %) and at the next highest concentration of drug (5.0 μg/ml) the inhibition measured for dog 3 was again below 50 % (43.28 %). At 10 μg/ml meclofenamic acid, the maximum drug concentration which was tested in all three dogs, inhibition exceeded 88 % in all animals and the mean inhibition of TxB_2 at this concentration was 95.55 %. In one animal (dog 3), drug concentrations of up to 38.55 μg/ml were tested. At this concentration the inhibition measured was little more than that produced by 10 μg/ml meclofenamic acid in blood from dog 2 (98.77 %), and was less than that produced by 10 μg/ml in dog 1 (99.09 %). The maximum mean inhibition was 98.98 %, this was produced by a solution of 38.55 μg/ml meclofenamic acid.

8.4.2.2 GOATS

The mean inhibition of TxB_2 in serum of goats at the minimum concentration of meclofenamic acid tested (0.001 μg/ml) was 6.52 ± 6.06 %. There was large inter-animal range in inhibition, and at this concentration there was no inhibition of TxB_2

production in one animal (goat 2). The first drug concentration at which inhibition in all animals exceeded 50 % was 0.25 μg/ml (mean inhibition 64.82 ± 2.84 %), however one animal (goat 2) had 51.92 % inhibition at a drug concentration of 0.1 μg/ml. Production of TxB_2 was reduced by 90 % or more in all animals at concentrations of 1.0 μg/ml of meclofenamic acid, and the mean inhibition at this concentration was 93.92 ± 1.29 %. At all higher concentrations tested, the inhibition of TxB_2 exceeded 90 % and at the maximum concentration tested in all animals (10 μg/ml) inhibition exceeded 99 % in all goats (mean 99.78 ± 0.06 %). Higher drug concentrations which were tested in two animals (goats 2 and 3) produced inhibition up to a maximum of 99.94 %.

8.4.2.3 HORSES

The mean inhibition of TxB_2 in serum of horses at the lowest concentration of meclofenamic acid tested (0.001 μg/ml) was 19.10 ± 14.62 %. No inhibition of TxB_2 production was detected for the serum from one animal (horse 2) at this concentration and this illustrates the large inter-animal variation which was recorded. A mean inhibition in excess of 50 % was produced by 0.25 μg/ml meclofenamic acid (69.81 ± 5.70 %), however inhibition in excess of 50 % had been noted with drug concentrations of 0.05 and 0.1 μg/ml for horse 1. A mean inhibition in excess of 90 % was measured for drug solutions of 0.75 μg/ml. However, at this concentration one animal (horse 3) had an inhibition of 86.20 %. Production of TxB_2 was reduced by 90 % or more in all animals at concentrations of 1.00 μg/ml of meclofenamic acid, and the mean inhibition at this concentration was 96.03 ± 1.84 %. At all higher concentrations tested the inhibition of TxB_2 production exceeded 95 % in all horses, and at the maximum concentration tested in all animals (20 μg/ml) inhibition exceeded 99 % (mean 99.73 ± 0.10 %). Drug concentrations of 38.55 and 133.33 μg/ml were also tested in two animals (horses 2 and 3).The maximum inhibition detected from these higher concentrations was 99.91 % by 38.55 μg/ml meclofenamic acid in blood from horse 2.

8.5 DISCUSSION

There are a number of advantages to the use of this in vitro test system. Firstly, it fulfils the previously stated criteria of being comparatively non-invasive. Secondly, it does not require the monitoring of inflicted pain or discomfort. Thirdly, it is based on the quantification of a compound, the assay of which has been fully validated, and requires no subjective

judgements such as those often required in clinical trails. Finally, it is a comparatively simple and cost effective process by which inter-species and inter-drug comparisons can be made.

A large inter-animal variation in serum TxB_2 inhibition was noted in all species and for both drugs. This was particularly apparent at lower concentrations of drug and it may be due to the difficulty of accurately measuring small degrees of inhibition. This variation may also have been caused by the existence of a threshold of activity for drug concentrations below which no inhibition occured and which showed inter-animal variation.

Examination of the raw data for individual animals and mean values showed that approximately 10-20 % inhibition of serum TxB_2 occured in most species in the presence of 0.001 μg/ml of either drug (ie approximately 0.002 μM flunixin and 0.003 μM meclofenamic acid). The exception to this was in the goat where 0.003 μM of meclofenamic acid produced a mean inhibition of only 6.52 %. At a drug concentration of 10 μg/ml (20.37 μM flunixin and 31.4 μM meclofenamic acid), inhibition exceeded 95 % for both drugs in goats and horses. In dogs, inhibition at 10 μg/ml meclofenamic acid ranged from 88-99 %. Examination of the concentration at which inhibition equalled or exceeded 50 % (IC_{50}) showed a variation between drugs and within species. Based on mean data the concentration of flunixin which produced 50 % or more inhibition of serum TxB_2 was 0.025, 0.020 and 0.025 μg/ml (0.051, 0.041 and 0.051 μM) for dog, goat and horse respectively. For meclofenamic acid the corresponding concentrations were 0.20, 0.25 and 0.25 μg/ml (0.628, 0.785 and 0.785 μM) and statistical examination of data shows that flunixin meglumine was significantly more effective in inhibiting serum TxB_2 than meclofenamic acid at a number of the concentrations tested (appendix 12.7). Computation of IC_{50} values from best fit curves for this data resulted in somewhat different values which are shown in table 8.10, this served to highlight the difficulties of making estimates from raw data. Figures for IC_{50} derived from the best fit curve showed that on a molar basis flunixin was a considerably poorer inhibitor of serum TxB_2 in vitro in dogs than in the two other species examined. It was 5.8 times more effective in producing inhibition of TxB_2 in the goat than in the dog, twice as effective an inhibitor in the goat than in the horse and 2.9 times more effective in the horse than the dog. Meclofenamic acid was equipotent in its actions in the blood of dogs and goats, however it was approximately 5.5 times more effective as an inhibitior of TxB_2 in the serum of horses.

Comparison of the in vitro inhibition of serum TxB_2 by each drug within species also revealed some major differences. In all cases flunixin produced superior inhibition of TxB_2.

In dogs and horses it was found to be approximately 7-8 times more effective on a molar basis than meclofenamic acid, and when tested in the blood of goats flunixin was more than 40 times more effective than meclofenamic acid.

Some studies, such as those described in earlier chapters of the present thesis, have examined drug induced inhibition of TxB_2 ex vivo. Soma et al (1992) administered flunixin intravenously at a dose rate of 1.1 mg/kg to sedentary horses and horses in training. This work showed that approximately 50 % inhibition of TxB_2 was detected when the concentration of flunixin in plasma was around 0.9 µg/ml. A study reported by Lees et al (1987), in which the same intravenous dose rate was administered to ponies, showed an inhibition of TxB_2 of about 50 % in the presence of less than 0.05 µg/ml of flunixin. It is difficult to draw comparison from these figures with those found in the present study. In both cases the blood platelets had been exposed to a much higher concentration of drug which was diminishing with time. In the present study the platelets were exposed to a single, stable, concentration of drug. It may be that the effects observed at specific time points in ex vivo studies are complicated by inhibitory effects produced by previous exposure to higher drug concentrations. Since the inhibitory effects of flunixin and meclofenamic acid on platelet cyclooxygenase are reversible, it is possible that the inhibition observed in the ex vivo study is a result of the combined effect of previous exposure and the concentration of drug to which the platelets are exposed at the time of sampling.

Large inter-species variations in the effectiveness of flunixin and meclofenamic acid to inhibit serum TxB_2 were demonstrated and these differences were significant at several concentrations of each drug (appendix 12.8 and 12.9). The results published by Lees et al (1987) were closer to those in this study than were the results of Soma et al (1992). This is interesting in view of the fact that both this study and the work of Soma et al (1992) used thoroughbred horses, where as the work of Lees et al (1987) was carried out in Welsh Mountain and New Forest ponies. All studies measured TxB_2 which had been generated in clotting blood, however, Soma et al (1992) used an incubation time of one hour, whereas both this study and that published by Lees et al (1987) used an incubation time of 90 minutes. In addition, Lees et al (1987) have demonstrated that blood which is incubated for one hour produces only 77 % of the TxB_2 which is produced if a 90 minute incubation is used. If this does in fact form the basis of the difference between the results of the two previous studies, then it seems likely that the similarity between the inhibition of TxB_2 measured in thoroughbreds in this study and ponies in the work of Lees et al (1987) indicates that the inter-species differences demonstrated may not extend to breeds.

A more recent study (Kankaanranta et al, 1993) has examined the in vitro inhibition of TxB_2 by flunixin and tolfenamic acid for human platelets. These workers reported that the IC_{50} values for serum TxB_2 inhibition using these drugs were 0.28 and 2.6 μM respectively for flunixin and tolfenamic acid. This was approximately 2, 15 and 8 times higher concentrations of flunixin than were required in the present study in the dog, goat and horse, respectively. Once more it is not possible to determine if this reflects true variation between man and other species or if these large differences are due to the short incubation period (30 minutes) which was used in the human study. It is likely that rather than producing higher IC_{50} values, a short incubation period would produce relatively lower IC_{50} concentrations since the interaction of drug with cyclooxygenase enzyme is rapid and a constant rate of inhibition would be expected after equilibration had occured.

There is little doubt that, despite the ambiguity introduced by different methodology in other studies, this work has shown that large inter-species variations exist in the inhibition of serum TxB_2 by NSAIDs. One explanation for this may be differences in affinity of the drug for enzymes in each species. This might result from intra-species structural differences in the cycloxygenase enzyme. Some evidence to support this theory exists in work on other enzymes such as phosphodiesterase and monoamine oxidase (Fuller, 1972; Horovitz et al, 1972; Squires, 1972; Yodim, 1972), where differential sensitivity of enzymes to drugs has been documented. Flower and Vane (1972) have reported that paracetamol showed widely differing activities against synthetase enzymes from dog spleen and rabbit brain, and it has been shown that antisynthetase activity to vary up to 1000 fold depending on the anatomical source of the same enzyme. These, and other unpublished studies, led Flower and Vane (1984) to speculate that at least one component protein of prostaglandin synthetase exists in more than one molecular form (isoenzymes) within an animal. Also, that each may catalyze a similar type of reaction, but has its own distinct biochemical and pharmacological profile, and this may relate to the function of prostaglandins in that specific tissue type. It is generally accepted, for the horse, that meclofenamic acid is more effective in the control of pain associated with movement and joints (eg laminitis) where as flunixin is more effective against pain associated with colic. These observations reinforce the case for the existence of different forms of cyclooxygenase in different tissues, as they may result from a differential sensitivity to particular NSAIDs amongst enzyme forms. In addition, recent work by Hla and Neilson (1992) has shown that expression and differential regulation of human cyclooxygenase in various cell types is encoded by two or more genes.

It has been reported (Egan et al, 1978) that the potency of fenamates in inhibiting cyclooxygenase is influenced by the presence of co-factors. The presence of these substances (such as phenol) has been shown to increase potency by up to 100 times, and it has been suggested that differential presence of naturally occuring co-factors in tissues may explain the tissue selective activity which has been reported for these drugs. In addition, fenamates are known to directly inhibit the action of some PGs (Collier and Sweatman,1968). These actions do not extend uniformly to all actions of a particular PG, and in addition the situation varies between species. This ability to inhibit some actions of PG could also explain inter-tissue differences in efficacy.

Another possible explanation for the different activity of flunixin and meclofenamic acid in different species is the variation in drug protein binding. Calculation of the likely concentration of unbound drug present at the IC_{50} concentrations of flunixin and meclofenamic acid in this study, based on work on protein binding described in chapter 7, resulted in the values shown in table 8.11. It is apparent from these figures that the inter-species differences in inhibition of meclofenamic acid and flunixin on the production of serum TxB_2 cannot simply be explained on the basis of the concentration of unbound drug. At the IC_{50}, the concentration of unbound flunixin is approximately 30 times less than the concentration of unbound meclofenamic acid for serum from goats. In dogs there is approximately 5 times less free flunixin. The concentration of flunixin required to produce the IC_{50} is approximately 40 times lower in the goat, and approximately 7 times lower in the dog than is required for meclofenamic acid. It is difficult to draw a similar comparison for these drugs in the horse as meclofenamate was estimated to be 100 % bound to serum protein. However, since less than 0.1 % meclofenamic acid was estimated to be unbound drug, then the concentration of unbound flunixin in serum from horses was more than 10 times greater than the concentration of unbound meclofenamic acid, on a molar basis. Examination of the recommended dose rates for flunixin and meclofenamic acid in the horse (1.1 and 2.2 mg/kg respectively) indicates that the relative clinical efficacy of flunixin in this species is twice that (three times on a molar basis) of meclofenamic acid. If the IC_{50} were determined simply on the basis of the concentration of free drug, then the results of the present study indicate that the dose rate of meclofenamic acid required to provide equivalent clinical efficacy would be approximately 10 times greater than the dose of flunixin. A further complication is the apparent differential effectiveness of these two drugs in different clinical situations, as outlined above.

8.6 TABLES AND FIGURES

	Phosphate Buffer		
	pH 7.0	**pH 7.5**	**pH 8.0**
0.85% NaCl	p<0.01	p<0.01	p<0.01

	Ethanol Solution				
	0.01%	**0.1%**	**1.0%**	**5.0%**	**10%**
0.85% NaCl	p>0.05	P>0.05	p>0.05	p<0.05	p<0.05

Table 8.1 Level of significance in differences in inhibition of in vitro serum TxB_2 produced by a range of buffers and ethanol solutions.when compared with 0.85 % NaCl.

Drug Conc.	Animal Number			
(μg/ml)	1	2	3	4
0	-	-	-	-
0.0005	16.50	NS	12.20	0
0.0010	10.54	17.56	14.06	0
0.0050	30.61	28.65	16.45	17.98
0.0075	22.28	17.19	6.90	12.12
0.0100	34.35	19.22	15.38	10.91
0.0200	54.44	9.61	23.87	25.86
0.0250	55.95	35.86	25.20	24.65
0.0500	69.39	51.39	48.81	50.71
0.0750	78.57	34.01	49.87	57.37
0.1000	75.51	59.89	56.23	63.84
0.2000	97.96	80.78	54.11	74.75
0.2500	95.58	74.31	77.98	78.79
0.5000	97.96	89.46	85.15	91.52
0.7500	95.41	88.17	96.29	92.53
1.0000	98.13	92.05	95.23	92.53
2.5000	98.81	96.12	96.82	96.57
5.0000	99.15	98.15	97.35	97.78
7.5000	99.15	96.67	98.67	97.37
10.0000	99.15	97.04	97.69	96.97

NS = No sample

Table 8.2 Inhibition (%) of thromboxane B_2 production in the serum of dogs. Blood clotting occured in the presence of flunixin.

Drug Conc	Animal Number		
(μg/ml)	1	2	3
0	-	-	-
0.0005	3.70	15.65	6.21
0.0010	6.76	14.54	11.97
0.0050	39.84	36.53	53.23
0.0075	38.66	42.43	48.02
0.0100	40.59	45.65	43.67
0.0200	59.62	58.05	76.55
0.0250	66.68	76.01	86.05
0.0500	81.75	87.25	94.82
0.0750	84.93	89.14	95.55
0.1000	96.93	93.16	97.84
0.2000	91.52	98.46	96.77
0.2500	91.69	98.12	98.08
0.5000	96.40	99.40	99.15
0.7500	98.92	99.66	98.51
1.0000	98.42	99.74	99.82
2.5000	98.96	99.91	99.73
5.0000	99.15	99.90	99.85
7.5000	99.03	99.95	99.70
10.0000	98.70	99.81	99.76

Table 8.3 Inhibition (%) of thromboxane B_2 production in the serum of goats. Blood clotting occured in the presence of flunixin.

Drug Conc.	Animal Number			
(μg/ml)	1	2	3	4
0	-	-	-	-
0.0005	NS	18.64	0	15.60
0.0010	3.89	27.09	0	32.39
0.0050	13.04	12.27	13.67	34.83
0.0075	NS	20.45	17.56	28.21
0.0100	72.54	51.45	30.22	54.23
0.0200	NS	58.55	24.22	58.97
0.0250	80.55	58.55	38.56	63.46
0.0500	93.14	66.00	63.11	82.78
0.0750	92.68	74.00	62.00	80.85
0.1000	97.71	83.36	79.00	76.84
0.2500	97.25	93.59	90.61	85.02
0.5000	98.63	97.18	96.22	71.18
0.7500	98.33	98.14	96.56	97.18
1.0000	98.33	98.07	96.62	97.58
2.5000	99.66	NS	NS	NS
5.0000	99.68	NS	98.64	97.25
10.0000	99.71	NS	98.29	97.85

NS = No sample

Table 8.4 Inhibition (%) of thromboxane B_2 production in the serum of horses. Blood clotting occured in the presence of flunixin.

Drug Conc. (μg/ml)	Dog		Goat		Horse	
	Mean	SEM	Mean	SEM	Mean	SEM
0	-	-	-	-	-	-
0.0005	9.57	4.94	8.52	3.64	11.41	5.78
0.0010	10.54	3.79	11.09	2.28	15.84	8.13
0.0050	23.42	3.62	43.20	5.10	18.45	5.47
0.0075	14.62	3.31	43.04	2.71	22.07	3.18
0.0100	19.96	5.09	43.30	1.47	52.11	8.67
0.0200	28.44	9.39	64.74	5.92	47.25	11.53
0.0250	35.41	7.31	76.25	5.59	60.28	8.64
0.0500	55.07	4.80	87.94	3.78	76.26	7.10
0.0750	54.95	9.26	89.87	3.08	77.38	6.42
0.1000	63.87	4.18	95.98	1.43	84.23	4.69
0.2000	76.90	9.05	95.58	2.08	NS	-
0.2500	81.66	4.74	95.96	2.13	91.62	2.58
0.5000	91.02	2.67	98.32	0.96	90.80	6.56
0.7500	93.10	1.83	99.03	0.33	97.55	0.42
1.0000	94.48	1.40	99.33	0.45	97.65	0.38
2.5000	97.08	0.59	99.53	0.29	99.66	-
5.0000	98.11	0.38	99.63	0.24	98.52	0.70
7.5000	97.96	0.57	99.56	0.27	NS	-
10.0000	97.71	0.51	99.42	0.36	98.57	0.57

NS = No sample

Table 8.5 Mean inhibition (%) of thromboxane B_2 production in the serum of dogs, goats and horses. Blood clotting occured in the presence of flunixin.

Drug Conc.	Animal Number		
(μg/ml)	1	2	3
0	-	-	-
0.0005	32.19	19.12	22.07
0.0010	35.84	26.96	16.03
0.0050	NS	14.95	33.79
0.0075	29.45	19.85	7.59
0.0100	34.70	20.34	5.69
0.0200	81.96	12.50	24.31
0.0250	NS	NS	10.52
0.0500	NS	21.57	17.41
0.0750	22.15	31.37	0
0.1000	58.68	25.25	24.31
0.2000	94.06	29.90	NS
0.2500	98.86	32.11	NS
0.5000	NS	64.71	32.59
0.7500	86.07	72.06	45.17
1.0000	96.35	NS	NS
2.5000	NS	82.11	80.34
5.0000	NS	97.30	43.28
7.5000	NS	98.53	76.55
10.0000	99.09	98.77	88.79
20.0000	NS	NS	95.00
38.5500	NS	NS	98.97

NS = No sample

Table 8.6 Inhibition (%) of thromboxane B_2 production in the serum of dogs. Blood clotting occured in the presence of meclofenamic acid.

Drug Conc.	Animal Number		
(μg/ml)	1	2	3
0	-	-	-
0.001	0.92	0	18.63
0.005	0	6.76	35.17
0.010	0	18.27	0
0.050	24.00	22.85	20.10
0.100	30.47	51.92	39.29
0.200	15.61	NS	NS
0.250	64.36	69.95	60.14
0.500	84.51	86.39	82.13
0.750	89.89	90.34	83.87
1.000	91.73	96.19	93.85
2.500	99.20	99.11	97.99
5.000	99.60	99.74	99.30
7.500	99.67	99.76	99.50
10.000	99.88	99.78	99.68
20.000	NS	99.82	99.88
38.550	NS	NS	99.94
133.330	NS	NS	99.94

NS = No sample

Table 8.7 Inhibition (%) of thromboxane B_2 production in the serum of goats. Blood clotting occured in the presence of meclofenamic acid.

Drug Conc.	Animal Number		
(μg/ml)	1	2	3
0	-	-	-
0.001	47.78	0	9.52
0.005	49.28	0	13.99
0.010	30.78	0	3.83
0.020	37.98	38.50	12.15
0.050	57.67	46.86	24.35
0.100	54.56	47.70	34.25
0.250	77.75	72.92	58.76
0.500	89.13	86.19	81.42
0.750	92.16	92.43	86.20
1.000	95.01	99.60	93.47
2.500	97.95	98.50	97.47
5.000	99.19	99.29	98.62
7.500	99.15	98.58	98.54
10.000	99.49	99.56	99.16
20.000	99.74	99.91	99.55
38.550	NS	99.91	99.63
133.330	NS	NS	99.86

NS = No sample

Table 8.8 Inhibition (%) of thromboxane B_2 production in the serum of horses. Blood clotting occured in the presence of meclofenamic acid.

Drug Conc. (µg/ml)	Dog		Goat		Horse	
	Mean	SEM	Mean	SEM	Mean	SEM
0	-	-	-	-	-	-
0.0005	22.46	3.96	NS	-	NS	-
0.0010	26.28	5.74	6.52	6.06	19.10	14.62
0.0050	24.35	9.48	13.98	10.78	21.09	14.68
0.0075	18.96	6.33	NS	-	NS	-
0.0100	20.24	8.38	6.09	6.09	11.54	9.70
0.0200	39.59	21.48	NS	-	29.54	8.71
0.0250	10.52	-	NS	-	NS	-
0.0500	19.49	2.09	22.32	1.16	42.96	9.82
0.0750	17.84	9.32	NS	-	NS	-
0.1000	36.08	7.99	40.56	6.23	45.50	5.97
0.2000	61.98	32.16	15.61	-	NS	-
0.2500	65.48	33.47	64.82	2.84	69.81	5.70
0.5000	48.64	16.11	84.34	1.23	85.58	2.25
0.7500	67.77	12.01	88.03	2.09	90.26	2.03
1.0000	96.35	-	93.92	1.29	96.03	1.84
2.5000	81.22	0.72	98.77	0.39	97.97	0.30
5.0000	70.29	27.09	99.55	0.13	98.73	0.21
7.5000	87.54	11.02	99.64	0.08	98.76	0.20
10.0000	95.55	3.38	99.78	0.06	99.40	0.12
20.0000	95.00	-	99.85	0.03	99.73	0.10
38.5500	98.98	-	99.94	-	99.77	0.14
133.3300	NS		99.94	-	99.86	-

NS = No sample

Table 8.9 Mean inhibition (%) of thromboxane B_2 production in the serum of dogs, goats and horses. Blood clotting occured in the presence of meclofenamic acid.

Species	Drug	Computed IC_{50}
Dog	Flunixin	0.051μg/ml (0.104μM)
	Meclofenamic Acid	0.225μg/ml (0.707μM)
Goat	Flunixin	0.009μg/ml (0.018μM)
	Meclofenamic Acid	0.241μg/ml (0.757μM)
Horse	Flunixin	0.018μg/ml (0.036μM)
	Meclofenamic Acid	0.095μg/ml (0.299μM)

Table 8.10 Computed IC_{50} values for flunixin and meclofenamic acid inhibition of serum thromboxane B_2 production in vitro for dogs, goats and horses.

Drug	Species		
	Dog	Goat	Horse
Flunixin	0.008	0.003	0.005
Meclofenamic Acid	0.044	0.098	0

Table 8.11 Estimation of the unbound concentration (μM) of flunixin and meclofenamic acid in test solutions which produced in vitro IC_{50} values for serum thromboxane B_2 generation in three animal species.

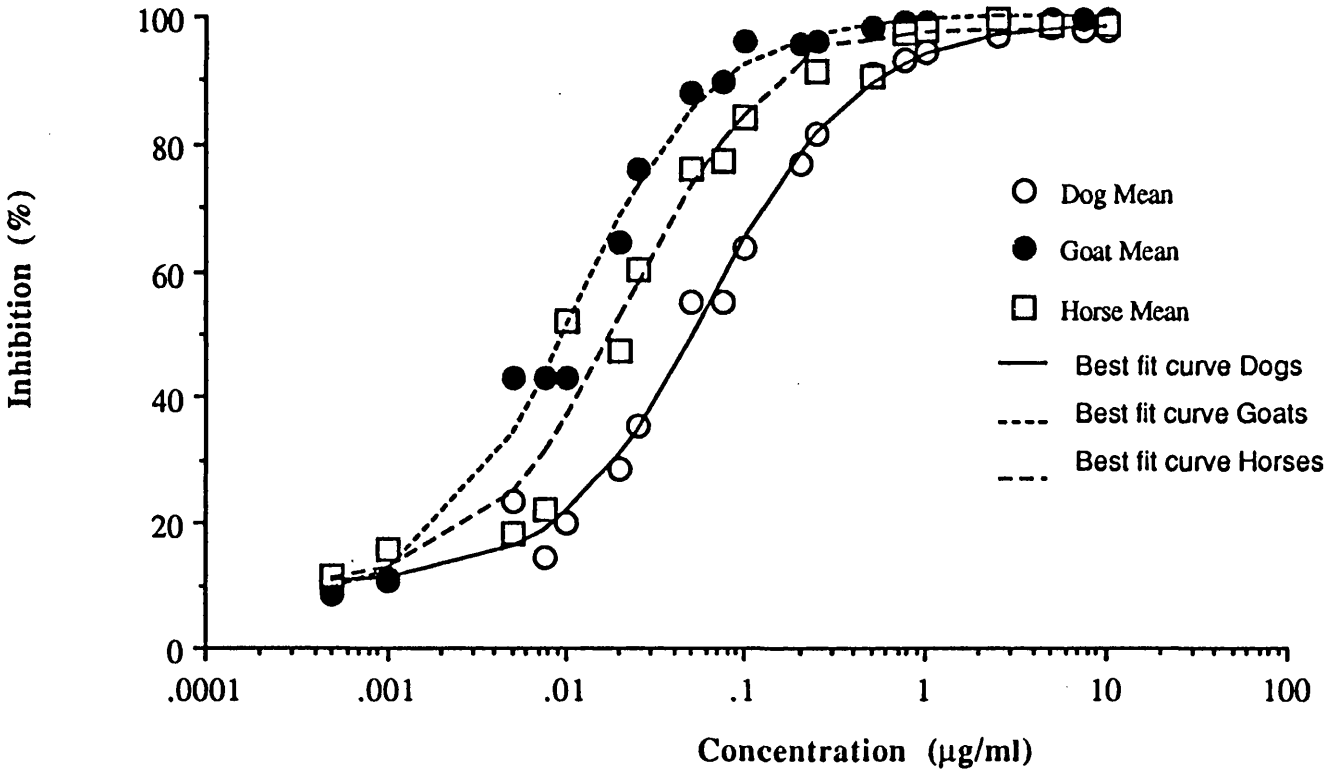


Figure 8.1 Mean inhibition (%) of thromboxane B_2 production in the serum of dogs, goats and horses. Blood clotting occured in the presence of flunixin.

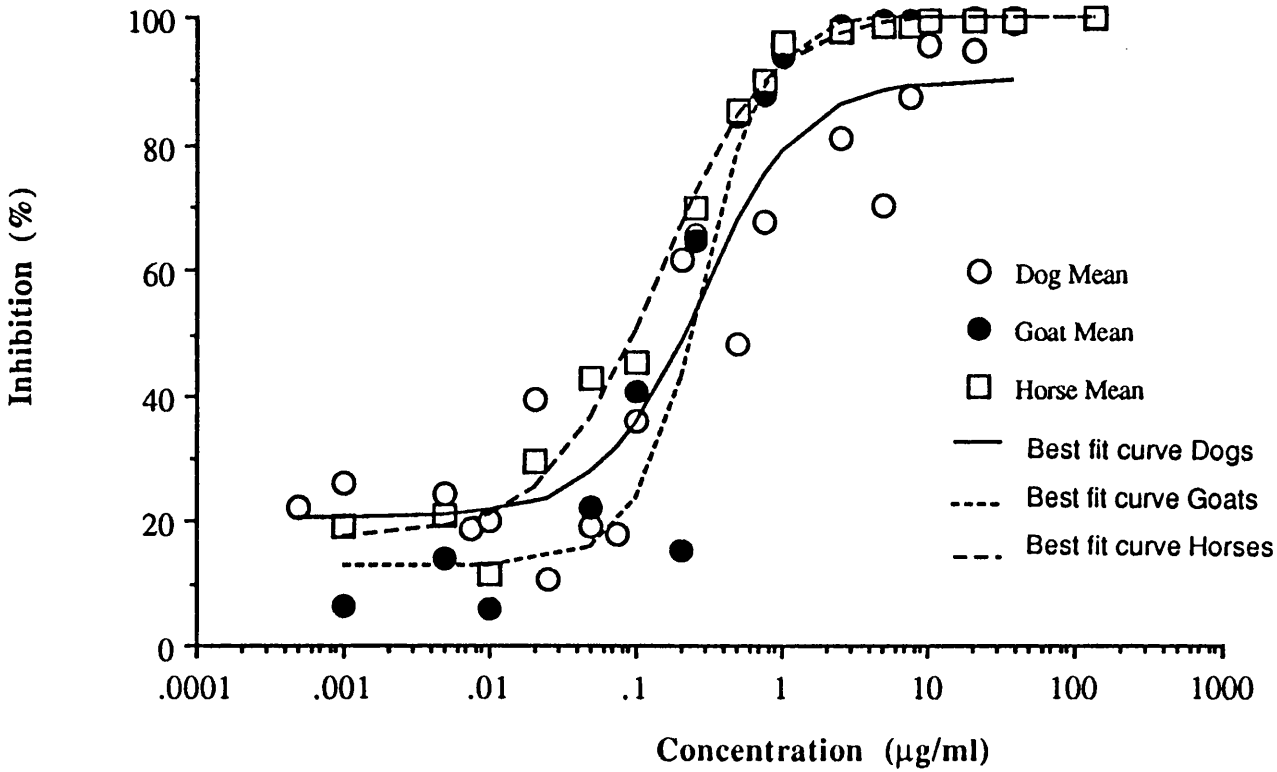


Figure 8.2 Mean inhibition (%) of thromboxane B_2 production in the serum of dogs, goats and horses. Blood clotting occured in the presence of meclofenamic acid.

Chapter 9

GENERAL DISCUSSION

The principal therapeutic uses for NSAIDs are in the control of pain and inflammation. Recently, as our understanding of the mechanism of action of these compounds has expanded, additional uses have included the control of platelet aggregation and the treatment of endotoxic shock. The ethical issues involved in the therapeutic use of any foreign substance, such as a drug, are increasingly under general discussion, and this issue is particularly emotive when it involves the use of drugs in animals. The debate surrounding the ethics of using NSAIDs in domestic animals where the control of pain and inflammation may prolong or enhance the competitive ability of an animal has been particularly heated (for example horse racing). The ability to recognise pain and discomfort in animals presents many difficulties, and our perception of these states in an animal is frequently based on comparable human experience. There is little doubt, however, that the ability of NSAIDs to relieve suffering is desirable, although the control of pain and inflammation should be used in support of a regimen which also addresses the underlying cause of these symptoms.

A wide variety of NSAIDs are available for use in humans. The development of these drugs has been encouraged by the enormous commercial return which can be derived from the production of a successful drug. The commercial value of veterinary drugs is considerably less, and as a result NSAIDs licensed for use in animals are fewer in number and frequently their use is restricted to a few specific species. The increasing concern for animal welfare, and a greater general affluence has gone some way towards changing this situation. As a result there is greater interest in exploring the possible uses of NSAIDs which were developed for human use. Inter-species, and occasionally age or sex related, differences in pharmacokinetics and disposition of NSAIDs and other drugs result in the necessity to elucidate the properties of a drug in each target species.

The good efficacy of any drug must be assessed in conjunction with the risks associated with drug therapy. It is now recognised that the mechanisms of action of NSAIDs are also the mechanisms responsible for adverse effects, the nature and extent of which also show inter-species variation. The adverse side effects of some NSAIDs have been discussed in some detail in previous chapters. The most common side effects of therapy with these drugs is gastrointestinal toxicity, frequently ulceration, and renal papillary necrosis. The duration of NSAID therapy is generally in the order of days, rather than weeks or months, and as a result the risk to life and the risk of permanent damage is outweighed by the benefit to be derived from their use.

The present studies have examined the pharmacokinetics and pharmacodynamics of four NSAIDs in dogs. Two of the drugs (flunixin and cinchophen) are currently licensed for veterinary use. The pharmacokinetics of the drugs studied varied widely, as can be seen from the estimates of $t^1/_2\beta$. After intravenous administration the $t^1/_2\beta$ of flunixin was shortest (2.97 hours), cinchophen and tolfenamic acid had slightly longer $t^1/_2\beta$ values (6.84 and 5.91 hours respectively). The $t^1/_2\beta$ of piroxicam after intravenous administration (40.16 hours) was more than ten times greater than that of flunixin and approximately six times longer than the $t^1/_2\beta$ of cinchophen and tolfenamic acid. Comparison of the $t^1/_2\beta$ estimates for NSAIDs in dogs in these studies with $t^1/_2\beta$ values reported in other species (eg for piroxicam in rats and man (Esteve et al, 1986; Nuotio and Makisara, 1978)) confirmed that large inter-species differences may occur and that it is necessary to determine the pharmacokinetics of each drug in the target species.

Very large differences also existed between the volumes of distribution for these drugs. The Vd_{ss} of cinchophen was smallest (110 ml/kg), flunixin and piroxicam had similar Vd_{ss} values (190 and 178 ml/kg respectively). The Vd_{ss} of tolfenamic acid was 1247 ml/kg - more than six times that of the other drugs and considerably larger than the figure of 160 ml/kg estimated for tolfenamic acid in man (Pentikaainen et al, 1981). Drugs, such as NSAIDs, which are highly protein bound typically have low volumes of distribution, and in man this figure is less than 200 ml/kg for many such drugs (Rowland and Tozer, 1989). The binding of tolfenamic acid in man, has been reported to be in excess of 99 % (Pentikaainen et al, 1981), which is similar to or higher than that for other NSAIDs. Examination of the serum protein binding of two NSAIDs in these studies have shown that there may be large inter-species variations. It may be that the level of binding of this drug is considerably lower in dogs. However, the binding of meclofenamic acid in this species was shown to be greater than 90 % and it seems likely that the chemically related tolfenamic acid would not exhibit a substantially different degree of binding although this remains to be proven.

In an attempt to determine the likely efficacy of the four drugs studied, inhibition of serum TxB_2 production was measured after intravenous administration. Comparison of drugs in these intravenous and other experiments was complicated by the preparation of cinchophen which was available. This drug was formulated in conjunction with the steroidal anti-inflammatory drug prednisolone. The possibility that the presence of prednisolone may have altered the pharmacodynamic properties of chinchophen cannot be ruled out. Although prednisolone does not directly inhibit cyclooxygenase, it may have

reduced serum TxB_2 production by inhibition of phospholipase. However, Seppala et al (1985) have shown that a single 20 mg dose of prednisolone did not significantly reduce the concentration of PGE_2, 6-keto-PGF_{1alpha} or TxB_2 in synovial fluid of patients with rheumatoid arthritis. Also, it was of interest to compare the effects of the combined preparation after intravenous and oral administration, to those obtained after administration of preparations containing only NSAID. Maximum inhibition of serum TxB_2 after intravenous administration was greater than 90 % for flunixin, piroxicam and tolfenamic acid (99.9, 96.8 and 91.3 % respectively). However, the maximum inhibition of TxB_2 after intravenous administration of cinchophen was only 71 %. Although the maximum inhibition produced by these drugs is important, their overall clinical usefulness is also linked to the duration of efficacy. The last time at which inhibition of TxB_2 was 50 % or greater was used to give some indication of this duration. The shortest duration was measured for tolfenamic acid and cinchophen (1 and 2 hours respectively). Flunixin had a duration of inhibition in excess of 50 % of 12 hours and the maximum duration was measured for piroxicam where 50 % inhibition was measured at 72 hours after drug administration. This approach can give only a very rough indication of the useful time scale for each drug as flunixin had 76 % inhibition at 12 hours but this had decreased to 18 % at the next sampling time of 24 hours. In addition the total area under the serum thromboxane inhibition versus time curve was measured for each drug after each route of administration. Although this does not give a direct measurement of duration of action, it does allow comparison of the likely overall effectiveness of the drugs when administered on a single occasion. The area under the TxB_2 inhibition curve was greatest after intravenous administration of piroxicam. This value was approximately twice that calculated for flunixin and tolfenamic acid, and was six times higher than that measured after intravenous administration of cinchophen. This indicates that piroxicam may be the drug of choice for single intravenous administration, although it has not been established if the area under the TxB_2 inhibition curve is positively correlated with the incidence of side effects of NSAIDs.

In many clinical situations the use of the intravenous route of administration for NSAIDs is impractical, and medication must be administered on more than one occasion. This can be achieved for some drugs by the use of oral preparations which can be administered by the owner of the animal. Alternatively, extended persistence of effect may be achieved by the use of intramuscular or subcutaneous preparations. Of the drugs considered in the present studies, flunixin, piroxicam and cinchophen were available as oral preparations.

Tolfenamic acid was available as a subcutaneous preparation and a preparation containing flunixin was also available for administration by this route.

Maximum concentrations of flunixin and cinchophen in plasma were measured between 1 and 2 hours after oral administration. The maximum concentration of piroxicam occured after approximately 3 hours. Mean residence time for plasma drug concentration after oral administration agreed with the ranking of $t^1/_2\beta$ values calculated for intravenous administration, that is flunixin had the shortest MRT, piroxicam had a considerably longer MRT and the values for cinchophen fell within these two extremes. The bioavailability of flunixin and cinchophen after oral administration was approximately 60 %. However, piroxicam had a much greater bioavailability of around 100 %.

Examination of the maximum serum TxB_2 inhibition and duration of inhibition may be used to assess the potency of each drug as a cyclooxygenase inhibitor in plasma. Flunixin, given orally at a dose rate of 1.1mg/kg, produced a maximum TxB_2 inhibition of 99 %. This is comparable to that produced by the same dose rate after intravenous administration and is considerably greater than the maximum inhibition measured after oral administration of piroxicam (74 %) and cinchophen (50 %). In the case of flunixin and cinchophen, these maximum values were measured at 1 hour after administration of the drug. Maximum inhibition of serum TxB_2 production after oral administration of piroxicam did not occur until 8 hours.

The last time at which inhibition of TxB_2 was 50 % or greater may be used to give an indication of the duration of action of drug. In all cases this duration of inhibition was shorter after oral than after intravenous administration. Cinchophen had the shortest duration of 1 hour, flunixin (1.1 mg/kg) was intermediate at 8 hours and piroxicam had the longest oral duration at 48 hours. Comparison of the area under the serum TxB_2 inhibition versus time curve for drugs administered orally demonstrated that flunixin (1.1 mg/kg) (5952 %.h) was was more effective than piroxicam (4067 %.h). The area measured after oral administration of cinchophen (636 %.h) was approximately 10 % of that for flunixin, indicating that flunixin had the greatest effect following oral administration.

It is of interest to note that despite the presence of prednisolone in the preparation of cinchophen which was administered, the level and duration of TxB_2 inhibition after both routes of administration indicate that it was a poor inhibitor of platelet cyclooxygenase. The clinical effectiveness of cinchophen may be attributable to other properties and it

would be of interest to examine the inhibition of TxB_2 following the separate administration of the two component drugs.

Subcutaneous administration of flunixin (1.1 mg/kg) and tolfenamic acid produced values for maximum inhibition of serum TxB_2 of 99 and 80 % respectively. These maxima occured at 1 and 2 hours. Measurement of persistence of this inhibition showed that at 12 hours inhibition was 78 % after subcutaneous flunixin and that the inhibition was 55 % at 4 hours after subcutaneous tolfenamic acid. These results support the findings of Epinasse et al, 1992), who reported that flunixin was significantly more effective than tolfenamic acid in inhibiting the production of PGE_2 in an experimental inflammatory model in cattle. In contrast, the area under the serum TxB_2 inhibition versus time curve after subcutaneous administration of flunixin and tolfenamic acid was approximately equal.

Examination of platelet numbers throughout each experiment showed that, with the exception of the intravenous administration of piroxicam, no consistent trend in platelet numbers was apparent. This suggests that the reduction in TxB_2 concentration seen after NSAID administration was due to inhibition of cyclooxygenase, rather than a reduction in the platelets which are the primary source of thromboxane. Platelet numbers were considerably lower than pre-drug administration numbers, in all dogs, after intravenous administration of piroxicam. This effect occurred rapidly (at the first sample time of 0.25 hours) and persisted, to some extent, throughout the sampling period. Blood disorders, including thrombocytopenia, have been recorded as a side effect of piroxicam (Bjornstad and Vik, 1986). However, similar reductions in the platelet counts were not observed in these dogs after oral administration of piroxicam. In addition, thrombocytopenia has been linked with alcoholism in man (Lindenbaum and Hargrove, 1968; Post and Desforges, 1968b; Cowan and Hines, 1971) and exposure to alcohol vapour in mice (Malik and Wickramasinge, 1986). In these cases effects were observed over prolonged periods of 20 days or more. It therefore seems unlikely that the reduction in platelet numbers was associated with administration of piroxicam or ethanol. Furthermore, some increase in platelet numbers was seen during the sampling period and this time scale would not be consistent with the replacement rate for circulating platelets. The possibility of the presence of ethanol interfering with the measurement of platelets also seems unlikely as some reduction in platelet number was still apparent at 72 hours.

The concentration of TxB_2 in the circulation under normal conditions is generally in the nanogram range. The biosynthetic capacity of platelets is 1000 fold greater than the normal rate of production in vivo and, as a result, a small residual enzyme activity can

produce relatively high concentrations of TxB_2 (Patrono et al, 1986; Patrono, 1990). Toivanen et al (1984) have estimated that only 2.5-10 % of the normal platelet number is required to maintain platelet function within the normal range. The relationship between NSAID concentration and TxB_2 inhibition in clinical situations is highly complex and depends on the degree to which the biosynthetic capacity of platelets has been 'stimulated' and the IC_{50} of the NSAID. The in vitro experiments carried out in the present study have been standardised by the application of rigid controls to the period of time over which blood was permitted to incubate. The incubation time applied in these studies was 90 minutes. Studies by Lees et al (1987) have shown that concentrations increase erratically over a period of 30 to 90 minutes, and that the maximum concentration, which was measured at 90 minutes was approximately twice that measured at 30 minutes.

It is interesting to note that the duration of inhibition of serum TxB_2 does not appear to relate directly to the $t^1/_2\beta$ of the drug. Ranking the drugs examined in order of $t^1/_2\beta$ and apparent duration of action results in

$t^1/_2\beta$

flunixin < tolfenamic acid < cinchophen < piroxicam

Duration of action (intravenous)

tolfenamic acid < cinchophen < flunixin < piroxicam

Duration of action (oral)

cinchophen < flunixin < piroxicam

Duration of action (subcutaneous)

tolfenamic acid < flunixin

This demonstrates that the duration of action is linked to a combination of the $t^1/_2\beta$ of the drug and the overall ability of the drug to produce inhibition of serum TxB_2 at the given dose rate. To compare the overall effect of $t^1/_2\beta$, maximum TxB_2 inhibition and duration of inhibition for each drug by each route of administration a factor was devised. This was the product of $t^1/_2\beta$, maximum mean TxB_2 inhibition (TxB_2max) and the AUC for TxB_2 inhibition (TxB_{2AUC}) divided by 1000.

$$\text{Efficacy factor} = \frac{t^1/_2\beta \times TxB_2max \times TxB_2AUC}{1000}$$

Table 9.1 shows the factor produced for each drug by each route of administration. This indicates that piroxicam is the drug of choice for single intravenous and subcutaneous administration, whilst tolfenamic acid would be the drug of choice for single oral administration. Controlled clinical trials would be required to validate this method of assessment.

A general assessment of the efficacy of these drugs can be proposed by comparison of the mean of the factors calculated above. Thus

Piroxicam > tolfenamic acid ≥ flunixin > cinchophen

This is in contrast to the findings of Kankaanranta et al (1993) who demonstrated that flunixin was approximately ten times more effective than tolfenamic acid (on a molar basis) in inhibiting the production of serum TxB_2 ex vivo.

In addition to the ex vivo studies which are described for four drugs, an in vitro model was used to investigate the inhibition of serum TxB_2 by two NSAIDs in different species. Solubility difficulties prevented the investigation of the full range of NSAIDs used in the in vivo studies. However, it was possible to carry out experiments using two widely used drugs (flunixin and meclofenamic acid) in dogs, horses, and goats. Throughout the study, concentrations of drug have been expressed primarily as µg/ml, rather than as molar concentrations. Although this may complicate inter-drug comparison, it allows ease of comparison with other in vivo/ex vivo studies. Where comparison of drug concentration was made in the in vitro experiments, values were expressed both as µg/ml and as molar concentrations. For the purposes of comparison, cinchophen and tolfenamic acid are of approximately equal molecular weights, piroxicam is approximately 1.3 times and flunixin meglumine is approximately 1.9 times the molecular weight of these drugs.

Approximate comparison of the degree of inhibition of serum TxB_2 measured ex vivo and corresponding drug concentrations, with the inhibition measured in vitro was possible for flunixin in the dog. Comparison of drug concentrations which coincided with maximum inhibition ex vivo with in vitro inhibitions for the same drug concentration gave values in excess of 90% inhibition in vitro. As these inhibitions were produced by drug

concentrations in serum and plasma which ranged from 2.4 - 8.7μg/ml flunixin, this simply illustrates that the concentration required to produce near maximum inhibition is less than these concentrations. The last time at which inhibition of TxB_2 was 50% or greater was also used for comparative purposes. In this case, the ex vivo inhibition was compared to that measured for the same drug concentration in vitro. In all but one experiment (0.55 mg/kg oral flunixin), the ex vivo inhibition of serum TxB_2 was greater than that measured in the presence of the same concentration of flunixin in the in vitro experiment. As ex vivo inhibition at this stage was measured against a background of decreasing concentration of flunixin in plasma, it is likely that the difference is due to inhibitory effects caused by the previously higher drug concentrations, the effect of which had not been fully reversed. It is possible, however, that the IC_{50} value of the drug is lower ex vivo than in vitro. The potency of fenamates has been shown to be influenced by the presence or absence of co-factors (Egan et al, 1978). It is possible that similar factors exist which influence the action of flunixin and that such factors were absent or diminished in concentration in the in vitro test system.

In vitro experiments demonstrated the existence of inter-species differences (some statistically significant) for each drug. In the case of meclofenamic acid, these may be attributable to a variation in the presence of co-factors in the blood. Examination of inter-species differences in the concentration of known co-factors may have confirmed whether inter-species differences in drug potency were related to different co-factor concentrations. In such a case a true inter-species comparison of absolute potency of a drug would only be possible in the presence of equal concentrations of such co-factors. It would also have been of interest to study the effect on in vitro inhibition of serum TxB_2 in each species after addition of known concentrations of various co-factors.

Experiments were carried out to compare the blood protein binding of flunixin and meclofenamic acid in dogs, horses and goats. Large inter-species differences were measured, particularly for meclofenamic acid. Based on these figures, estimations of unbound drug which might be in the circulation were calculated for a given concentration of drug. The differences in unbound drug were particularly large when considered in relative terms (eg more than twice the unbound meclofenamate would be circulating in a goat than in a dog at normal therapeutic concentrations), although these differences may be of little significance if a process of enhanced dissociation of protein bound drugs exists (Pardridge, 1985). Also, the situation at the site of inflammation may be of greater relevance than that in the circulation. It has been established that an increase in protein

concentration occurs in inflammatory exudate (Lees and Higgins, 1984). Since NSAIDs are principally bound to albumin, it would be of interest to examine the relative proportions of the various proteins in exudate. In addition, determination of the extent of binding of NSAIDs in exudate of a variety of animal species may give a more complete knowledge of whether the increase in plasma/exudate drug ratios observed by Lees and Higgins (1984) resulted from the measured increased in protein concentration, or from some other, as yet unidentified, mechanism.

If serum TxB_2 was only inhibited by unbound NSAID then the in vitro inhibition versus drug concentration curve should be displaced to the right for species or drugs where there is a higher protein binding. It is of interest to note that this effect is not clearly apparent when the results of the in vitro TxB_2 and protein binding studies are compared. This may simply be due to insufficient data. However, it may also indicate that drug which is bound to protein is capable of causing inhibition or may result from the dynamic nature of in vivo drug protein binding which may in part explain the clinical effectiveness and ability to inhibit serum TxB_2 of drugs, such as meclofenamic acid, which are very highly protein bound.

Several studies (Hardee and Moore, 1986; Lees et al, 1987) have reported unexplained increases in TxB_2 compared with pre drug administration concentrations after NSAID inhibition. This was also noted for some animals in the present studies. Hardee and Moore (1986) proposed several explanations for this observation. They suggested that the increase could have resulted from an increase in the number of circulating platelets. Since platelet numbers were estimated at a large number of sample times in this present study it was possible to examine this theory. Although in some cases TxB_2 'rebound' coincided with increased platelet numbers this was not observed on a sufficient number of occasions to confirm the theory. Other proposed explanations were that cyclooxygenase, Tx synthetase or phospholipase activity was enhanced after the inhibitor was removed, or that there was a build up of arachidonic acid acid in the membranes as a result of continuing phospholipase activity during cyclooxygenase inhibition. It would be of interest to examine the relative concentrations of prostaglandins, thromboxane and leukotrienes prior to, during and after NSAID administration as this may give some indication of the cause of these observations. Lees et al (1987) examined the possibilities of circadian variations in TxB_2 production. They concluded that although no clear circadian rhythm was apparent for TxB_2 generation, there were significant variations in the normal TxB_2 concentrations

between samples. These observations suggest that 'rebound' effects which are observed are normal variations which have occured by chance at these sample times .

With the exception of the studies on piroxicam described earlier, no clear pattern of abnormal platelet numbers was seen with any of the NSAIDs studied. Increased bleeding times have been widely reported after administration of acetylsalicylic acid in man (Ivy et al, 1941; Meilke et al, 1969) and in horses (Judson and Barton, 1981; Trujillo et al, 1981). However, prolonged bleeding time was not evident in any of the experiments performed in this study, where the measurement used was the time taken for blood to clot. This is a comparatively crude test in comparison to the venostasis template technique devised by Ivy et al (1941). Kopp et al (1985) used this technique, which was standardised for use in humans, to investigate bleeding times in horses after NSAID administration. Although a 46% increase was measured after administration of flunixin meglumine at a dose rate of 0.5 mg/kg this change was not statistically significant. Examination of bleeding times measured prior to drug administration showed a very large inter-animal variation (range 2.5-14 minutes). Any study in which a small sample size is examined would be unlikely to reveal small underlying effects on bleeding times.

The present study has allowed comparison of the pharmacokinetics of some NSAIDs which may be suitable for use in the dog, and has given some insight into their potential to control clinical disorders through the inhibition of cyclooxygenase. Many questions about the clinical effectiveness and therapeutic differences between these drugs remain unanswered, and future studies on the use of NSAIDs in conjunction with appropriate models of inflammation will provide further valuable information.

Drug	Route of Administration		
	Intravenous	Oral	Subcutanoeus
Flunixin	637.3	1746.5	811.5
Piroxicam	20641.0	12145.1	--
Cinchophen	403.3	209.9	--
Tolfenamic Acid	1002.0	--	1322.6

Table 9.1 Calculated factors for the prediction of the efficacy of four NSAIDs in the dog.
Efficacy is predicted to be linearly related to the factor and was calculated using the formula:

$$\text{Efficacy factor} = \frac{t^1/_2\beta \times TxB_2max \times TxB_2AUC}{1000}$$

where t1/2ß is the intravenous half life of the drug, TxB_2max is the maximum inhibition of serum thromboxane B_2 and TxB_2AUC is the area under the serum thromboxane B_2 versus time curve.

APPENDIX 1

EXTRACTION RECOVERIES OF DRUGS

Concentration Flunixin in Plasma (µg/ml)	Mean ± SD Intra-assay Recovery (%)	Intra-assay Coefficient Variation (%)	Mean ± SD Inter-assay Recovery (%)	Inter-assay Coefficient Variation (%)
0.10	92.5 ± 3.9	4.2	88.0 ± 6.8	7.8
0.50	93.6 ± 1.1	1.2	NS	NS
1.00	95.2 ± 2.1	2.2	90.2 ± 6.3	7.0
5.00	90.2 ± 4.7	5.2	90.2 ± 2.5	2.8

NS = No sample

Appendix 1.1 Mean (±SD) recovery, intra-assay and inter-assay variation in recovery of flunixin from plasma fortified at known concentrations.

Concentration Piroxicam in Plasma (µg/ml)	Mean ± SD Intra-assay Recovery (%)	Intra-assay Coefficient Variation (%)	Mean ± SD Inter-assay Recovery (%)	Inter-assay Coefficient Variation (%)
0.10	70.21± 2.37	2.4	70.33± 6.18	8.8
0.25	NS		69.04± 4.87	7.0
0.50	66.58± 2.51	3.8	72.08±13.46	18.7
1.00	NS		68.49± 6.65	7.4
2.00	72.44± 1.10	1.5	68.49± 6.65	9.7

NS = No sample

Appendix 1.2 Mean (±SD) recovery, intra-assay and inter-assay variation in recovery of piroxicam from plasma fortified at known concentrations.

Concentration Cinchophen in Plasma (µg/ml)	Mean ± SD Intra-assay Recovery (%)	Intra-assay Coefficient Variation (%)	Mean ± SD Inter-assay Recovery (%)	Inter-assay Coefficient Variation (%)
1.0	91.99 ± 3.29	3.6	92.20 ± 5.12	5.5
5.0	NS		86.77 ± 4.18	4.8
10.0	92.18 ± 5.07	5.5	91.02 ± 1.68	1.8
20.0	NS		92.90 ± 3.30	3.5
50.0	91.70 ± 4.84	5.3	91.40± 6.34	6.9

NS = No sample

Appendix 1.3 Mean (±SD) recovery, intra-assay and inter-assay variation in recovery of cinchophen from plasma fortified at known concentrations.

Concentration Tolfenamic acid in Plasma (µg/ml)	Mean ± SD Intra-assay Recovery (%)	Intra-assay Coefficient Variation (%)	Mean ± SD Inter-assay Recovery (%)	Inter-assay Coefficient Variation (%)
0.10	65.8 ± 0.5	2.0	66.1 ± 2.4	3.6
0.25	66.8 ± 1.7	6.1	NS	NS
0.50	74.7 ± 2.4	7.9	73.06 ± 4.5	6.2
1.00	71.3 ± 1.1	3.8	NS	NS
5.00	77.9 ± 2.4	7.6	76.7 ± 4.6	6.0

NS = No sample

Appendix 1.4 Mean (±SD) recovery, intra-assay and inter-assay variation in recovery of tolfenamic acid from plasma fortified at known concentrations.

APPENDIX 2

FLUNIXIN ORAL ADMINISTRATION TABLES

Occasion	Dose Rate (mg/kg)		
	0.55	**1.10**	**1.65**
1	Dog 1 & 2	Dog 3 & 5	Dog 4 & 6
2	Dog 3 & 5	Dog 4 & 6	Dog 1 & 2
3	Dog 4 & 6	Dog 1 & 2	Dog 3 & 5

Appendix 2.1 Dose rate (mg/kg) of flunixin adminisered to each dog on each occasion.

Time	Animal Number					
(h)	1	2	3	4	5	6
Pre	0	0	0	0	0	0
0.50	0	0.11	3.04	2.52	3.69	2.24
1.00	0	2.25	5.23	1.88	3.05	1.99
2.00	0	0.85	4.36	0.61	1.62	1.21
4.00	0.67	0.56	0.35	0.13	1.11	0.46
8.00	0	0.11	1.17	0	0.43	0.20
12.00	0	0	0.43	0	0.41	0.21
24.00	0	0	0	0	0	0
48.00	0	0	0	0	0	0
72.00	0	0	0	0	0	0
96.00	0	0	0	0	0	0
168.00	0	0	0	0	0	0

Appendix 2.2 Concentration (μg/ml) of flunixin in plasma of dogs after administration at a dose rate of 0.55 mg/kg by the oral route.

Time	Animal Number					
(h)	1	2	3	4	5	6
Pre	0	0	0	0	0	0
0.50	4.39	3.07	0.31	0.47	3.38	0.06
1.00	2.89	9.21	2.20	2.15	5.62	5.37
2.00	1.05	4.43	3.42	1.19	3.16	3.74
4.00	0.44	2.05	0.91	0.65	1.03	2.92
8.00	0.10	0.70	0.23	0	0.58	1.05
12.00	0	0.14	0	0	0.81	0.35
24.00	0	0	0	0	0.10	0
48.00	0	0	0	0	0	0
72.00	0	0	0	0	0	0
96.00	0	0	0	0	0	0
168.00	0	0	0	0	0	0

Appendix 2.3 Concentration (μg/ml) of flunixin in plasma of dogs after administration at a dose rate of 1.10 mg/kg by the oral route.

Time	Animal Number					
(h)	1	2	3	4	5	6
Pre	0	0	0	0	0	0
0.50	0.36	6.73	2.07	0.86	7.20	0.40
1.00	1.18	5.79	5.60	7.45	7.91	16.59
2.00	1.47	9.80	4.20	4.91	4.13	5.41
4.00	1.67	2.30	1.59	1.07	1.75	1.46
8.00	0.26	0.71	0.61	0.30	1.11	0.63
12.00	0	0.21	0.19	0.19	0.66	0.63
24.00	0	0	0	0	0.25	0.16
48.00	0	0	0	0	0	0.10
72.00	0	0	0	0	0	0
96.00	0	0	0	0	0	0
168.00	0	0	0	0	0	0

Appendix 2.4 Concentration (μg/ml) of flunixin in plasma of dogs after administration at a dose rate of 1.65 mg/kg by the oral route.

0.55mg/kg

Time	Animal Number						
(h)	1	2	3	4	5	6	Mean
Pre	1.600	1.240	1.440	1.230	0.920	1.100	1.362
0.50	0.760	0.440	0.015	0.018	0.018	0.040	0.215
1.00	0.660	0.010	0.006	0.045	0.013	0.040	0.127
2.00	0.960	<0.220	0.011	0.028	0.066	0.240	<0.254
4.00	0.250	0.340	0.120	0.022	0.092	0.480	0.217
8.00	0.370	0.880	0.066	0.054	0.164	0.640	0.445
12.00	1.120	0.400	0.116	0.900	0.180	0.620	0.606
24.00	1.322	0.660	0.600	0.640	0.560	1.040	0.803
48.00	1.040	1.240	1.120	1.360	1.040	1.240	1.173
72.00	0.770	0.880	1.360	0.780	2.000	0.720	1.103
96.00	0.600	1.000	1.480	0.420	1.020	0.520	0.840
168.00	1.680	0.920	1.600	0.340	1.240	0.620	1.067

1.10 mg/kg

Time	Animal Number						
(h)	1	2	3	4	5	6	Mean
Pre	1.360	1.320	1.560	0.830	2.000	1.600	1.412
0.50	0.012	0.030	0.480	0.760	0.004	0.005	0.215
1.00	0.035	0.004	<0.001	0.024	<0.001	0.015	<0.013
2.00	0.152	0.029	0.014	0.240	0.005	0.092	0.089
4.00	0.440	<0.010	0.112	0.056	0.026	0.026	<0.110
8.00	0.700	0.380	0.440	1.000	0.042	0.160	0.454
12.00	1.200	0.680	1.640	0.740	0.132	0.940	0.888
24.00	1.200	1.160	1.040	1.280	0.860	0.640	1.030
48.00	1.040	1.320	1.040	1.480	0.620	2.000	1.205
72.00	0.800	0.820	0.820	1.160	0.680	1.160	0.907
96.00	0.400	0.460	0.900	1.240	0.740	1.360	0.872
168.00	0.560	0.640	1.440	1.230	0.920	1.100	0.981

1.65 mg/kg

Time	Animal Number						
(h)	1	2	3	4	5	6	Mean
Pre	1.680	0.920	1.600	1.600	1.240	0.620	1.277
0.50	0.520	0.010	0.044	0.184	<0.001	0.820	<0.263
1.00	0.270	0.011	0.055	0.001	<0.001	<0.001	<0.048
2.00	0.016	0.004	0.019	0.003	0.005	0.029	0.013
4.00	0.120	0.019	0.066	0.136	<0.010	0.052	<0.065
8.00	0.360	0.160	0.260	0.380	0.078	0.240	0.246
12.00	1.720	0.520	0.800	0.350	0.260	0.600	0.708
24.00	0.980	0.880	0.780	0.680	0.250	0.880	0.741
48.00	1.560	0.940	1.040	0.560	0.620	0.760	0.913
72.00	0.640	1.720	0.980	0.720	0.620	1.000	0.947
96.00	1.720	0.940	0.600	0.380	0.420	1.240	0.883
168.00	1.360	1.320	0.480	0.700	0.340	1.400	0.955

Appendix 2.5 Thromboxane B_2 concentration (µg/ml) in serum of dogs after administration of flunixin at three dose rates by the oral route.

0.55mg/kg

Time	Animal Number							
(h)	1	2	3	4	5	6	Mean	± SEM
Pre	706	608	358	313	278	405	445	± 70.5
0.50	650	514	351	310	265	339	405	± 59.9
1.00	531	744	269	319	248	375	414	± 77.8
2.00	548	484	267	290	319	310	370	± 47.6
4.00	694	544	400	268	316	323	424	± 66.8
8.00	498	434	235	287	340	326	353	± 39.5
12.00	648	593	349	220	314	336	410	± 69.4
24.00	614	802	598	188	308	274	464	± 98.5
48.00	517	616	229	261	288	275	364	± 65.7
72.00	543	509	345	300	405	369	412	± 38.9
96.00	598	565	460	300	292	362	429	± 54.1
168.00	576	503	382	297	323	418	416	± 43.6

1.10mg/kg

Time	Animal Number							
(h)	1	2	3	4	5	6	Mean	± SEM
Pre	564	534	386	300	275	381	407	± 48.6
0.50	547	503	399	233	274	367	387	± 50.4
1.00	503	479	402	369	253	337	391	± 37.8
2.00	547	523	384	332	295	337	403	± 43.4
4.00	655	506	354	257	296	404	412	± 60.2
8.00	472	506	349	504	215	358	401	± 46.8
12.00	435	439	386	425	299	392	396	± 21.4
24.00	449	405	304	419	245	361	364	± 31.5
48.00	449	493	379	368	244	294	371	± 37.9
72.00	479	462	360	245	271	240	345	± 44.2
96.00	412	547	369	244	268	345	366	± 43.6
168.00	513	554	358	271	278	405	404	± 45.0

1.65mg/kg

Time	Animal Number							
(h)	1	2	3	4	5	6	Mean	± SEM
Pre	576	503	382	277	323	400	410	± 45.6
0.50	561	432	389	247	268	368	378	± 46.9
1.00	564	485	382	316	326	347	403	± 40.7
2.00	565	551	392	231	291	310	390	± 57.2
4.00	579	514	375	256	271	358	392	± 53.0
8.00	636	217	349	245	274	329	342	± 62.3
12.00	454	509	287	230	245	289	336	± 47.6
24.00	644	583	NS	301	213	312	411	± 85.1
48.00	603	463	313	241	300	329	375	± 54.5
72.00	622	566	379	266	323	359	419	± 57.9
96.00	58	437	391	288	389	305	415	± 48.8
168.00	564	534	415	300	323	323	410	± 47.0

Appendix 2.6 Platelet numbers ($x10^9/l$).in blood of dogs after administration of flunixin at three dose rates by the oral route.

APPENDIX 3

FLUNIXIN INTRAVENOUS ADMINISTRATION TABLES

Time	Animal Number					
(h)	1	2	3	4	5	6
Pre	0	0	0	0	0	0
0.25	10.36	9.91	12.43	16.78	12.76	14.56
0.50	7.61	6.68	8.09	9.76	10.21	9.94
1.00	4.31	4.91	4.45	5.49	6.35	6.16
2.00	1.75	2.26	2.35	1.50	3.02	2.10
4.00	0.53	0.96	1.13	1.44	2.09	0.59
8.00	0	0.34	0.33	0.35	0.59	0.43
12.00	0	0.31	0	0.19	0.71	0.15
24.00	0	0.13	0	0.16	0.09	0.17
48.00	0	0	0	0.13	0	0.14
72.00	0	0	0	0.11	0	0

Appendix 3.1 Concentration (μg/ml) of.flunixin in plasma of dogs after administration at a dose rate of 1.10 mg/kg by the intravenous route.

Time (h)	Animal Number 1	2	3	4	5	6	Mean	±	SEM
0	1.1325	1.0430	1.0930	1.1040	0.8290	1.4880	1.1149	±	0.0870
0.25	0.0016	0.0008	0.0008	0.0010	0.0003	0.0030	0.0012	±	0.0004
0.50	0.011	0.0057	0.0013	0.0015	0.0006	0.0040	0.0024	±	0.0008
1.00	0.0235	0.0032	0.0037	0.0093	0.0010	0.0060	0.0078	±	0.0033
2.00	0.0390	0.0364	0.0124	0.0436	0.0127	0.0170	0.0268	±	0.0058
4.00	0.0351	0.0720	0.0615	0.0511	0.0277	0.2636	0.0852	±	0.0363
6.00	0.1822	0.1420	0.0820	0.1538	0.0585	0.4224	0.1735	±	0.0532
8.00	0.4833	0.2167	0.1362	0.3680	0.0462	0.3595	0.2683	±	0.0668
12.00	0.1532	0.0580	0.1347	0.2985	0.0397	0.0860	0.1283	±	0.0384
24.00	1.0660	1.2230	0.7570	0.8260	0.4440	1.6690	0.9975	±	0.1732
48.00	1.3460	0.7210	1.2460	0.7590	0.6870	1.4170	1.0293	±	0.1394
72.00	1.5500	1.3510	1.0620	1.2900	0.9620	1.4910	1.2793	±	0.0985

Appendix 3.2 Thromboxane B_2 concentration (μg/ml) in serum of dogs after administration of flunixin at at a dose rate of 1.10 mg/kg by the intravenous route.

Time	Animal Number								
(h)	1	2	3	4	5	6	Mean	±	SEM
Pre	326	323	284	210	232	274	274.8	±	19.2
0.25	405	382	271	245	258	NS	312.2	±	33.7
0.50	NS	175	280	178	258	271	232.4	±	23.2
1.00	375	362	248	220	242	251	283.0	±	27.4
2.00	346	319	280	200	NS	251	279.2	±	25.7
4.00	326	336	239	191	229	264	2642	±	23.2
6.00	310	319	293	220	271	287	283.3	±	14.4
8.00	336	178	251	200	213	172	225.0	±	25.0
12.00	293	323	300	166	NS	226	261.6	±	21.8
24.00	NS	326	280	210	232	220	253.6	±	21.8
48.00	352	349	277	188	204	235	217.6	±	58.2
72.00	306	316	277	182	188	242	251.8	±	23.6

Appendix 3.3 Platelet numbers ($x10^9/l$) in blood of dogs after administration of flunixin at a dose rate of 1.10 mg/kg by the intravenous route.

Time	Animal Number						
(h)	1	2	3	4	5	6	Mean
Pre	2.99	3.82	3.71	4.52	4.52	5.00	4.09
1.00	3.68	3.59	3.79	4.08	3.12	2.98	3.54
2.00	3.76	3.52	2.95	3.96	2.64	2.86	3.28
4.00	3.34	4.07	4.23	3.68	4.10	4.10	3.92
6.00	3.34	3.30	3.95	4.14	4.52	3.80	3.84
8.00	0.59	3.22	3.67	3.18	2.70	2.97	2.72
24.00	3.65	4.13	3.70	1.58	4.02	2.52	3.27
48.00	3.27	3.88	3.48	3.57	3.13	3.66	2.85
72.00	4.32	3.39	3.65	4.08	3.48	2.95	3.64

Appendix 3.4 Clotting time (min.) of blood from dogs after administration of flunixin at a dose rate of 1.10 mg/kg by the intravenous route.

Time (h).	Pen Number 1	2	3
Pre	-/-	-/-	-/-
24	-/-	-/-	+/-
48	-/-	-/-	-/-
72	-/-	-/-	-/-
96	-/-	-/-	-/-
168	+/-	-/-	-/-

- = Negative

Appendix 3.5 Occult blood in faeces of dogs after administration of flunixin at a dose rate of 1.10 mg/kg by the intravenous route.

APPENDIX 4

FLUNIXIN SUBCUTANEOUS ADMINISTRATION TABLES

Time	Animal Number					
(h)	1	2	3	4	5	6
Pre	0	0	0	0	0	0
0.25	2.49	3.68	3.79	4.14	2.86	1.94
0.50	4.12	5.04	5.75	7.91	4.95	4.10
1.00	3.82	6.37	6.96	8.55	6.50	5.67
2.00	1.38	4.86	6.29	6.17	3.33	3.20
4.00	0.43	1.80	2.47	2.64	1.00	1.01
8.00	0.15	0.53	0.53	0.50	0.19	0.47
12.00	0.12	0.29	0.36	0.27	0.08	0.95
24.00	0	0	0	0.08	0	0.18
48.00	0	0	0	0.07	0	0.11
72.00	0	0	0	0	0	0.21
96.00	0	0	0	0	0	0.14
168.00	0	0	0	0	0	0

Appendix 4.1 Concentration (µg/ml) of.flunixin in plasma of dogs after administration at a dose rate of 1.10 mg/kg by the subcutaneous route body weight.

Time (h)	Animal Number 1	2	3	4	5	6	Mean	±	SEM
0	1.3200	1.2600	1.0220	0.8030	0.9290	1.5830	1.1528	±	0.1174
0.25	0.0150	0.0025	0.0016	0.0032	0.0069	0.0090	0.0064	±	0.0021
0.50	0.0162	0.0012	0.0007	0.0011	0.0040	0.0023	0.0042	±	0.0024
1.00	0.0020	0.0030	0.0028	0.0032	0.0026	0.0041	0.0029	±	0.0003
2.00	0.0800	0.0150	0.0012	0.0024	0.0110	0.0106	0.0200	±	0.0122
4.00	0.0940	0.0390	0.0073	0.0089	0.0593	0.3559	0.0941	±	0.0540
8.00	0.1750	0.1614	0.1750	0.0885	0.3731	0.2787	0.2086	±	0.0412
12.00	0.3350	0.2533	0.1402	0.1255	0.4853	0.0710	0.2787	±	0.0654
24.00	0.9600	0.8930	0.9300	0.4135	0.8880	0.6510	0.7897	±	0.0877
48.00	1.2470	1.3160	1.2080	0.6680	1.0430	1.0220	1.0840	±	0.0956
72.00	1.1830	1.1520	1.1710	0.8700	1.3640	1.2500	1.1650	±	0.0669
96.00	1.4030	1.4210	1.2000	0.7650	1.3500	1.4090	1.2580	±	0.1041
168.00	1.1780	1.5500	1.3690	0.7410	1.1000	1.1760	1.1857	±	0.1113

Appendix 4.2 Thromboxane B_2 concentration (μg/ml) in serum of dogs after administration of flunixin at a dose rate of 1.10 mg/kg by the subcutaneous route.

Time	Animal Number								
(h)	1	2	3	4	5	6	Mean	±	SEM
Pre	430	370	230	200	380	300	318.3	±	37.0
0.25	350	340	260	100	190	300	256.7	±	39.4
0.50	350	370	300	NS	350	310	336.0	±	13.4
1.00	410	370	280	60	410	270	300.0	±	54.1
2.00	20	340	260	120	400	300	240.0	±	58.4
4.00	380	390	260	130	400	170	288.3	±	48.7
8.00	NS	280	70	240	340	170	220.0	±	46.7
12.00	250	330	220	130	270	260	243.3	±	27.0
24.00	310	330	280	90	340	200	258.3	±	39.4
48.00	310	320	300	150	330	310	286.7	±	27.6
72.00	320	200	230	330	280	160	257.3	±	27.8
96.00	380	280	330	180	370	140	280.0	±	40.9
168	350	450	290	210	1330	300	48.3	±	171.3

Appendix 4.3 Platelet numbers in blood of dogs after administration of flunixin at a dose rate of 1.10 mg/kg by the subcutaneous route

Time	Animal Number						
(h)	1	2	3	4	5	6	Mean
Pre	1.75	2.45	2.63	2.33	2.08	2.58	2.30
0.25	2.77	2.55	3.03	2.54	2.53	1.56	2.50
0.50	0.96	3.68	2.71	1.80	1.67	2.87	2.28
1.00	2.53	3.33	2.31	3.18	2.28	3.08	2.79
2.00	2.89	2.47	3.78	3.22	3.28	3.89	3.26
4.00	2.02	3.19	2.94	2.17	2.83	2.58	2.62
6.00	2.93	4.00	3.33	2.88	3.78	3.99	3.49
8.00	4.00	3.00	4.00	2.00	0.67	1.33	2.50
24.00	3.50	4.22	2.02	3.78	3.06	3.03	3.27
48.00	3.94	3.85	3.67	4.78	3.70	3.81	3.96
72.00	2.67	3.01	3.18	2.31	2.60	3.56	2.89
168.00	3.07	2.23	2.83	1.94	2.85	3.00	2.65

Appendix 4.4 Clotting time (min.) of blood from dogs after administration of flunixin at a dose rate of 1.10 mg/kg by the subcutaneous route.

Time (h).	Pen Number 1	2	3
Pre	-/-	-/-	-/-
24	-/-	-/-	-/-
48	-/-	-/-	-/-
72	-/-	-/-	-/-

- = Negative

Appendix 4.5 Occult blood in faeces of dogs after administratioon of flunixin at a dose rate of 1.10 mg/kg by the subcutaneous route.

APPENDIX 5

PIROXICAM INTRAVENOUS TABLES

Time (h)	Animal Number 1	2	3	4	5	6
Pre	0	0	0	0	0	0
0.25	0.99	1.06	1.11	1.25	0.98	1.25
0.50	0.87	0.80	0.86	1.06	0.75	0.84
1.00	0.86	0.90	0.85	1.45	0.75	0.75
2.00	0.81	0.94	0.97	1.68	0.78	0.71
4.00	0.76	0.89	1.37	1.71	1.15	0.84
6.00	0.83	0.87	1.29	1.58	0.84	0.96
8.00	0.92	0.88	0.91	1.16	0.97	0.82
12.00	0.77	0.71	0.77	0.92	0.87	0.67
24.00	0.51	0.79	0.97	0.99	0.99	0.68
48.00	0.38	0.49	0.45	0.46	0.59	0.36
72.00	0.16	0.33	0.35	0.42	0.30	0.22

Appendix 5.1 Concentration (μg/ml) of piroxicam in plasma of dogs after administration at a dose rate of 0.3 mg/kg by the intravenous route

Time (h)	Animal Number 1	2	3	4	5	6	Mean
Pre	1.321	0.997	1.380	1.127	0.685	1.202	1.119
0.25	0.087	0.059	<0.007	<0.007	0.026	0.018	<0.034
0.50	0.158	0.092	<0.007	<0.009	0.017	0.028	<0.052
1.00	0.138	0.141	<0.007	0.007	0.014	0.077	<0.064
2.00	0.138	0.392	0.047	0.073	0.024	0.199	0.148
4.00	0.176	<0.009	0.038	0.119	0.043	0.168	<0.092
6.00	0.221	0.197	0.098	0.053	0.017	0.126	0.127
8.00	0.141	0.121	0.107	0.057	0.045	0.126	0.099
12.00	0.248	0.181	NS	NS	NS	NS	0.214
24.00	0.305	0.319	0.113	0.182	0.141	0.206	0.211
48.00	0.492	0.457	0.133	0.205	0.186	0.430	0.317
72.00	0.613	0.632	0.681	0.506	0.285	0.638	0.558

Appendix 5.2 Thromboxane B_2 concentration (μg/ml) in serum of dogs after administration of piroxicam at a dose rate of 0.3 mg/kg by the intravenous route.

Time (h)	Animal Number 1	2	3	4	5	6	Mean ± SEM
Pre	457	419	395	318	269	276	354.0 ± 31.8
0.25	128	144	30	27	21	18	61.3 ± 23.8
0.50	216	235	76	51	48	48	112.3 ± 36.1
1.00	255	239	113	169	91	88	159.2 ± 30.3
2.00	239	300	144	119	76	125	167.2 ± 34.5
4.00	271	319	NS	131	153	NS	218.5 ± 45.5
6.00	258	274	182	131	128	172	190.8 ± 25.4
8.00	147	310	169	116	94	76	152.0 ± 34.5
12.00	150	226	138	119	79	91	133.8 ± 21.5
24.00	213	206	128	104	113	150	152.3 ± 19.2
48.00	226	249	168	115	85	149	165.3 ± 25.8
72.00	226	284	163	122	147	131	178.8 ± 25.8

Appendix 5.3 Platelet numbers ($x10^9/l$).in blood of dogs after administration of piroxicam at at a dose rate of 0.3 mg/kg by the intravenous route.

Time (h)	Animal Number 1	2	3	4	5	6	Mean
Pre	2.28	2.75	2.68	3.05	5.01	2.78	3.09
0.5	3.98	2.53	3.93	2.83	2.98	2.43	3.11
1.0	3.7	4.28	3.45	2.83	2.88	2.58	3.20
2.0	2.57	3.28	3.17	1.00	2.97	3.13	2.69
4.0	3.63	4.00	1.38	3.48	3.48	1.52	2.91
6.0	2.93	4.33	3.23	1.17	3.02	3.25	2.99
8.0	3.62	4.72	3.00	1.08	4.82	3.17	3.40
24.0	2.15	3.38	2.20	3.28	1.50	3.53	2.67
48.0	3.75	4.25	3.65	3.27	2.92	3.98	3.64
72.0	3.58	3.98	2.83	3.03	3.03	1.63	3.01

Appendix 5.4 Clotting time (min.) of blood from of dogs after administration of piroxicam at a dose rate of 0.3 mg/kg by the intravenous route.

Time (h).	Pen Number 1	2	3
Pre	-/-	-/-	-/-
24	-/-	-/-	-/-
48	-/-	-/-	-/-
72	-/-	-/+	-/-

\- = Negative
\+ = Definite colour change

Appendix 5.5 Occult blood in faeces of dogs after administration of piroxicam at a dose rate of 0.3 mg/kg by the intravenous route.

APPENDIX 6

PIROXICAM ORAL ADMINISTRATION TABLES

Time	Animal Number					
(h)	1	2	3	4	5	6
Pre	0	0	0	0	0	0
0.50	0.52	0.71	1.27	0.99	0.27	0.87
1.00	0.68	1.17	1.21	1.11	1.26	1.86
2.00	0.88	1.12	1.10	1.24	1.41	1.86
4.00	1.01	0.98	1.11	1.14	1.45	1.81
8.00	0.87	1.29	0.68	0.85	1.10	1.24
12.00	0.68	0.74	0.52	0.77	0.83	1.09
24.00	0.64	0.76	0.70	0.73	1.00	0.96
48.00	0.31	0.49	0.44	0.40	0.52	0.49
72.00	0.23	0.45	0.30	0.26	0.56	0.53

Appendix 6.1 Concentration (μg/ml) of piroxicam in plasma of dogs after administration at a dose rate of 0.3 mg/kg by the oral route

Time	Animal Number								
(h)	1	2	3	4	5	6	Mean	±	
Pre	1.072	1.796	1.905	1.579	1.377	2.071	1.633	±	0.150
0.50	0.488	0.383	0.531	0.392	0.758	0.768	0.553	±	0.070
1.00	0.391	0.423	0.755	0.341	0.283	0.486	0.448	±	0.068
2.00	0.343	0.378	0.626	0.406	0.314	0.696	0.460	±	0.065
4.00	0.420	0.335	0.661	0.439	0.323	0.643	0.470	±	0.060
8.00	0.301	0.323	0.728	0.355	0.290	0.540	0.423	±	0.072
12.00	0.501	0.431	0.675	0.385	0.473	0.804	0.545	±	0.066
24.00	0.546	0.616	0.878	0.576	0.445	0.945	0.668	±	0.081
48.00	0.525	0.658	1.124	0.593	0.460	0.944	0.717	±	0.106
72.00	1.111	1.090	1.073	0.865	0.828	1.164	1.022	±	0.057

Appendix 6.2 Thromboxane B_2 concentration (μg/ml) in serum of dogs after administration of piroxicam at at a dose rate of 0.3 mg/kg by the oral route.

Time	Animal Number								
(h)	1	2	3	4	5	6	Mean	±	SEM
Pre	412	415	264	389	306	355	356.8	±	24.9
0.5	333	402	395	239	235	310	317.3	±	28.8
1.0	398	429	408	251	255	310	341.8	±	32.6
2.0	333	369	349	239	268	303	276.8	±	14.3
4.0	375	379	429	248	245	313	331.5	±	30.8
8.0	210	306	336	194	200	226	245.3	±	24.6
12.0	316	323	NS	226	300	345	302.0	±	20.3
24.0	313	329	372	210	255	284	293.8	±	23.3
48.0	287	389	339	223	264	306	301.3	±	23.7
72.0	422	395	379	242	290	316	340.7	±	28.2

Appendix 6.3 Platelet numbers ($x10^9$/l).in blood of dogs after oral administration of piroxicam at at a dose rate of 0.3 mg/kg by the oral route.

Time	Animal Number						
(h)	1	2	3	4	5	6	Mean
Pre	1.95	3.18	2.40	1.87	3.60	3.03	2.67
0.5	2.11	3.50	2.08	2.08	2.75	2.86	2.57
1.0	2.48	3.53	1.43	2.88	3.28	2.67	2.72
2.0	3.65	3.68	2.83	3.82	1.93	2.15	3.02
4.0	2.27	3.18	2.50	3.32	3.20	2.08	2.77
8.0	3.38	4.08	3.00	3.92	3.72	3.88	3.67
24.0	3.26	3.23	3.33	2.52	2.28	3.57	3.07
48.0	2.88	3.57	3.52	3.07	3.42	4.29	3.50
72.0	2.92	2.50	2.47	2.97	2.77	3.07	2.78

Appendix 6.4 Clotting time (min.) of blood from of dogs after administration of piroxicam at a dose rate of 0.3 mg/kg by the oral route.

Time (h).	Pen Number 1	2	3
Pre	-/-	-/-	-/-
24	-/-	-/-	-/-
48	-/NS	-/-	-/NS
72	-/NS	-/-	-/-

\- = Negative
NS = No sample

Appendix 6.5 Occult blood in faeces of dogs after administration of piroxicam at a dose rate of 0.3 mg/kg by the oral route.

APPENDIX 7

CINCHOPHEN INTRAVENOUS ADMINISTRATION TABLES

Time	Animal Number					
(h)	1	2	3	4	5	6
Pre	0	0	0	0	0	0
0.08	135.15	143.95	132.47	159.01	89.28	61.58
0.17	116.29	135.33	128.47	155.76	87.20	73.89
0.33	111.60	135.78	117.75	140.51	86.68	82.02
0.50	112.28	111.87	113.88	136.33	91.97	77.79
0.75	96.24	113.15	102.35	138.92	84.48	92.09
1.00	89..41	112.06	90.06	135.31	84.40	88.36
2.00	79.53	94.58	76.51	99.26	67.52	82.59
4.00	50.42	80.28	55.13	77.02	70.36	73.12
8.00	28.95	61.39	35.65	43.99	50.33	53.71
12.00	15.79	35.68	29.13	40.81	32.13	47.36
24.00	2.75	13.57	8.96	9.66	12.25	20.35
30.00	1.14	8.22	5.48	8.92	7.44	13.79
48.00	0.08	1.74	0.92	1.20	1.49	2.60
55.00	0	1.04	0.48	0.52	0.94	1.35

Appendix 7.1 Concentration (μg/ml) of cinchophen in plasma of dogs after administration at a dose rate of 12.5 mg/kg by the intravenous* route.
(* in combination with 0.15 mg/kg prednisolone.)

Time	Animal Number								
(h)	1	2	3	4	5	6	Mean	±	SEM
Pre	1.494	1.157	1.336	0.616	0.848	0.917	1.061	±	0.133
0.08	0.239	0.247	0.589	0.140	0.226	0.504	0.324	±	0.072
0.17	0.173	0.279	0.237	0.104	0.305	0.605	0.284	±	0.071
0.33	0.381	0.215	0.345	0.209	0.319	0.386	0.309	±	0.032
0.50	0.292	0.280	1.090	0.194	0.204	0.699	0.460	±	0.147
0.75	0.372	0.317	0.238	0.338	0.265	0.821	0.392	±	0.088
1.00	0.362	0.456	0.609	0.556	0.546	0.694	0.537	±	0.047
2.00	0.657	0.476	0.519	0.282	0.434	0.546	0.486	±	0.051
4.00	0.722	0.561	0.580	0.261	0.632	1.050	0.634	±	0.104
8.00	0.639	0.997	0.472	0.229	0.603	0.362	0.550	±	0.109
12.00	0.956	1.146	1.070	0.506	0.975	1.427	1.013	±	0.123
24.00	0.775	1.161	NS	0.329	0.612	1.911	0.954	±	0.274
30.00	1.138	NS	1.367	0.679	1.108	1.930	1.244	±	0.204
48.00	NS	NS	NS	NS	NS	1.367	1.367	±	-

Appendix 7.2 Thromboxane B_2 concentration (µg/ml) in serum of dogs after administration of cinchophen (in combination with 0.15 mg/kg prednisolone) at a dose rate of 12.5 mg/kg by the intravenous route.

Time	Animal Number								
(h)	1	2	3	4	5	6	Mean	±	SEM
Pre	460	420	250	210	400	320	343.3	±	40.7
0.08	390	220	300	190	NS	370	294.0	±	39.5
0.17	390	330	320	160	380	330	318.3	±	33.8
0.33	400	350	330	160	380	330	325.0	±	34.9
0.50	290	320	290	130	320	280	271.7	±	29.1
0.75	380	370	320	100	360	380	318.3	±	44.6
1.00	310	330	NS	160	NS	320	280.0	±	40.2
2.00	350	380	310	150	300	320	301.7	±	32.6
4.00	250	360	260	200	390	210	278.3	±	32.2
8.00	460	440	320	180	380	380	360.0	±	41.3
12.00	380	410	350	190	390	150	311.7	±	45.8
24.00	290	320	290	170	340	310	286.7	±	24.6
30.00	360	360	300	160	380	260	303.3	±	34.0
48.00	250	170	310	190	320	320	260.0	±	27.6

Appendix 7.3 Platelet numbers($x10^9$/l) in blood of dogs after administration of cinchophen (in combination with 0.15 mg/kg prednisolone) at a dose rate of 12.5 mg/kg by the intravenous route.

Time	Animal Number						
(h)	1	2	3	4	5	6	Mean
Pre	3.10	3.75	2.44	2.08	3.08	3.46	2.98
0.5	2.42	2.63	3.93	3.30	3.73	3.35	3.23
1.0	2.85	4.65	3.10	4.04	4.04	4.42	3.85
2.0	3.37	2.95	3.69	3.50	3.73	3.63	3.48
4.0	4.26	3.07	3.74	3.23	3.14	4.78	3.70
8.0	3.50	1.66	2.75	1.71	3.06	3.53	2.70
24.0	3.04	4.12	3.50	1.38	3.50	3.67	3.20
30.0	3.00	1.17	3.76	3.37	4.04	3.70	3.17
48.0	1.69	2.00	3.50	2.83	3.33	NS	2.67

Appendix 7.4 Clotting time (min.) of blood from dogs after administration of cinchophen (in combination with 0.15 mg/kg prednisolone) at a dose rate of 12.5 mg/kg by the intravenous route.

Time (h).	Pen Number 1	2	3
Pre	-/-	-/NS	-/NS
24	-/NS	-/NS	NS/NS
48	-/NS	-/-	-/NS

\- = Negative
NS = No sample

Appendix 7.5 Ocult blood in faeces of dogs after administration of cinchophen (in combination with 0.15 mg/kg prednisolone) at a dose rate of 12.5 mg/kg by the intravenous route.

APPENDIX 8

CINCHOPHEN ORAL ADMINISTRATION TABLES

Time	Animal Number					
(h)	1	2	3	4	5	6
Pre	0	0	0	0	0	0
0.08	0	0.17	0	0.06	0	0
0.17	0.11	12.22	0.07	21.21	0.06	0.14
0.33	0.25	67.35	0.29	69.22	1.14	0.23
0.50	0.48	93.58	75.18	72.80	3.18	0.62
0.75	10.98	94.60	99.56	61.84	4.81	0.97
1.00	66.64	92.02	90.36	62.32	16.33	1.43
2.00	68.78	87.76	74.83	80.29	61.47	78.06
4.00	37.92	72.24	66.35	54.46	38.28	78.53
8.00	25.17	55.91	41.33	18.26	44.74	50.99
12.00	10.07	41.45	29.91	21.01	32.56	40.23
24.00	1.09	22.52	15.88	4.98	15.31	19.09
30.00	1.13	24.41	11.31	2.43	12.28	10.47
48.00	0.71	7.37	2.65	0.38	2.33	3.67
50.00	0.33	5.41	1.55	0.26	1.44	3.30

Appendix 8.1 Concentration (μg/ml) of cinchophen in plasma of dogs after administartion at a dose rate of 12.5 mg.kg by the oral* route. (* in combination with 0.15 mg/kg prednisolone.)

Time	ANIMAL NUMBER								
(h)	1	2	3	4	5	6	Mean	±	SEM
Pre	1.494	1.404	1.178	1.369	1.345	1.738	1.423	±	0.076
0.08	1.018	1.550	1.184	0.996	1.725	1.517	1.332	±	0.125
0.17	1.192	1.530	1.124	1.505	1.060	1.605	1.336	±	0.097
0.33	1.405	1.080	1.118	0.513	1.179	2.037	1.222	±	0.203
0.50	1.408	0.957	0.687	0.834	1.395	1.534	1.136	±	0.144
0.75	1.926	0.500	0.345	0.765	1.340	1.271	1.024	±	0.243
1.00	0.747	0.507	0.391	1.886	1.268	0.668	0.991	±	0.231
2.00	0.627	0.645	0.526	0.622	1.153	1.205	0.796	±	0.122
4.00	0.978	1.173	2.340	1.647	0.843	1.365	1.391	±	0.222
8.00	0.827	1.291	1.264	1.647	0.777	2.082	1.315	±	0.020
12.00	1.396	1.525	2.810	1.385	0.833	1.435	1.564	±	0.269
24.00	1.442	1.147	1.399	1.134	2.227	1.706	1.509	±	0.167
30.00	1.108	1.475	1.793	0.945	1.486	2.163	1.495	±	0.181
48.00	1.311	2.165	1.510	0.827	1.184	3.735	1.789	±	0.429
50.00	1.312	1.494	1.248	1.442	1.203	3.528	1.704	±	0.367

Appendix 8.2 Thromboxane B_2 concentration (μg/ml) in serum of dogs after administration of cinchophen (in combination with 0.06 mg/kg prednisolone) at a dose rate of 12.5 mg/kg by the oral route.

Time	Animal Number								
(h)	1	2	3	4	5	6	Mean	±	SEM
Pre	500	370	310	170	330	390	345.0	±	44.2
0.08	450	410	370	130	330	NS	338.0	±	55.6
0.16	420	450	410	200	370	270	353.3	±	39.9
0.33	430	390	370	240	350	240	336.7	±	32.4
0.50	420	420	370	210	360	NS	356.0	±	38.5
0.75	460	400	360	220	300	390	355.0	±	34.4
1.00	NS	400	260	160	350	310	296.0	±	41.0
2.00	290	350	370	190	310	350	276.6	±	25.1
4.00	NS	420	330	220	360	350	336.0	±	32.6
8.00	350	400	160	200	270	310	281.7	±	37.0
12.00	400	190	350	250	350	290	305.0	±	31.4
24.00	430	410	320	210	280	280	321.7	±	34.4
30.00	370	410	350	250	320	360	343.3	±	22.2
48.00	360	130	330	220	300	350	281.7	±	36.6
50.00	430	420	360	160	330	240	323.3	±	43.1

Appendix 8.3 Platelet numbers ($x10^9/l$) in blood of dogs after administration of cinchophen (in combination with 0.06 mg/kg prednisolone) at a dose rate of 12.5 mg/kg by the oral route.

Time	Animal Number						
(h)	1	2	3	4	5	6	Mean
Pre	2.67	2.50	3.92	3.60	3.23	3.65	3.26
0.5	1.83	3.08	1.92	2.83	3.83	2.75	2.71
1.0	3.55	3.78	3.57	2.92	3.27	2.50	3.26
2.0	3.17	4.00	3.48	2.58	3.00	2.67	3.15
4.0	2.33	3.66	2.94	4.04	3.46	3.89	3.39
8.0	4.17	3.50	2.67	4.17	3.75	4.00	3.71
24.0	1.67	1.58	2.12	3.08	2.00	2.33	2.13
30.0	2.32	3.32	3.26	3.75	3.60	2.73	3.16
48.0	2.67	2.89	3.42	2.50	2.50	2.61	2.76

Appendix 8.4 Clotting time (min.) of blood from dogs after administration of cinchophen (in combination with 0.06 mg/kg prednisolone) at a dose rate of 12.5 mg/kg by the oral route.

Time (h).	Pen Number 1	2	3
Pre	-/NS	-/-	NS/NS
12	-/-	-/-	-/-
24	-/-	-/-	-/-
48	NS/NS	NS/NS	NS/NS

\- = Negative
NS = No sample

Appendix 8.5 Occult blood in faeces of dogs after administration of cinchophen (in combination with 0.06 mg/kg prednisolone) at a dose rate of 12.5 mg/kg by the oral route.

APPENDIX 9

TOLFENAMIC ACID INTRAVENOUS ADMINISTRATION TABLES

Time	Animal Number					
(h)	1	2	3	4	5	6
Pre	0	0	0	0	0	0
0.08	14.96	23.92	24.47	14.50	30.48	44.13
0.17	8.67	13.07	23.63	11.55	24.05	28.10
0.33	6.65	6.82	12.73	6.23	9.91	17.21
0.50	0.92	2.15	7.34	4.81	5.03	9.66
0.75	1.45	2.25	4.11	3.92	3.07	6.05
1.00	1.25	0.55	2.04	2.14	1.11	2.60
2.00	0	0	0.63	1.32	0.21	0.30
4.00	0.20	0.32	1.65	2.34	0.08	0.44.
8.00	0.47	0.43	0.41	1.17	0.16	0.33
12.00	0.27	0.34	0.24	0.52	0.08	0.21
24.00	0.05	0	0.11	0.20	0	0.06
48.00	0	0	0.10	0.12	0	0
72.00	0	0	0	0.06	0	0
96.00	0	0	0	0.06	0	0
168.00	0	0	0	0	0	0

Appendix 9.1 Concentration (μg/ml) of tolfenamic acid in plasma of dogs after administration at a dose rate of 4.0 mg/kg by the intravenous route.

Time (h)	Animal Number 1	2	3	4	5	6	Mean ± SEM
Pre	1.21	1.79	1.35	0.62	1.15	1.43	1.26 ± 0.16
0.50	0.17	0.08	0.07	0.09	0.10	0.08	0.10 ± 0.01
1.00	1.05	0.42	0.36	0.21	0.85	0.41	0.55 ± 0.13
2.00	1.24	0.96	1.55	0.43	0.76	1.04	0.99 ± 0.16
4.00	1.12	0.89	0.40	0.27	1.19	1.34	0.87 ± 0.18
8.00	1.11	0.94	0.84	0.36	1.40	1.14	0.96 ± 0.14
12.00	0.96	0.89	0.67	0.54	1.06	1.20	0.89 ± 0.10
24.00	1.28	0.92	1.07	0.48	0.98	1.23	0.99 ± 0.12
48.00	0.79	1.35	1.12	0.82	1.49	1.17	1.12 ± 0.11
72.00	1.52	1.48	1.43	0.92	0.74	1.05	1.19 ± 0.13
96.00	1.06	1.53	1.15	0.92	0.69	0.78	1.02 ± 0.12
168.00	1.45	1.90	2.25	1.55	1.42	1.18	1.62 ± 0.16

Appendix 9.2 Thromboxane B_2 concentration (μg/ml) in serum of dogs after administration of tolfenamic acid at a dose rate of 4.0 mg/kg by the intravenous route.

	Animal Number								
(h)	**1**	**2**	**3**	**4**	**5**	**6**	**Mean**	±	**SEM**
Pre	341	190	NS	102	93	207	186.6	±	44.7
0.25	311	353	317	194	348	227	291.7	±	26.9
0.50	398	367	299	197	294	253	301.3	±	30.0
1.00	342	322	210	196	282	211	260.5	±	25.8
2.00	361	314	262	150	303	110	250.0	±	40.4
4.00	388	NS	NS	NS	328	NS	358.0	±	30.1
6.00	NS	321	258	92	321	NS	248.0	±	54.1
8.00	264	153	208	268	221	217	221.8	±	17.2
12.00	224	350	357	213	391	266	300.2	±	30.8
24.00	349	410	278	245	313	326	320.2	±	23.4
48.00	NS	284	197	NS	NS	NS	240.5	±	43.6
72.00	293	319	287	185	248	207	256.5	±	21.4

Appendix 9.3 Platelet numbers ($x10^9/l$) in blood of dogs after administration of tolfenamic acid at a dose rate of 4.0 mg/kg by the intravenous route.

Time (h)	Animal Number 1	2	3	4	5	6	Mean
Pre	2.42	3.90	3.72	2.22	2.35	3.99	3.10
0.5	3.56	3.83	2.78	2.18	4.44	3.48	3.38
1.0	3.08	4.17	3.44	4.30	3.17	3.50	3.61
2.0	1.50	2.17	1.50	3.33	3.00	4.33	2.64
4.0	4.00	2.67	3.83	1.50	3.83	2.00	2.97
8.0	3.67	3.17	3.83	3.17	3.00	3.83	3.44
24.0	3.68	1.66	3.00	2.88	3.55	4.03	3.13
48.0	2.67	2.78	2.72	2.75	4.19	2.14	2.88
72.0	3.14	4.33	2.75	2.83	2.50	2.75	3.05
96.0	2.83	3.00	1.12	1.67	2.45	3.62	2.45
168.0	4.06	3.30	2.50	3.00	2.25	3.64	3.29

Appendix 9.4 Clotting time (min.) of blood from dogs after administration of tolfenamic acid at a dose rate of 4.0 mg/kg by the intravenous route.

Time (h).	Pen Number 1	2	3
Pre	-/-	-/-	-/-
24	-/-	-/-	-/-
48	NS	-/-	-/-
72	-/NS	-/NS	TR/NS
96	NS	NS	-/-
168	-/NS	-/-	-/NS

\- = Negative
TR = Trace of colour change
NS = No sample

Appendix 9.5 Occult blood in faeces of dogs after administration of tolfenamic acid at a dose rate of 4.0 mg/kg by the intravenous route.

APPENDIX 10

TOLFENAMIC ACID SUBCUTANEOUS ADMINISTRATION TABLES

Time	Animal Number				
(h)	1	2	4	5	6
Pre	0	0	0	0	0
0.25	1.24	2.02	0.05	1.60	0.64
0.50	3.17	2.44	1.27	2.11	1.82
1.00	4.14	4.04	5.73	2.63	3.64
2.00	3.47	3.23	5.76	2.39	5.40
4.00	1.41	0.90	3.05	1.06	1.58
8.00	0.19	0.68	0.33	0.40	0.99
12.00	1.81	1.05	1.54	0.15	1.28
24.00	0	0.11	0.07	0	0.36
48.00	0.06	0	0	0	0.07
72.00	0	0	0	0	0
96.00	0	0	0	0	0
168.00	0	0	0	0	0

Appendix 10.1 Concentration (µg/ml) of tolfenamic acid in plasma of dogs after administration at a dose rate of 4.0 mg/kg by the subcutaneous route.

Time (h)	Animal Number 1	2	3	4	5	6	Mean ± SEM
Pre	1.64	1.23	1.44	1.22	1.64	1.74	1.48 ± 0.09
0.25	0.42	0.38	0.31	0.09	0.61	1.18	0.50 ± 0.15
0.50	0.21	0.21	0.14	0.13	0.55	0.79	0.34 ± 0.11
1.00	0.18	0.23	0.22	0.18	0.37	0.74	0.32 ± 0.09
2.00	0.27	0.30	0.31	0.25	0.28	0.30	0.29 ± 0.01
4.00	0.47	0.74	0.53	0.60	0.78	0.79	0.65 ± 0.05
8.00	0.65	0.71	0.78	0.60	1.29	0.85	0.81 ± 0.10
12.00	0.67	0.63	1.45	0.71	1.65	1.25	1.06 ± 0.18
24.00	1.30	1.11	1.00	0.67	1.50	1.48	1.18 ± 0.13
48.00	1.05	0.96	1.22	0.78	1.43	1.97	1.24 ± 0.17
72.00	1.16	1.23	1.45	0.96	1.16	1.45	1.24 ± 0.08
96.00	0.83	1.42	1.55	1.11	1.81	1.48	1.37 ± 0.14
168.00	1.29	1.04	1.48	1.19	1.23	1.58	1.30 ± 0.08

Appendix 10.2 Thromboxane B_2 concentration (μg/ml) in serum of dogs after administration of tolfenamic acid at a dose rate of 4.0 mg/kg by the subcutaneous route.

Time	Animal Number								
(h)	1	2	3	4	5	6	Mean	±	SEM
Pre	419	439	482	NS	464	366	434.0	±	20.1
0.25	385	303	428	252	430	325	353.8	±	29.5
0.50	409	411	448	275	412	NS	391.0	±	29.8
1.00	372	414	444	289	452	118	348.2	±	52.1
2.00	302	274	280	267	351	318	298.7	±	13.0
4.00	391	439	361	290	NS	394	375.0	±	24.6
8.00	379	391	301	257	330	310	328.0	±	20.5
12.00	329	312	421	255	421	209	324.5	±	35.1
24.00	400	380	302	NS	341	NS	355.7	±	21.7
48.00	NS	367	312	198	252	265	278.8	±	28.5
72.00	390	374	399	246	394	255	343.0	±	29.5
96.00	242	251	274	175	209	100	208.5	±	25.9
168.00	353	391	311	253	382	221	318.5	±	28.5

Appendix 10.3 Platelet numbers ($x10^9/l$) in blood of dogs after administration of tolfenamic acid at a dose rate of 4.0 mg/kg by the intravenous route.

Time	Animal Number						
(h)	1	2	3	4	5	6	Mean
Pre	2.33	3.33	3.30	2.50	2.78	3.33	2.93
0.5	3.33	3.67	2.86	3.92	2.37	3.27	3.24
1.0	1.33	1.66	2.33	3.10	3.48	1.93	2.29
2.0	2.67	4.08	3.28	3.53	3.17	3.73	3.41
4.0	2.00	2.92	3.12	2.83	2.50	2.67	2.67
8.0	3.50	2.67	3.17	3.24	2.98	2.50	3.01
24.0	1.83	3.33	3.07	2.00	2.15	4.13	2.75
48.0	2.17	2.83	2.50	2.83	2.00	3.22	2.59
72.0	2.50	3.52	2.46	2.87	3.07	1.83	2.71
96.0	2.67	2.67	2.62	1.47	2.01	3.17	2.44
168.0	4.17	3.00	2.77	2.57	2.20	2.90	2.93

Appendix 10.4 Clotting time (min.) of blood from dogs after administration of tolfenamic acid at a dose rate of 4.0 mg/kg by the subcutaneous route.

Time (h).	Pen Number 1	2	3
Pre	-/-	-/-	-/-
24	-/NS	-/NS	-/NS
48	-/NS	TR/NS	+/NS
72	-/-	-/-	-/-
96	NS	NS	NS
168	-/-	-/-	-/-

\- = Negative
TR = Trace of colour change
NS = No sample

Appendix 10.5 Occult blood in faeces of dogs after administration of tolfenamic acid at a dose rate of 4.0 mg/kg by the subcutaneous route.

APPENDIX 11

PROTEIN BINDING TABLES

Species	Sample	Dialysed Ratio	Undialysed Ratio
Dog	**1**	1.29	1.37
	2	1.21	1.35
	3	1.32	1.36
	4	1.30	1.52
	5	1.24	1.42
	6	1.28	1.29
	Mean	**1.27**	**1.38**
	SEM	**0.02**	**0.03**
Goat	**1**	NS	1.18
	2	1.22	1.40
	3	1.15	1.34
	4	1.13	1.30
	5	1.13	1.36
	6	1.06	1.31
	Mean	**1.14**	**1.31**
	SEM	**0.03**	**0.03**
Horse	**1**	1.24	1.47
	2	1.20	1.46
	3	1.22	1.37
	4	1.23	1.35
	5	1.25	1.39
	6	1.24	1.47
	Mean	**1.23**	**1.42**
	SEM	**0.01**	**0.02**

Appendix 11.1 Ratios of sample to standard HPLC peak heights for flunixin in dialysed and undialysed serum from three animal species.

Species	Sample	Dialysed Ratio	Undialysed Ratio
Dog	**1**	0.91	0.97
	2	0.85	1.04
	3	0.95	1.00
	4	0.92	1.00
	5	0.93	1.07
	6	0.93	1.01
	Mean	**0.91**	**0.97**
	SEM	**0.01**	**0.05**
Goat	**1**	0.82	1.04
	2	0.83	0.89
	3	0.83	0.94
	4	0.87	1.09
	5	0.89	1.06
	6	0.89	1.06
	Mean	**0.85**	**1.01**
	SEM	**0.01**	**0.03**
Horse	**1**	1.29	1.07
	2	1.02	1.14
	3	0.99	1.16
	4	1.04	0.96
	5	1.02	1.13
	6	1.10	1.05
	Mean	**1.08**	**1.08**
	SEM	**0.04**	**0.03**

Appendix 11.2 Ratios of sample to standard HPLC peak heights for meclofenamic acid in dialysed and undialysed serum from three animal species.

APPENDIX 12

IN VITRO INHIBITION OF SERUM THROMBOXANE TABLES

Drug Conc.	Animal Number			
(µg/ml)	1	2	3	4
0	0.588	0.541	0.377	0.495
0.0005	0.491	NS	0.331	0.520
0.0010	0.526	0.446	0.324	0.505
0.0050	0.408	0.386	0.315	0.406
0.0075	0.457	0.448	0.351	0.435
0.0100	0.386	0.437	0.319	0.441
0.0200	0.262	0.489	0.287	0.367
0.0250	0.259	0.347	0.282	0.373
0.0500	0.180	0.263	0.193	0.244
0.0750	0.126	0.357	0.189	0.211
0.1000	0.144	0.217	0.165	0.179
0.2000	0.012	0.104	0.173	0.125
0.2500	0.026	0.139	0.083	0.105
0.5000	0.012	0.057	0.056	0.042
0.7500	0.027	0.064	0.014	0.037
1.0000	0.011	0.043	0.018	0.037
2.5000	0.007	0.021	0.012	0.017
5.0000	0.005	0.010	0.010	0.011
7.5000	0.005	0.018	0.005	0.013
10.0000	0.005	0.016	0.009	0.015

NS = No sample

Appendix 12.1 Concentration (µg/ml) of thromboxane B_2 in the serum of dogs. Blood clotting occured in the presence of flunixin.

Drug Conc	Animal Number		
(μg/ml)	1	2	3
0	0.04247	0.05845	0.03284
0.0005	0.04090	0.04930	0.03080
0.0010	0.03960	0.04995	0.02891
0.0050	0.02555	0.03710	0.01536
0.0075	0.02605	0.03365	0.01707
0.0100	0.02523	0.03177	0.01850
0.0200	0.01715	0.02452	0.00770
0.0250	0.01415	0.01402	0.00468
0.0500	0.00775	0.00745	0.00170
0.0750	0.00640	0.00635	0.00146
0.1000	0.00555	0.00400	0.00071
0.2000	0.00360	0.00090	0.00106
0.2500	0.00353	0.00110	0.00063
0.5000	0.00153	0.00035	0.00028
0.7500	0.00046	0.00020	0.00049
1.0000	0.00067	0.00015	0.00006
2.5000	0.00044	0.00005	0.00009
5.0000	0.00036	0.00006	0.00005
7.5000	0.00041	0.00003	0.00010
10.0000	0.00055	0.00011	0.00008

Appendix 12.2 Concentration (μg/ml) of thromboxane B_2 in the serum of goats. Blood clotting occured in the presence of flunixin.

Drug Conc.	Animal Number			
(μg/ml)	1	2	3	4
0	0.0437	0.0110	0.0090	0.0234
0.0005	NS	0.00895	0.0105	0.01975
0.0010	0.0420	0.00802	0.01025	0.01582
0.0050	0.0380	0.00965	0.00777	0.01525
0.0075	NS	0.00875	0.00742	0.01680
0.0100	0.0280	0.00534	0.00628	0.01071
0.0200	NS	0.00456	0.00682	0.00960
0.0250	0.0120	0.00456	0.00553	0.00855
0.0500	0.0085	0.00374	0.00332	0.00403
0.0750	0.003	0.00286	0.00342	0.00488
0.1000	0.0030	0.00183	0.00189	0.00542
0.2500	0.0010	0.00070	0.00845	0.00350
0.5000	0.0012	0.00031	0.00034	0.00674
0.7500	0.0006	0.00020	0.00031	0.00066
1.0000	0.00073	0.00021	0.00030	0.00057
2.5000	0.00015	NS	NS	NS
5.0000	0.00014	NS	0.00012	0.00066
10.0000	0.00013	NS	0.00015	0.00050

NS = No sample

Appendix 12.3 Concentration (μg/ml) of thromboxane B_2 in the serum of horses. Blood clotting occured in the presence of flunixin.

Drug Conc.	Animal Number		
(μg/ml)	1	2	3
0	0.438	0.408	0.580
0.0005	0.297	0.330	0.452
0.0010	0.281	0.298	0.487
0.0050	NS	0.347	0.384
0.0075	0.309	0.327	0.536
0.0100	0.286	0.325	0.547
0.0200	0.079	0.357	0.439
0.0250	NS	NS	0.519
0.0500	NS	0.320	0.479
0.0750	0.341	0.280	0.617
0.1000	0.181	0.305	0.439
0.2000	0.026	0.286	NS
0.2500	0.005	0.277	NS
0.5000	NS	0.144	0.391
0.7500	0.061	0.114	0.318
1.0000	0.016	NS	NS
2.5000	NS	0.073	0.114
5.0000	NS	0.011	0.329
7.5000	NS	0.006	0.136
10.0000	0.004	0.005	0.065
20.0000	NS	NS	0.029
38.0000	NS	NS	0.006

NS = No sample

Appendix 12.4 Concentration (μg/ml) of thromboxane B_2 in the serum of dogs. Blood clotting occured in the presence of meclofenamic acid.

Drug Conc	Animal Number		
(μg/ml)	1	2	3
0	0.04234	0.08800	0.07767
0.001	0.04195	0.09295	0.06320
0.005	0.04425	0.08205	0.05035
0.010	0.04970	0.07192	0.07830
0.050	0.03218	0.06789	0.06206
0.100	0.02944	0.04231	0.04715
0.200	0.03573	NS	NS
0.250	0.01509	0.02644	0.03096
0.500	0.00656	0.01198	0.01388
0.750	0.00428	0.00850	0.01253
1.000	0.00350	0.00335	0.00478
2.500	0.00034	0.00078	0.00156
5.000	0.00017	0.00023	0.00054
7.500	0.00014	0.00021	0.00039
10.000	0.00005	0.00019	0.00025
20.000	NS	0.00016	0.00009
38.550	NS	NS	0.00005
133.330	NS	NS	0.00005

NS = No sample

Appendix 12.5 Concentration (μg/ml) of thromboxane B_2 in the serum of goats. Blood clotting occured in the presence of meclofenamic acid.

Drug Conc.	Animal Number		
(µg/ml)	1	2	3
0	0.02346	0.02260	0.05145
0.001	0.01225	0.03040	0.04655
0.005	0.01190	0.02745	0.04425
0.010	0.01624	0.02458	0.04948
0.020	0.01455	0.01390	0.04520
0.050	0.00993	0.01201	0.03892
0.100	0.01066	0.01182	0.03383
0.250	0.00522	0.00612	0.02122
0.500	0.00255	0.00312	0.00956
0.750	0.00184	0.00171	0.00710
1.000	0.00117	0.00009	0.00336
2.500	0.00048	0.00034	0.00130
5.000	0.00019	0.00016	0.00071
7.500	0.00020	0.00032	0.00075
10.000	0.00012	0.00010	0.00043
20.000	0.00006	0.00002	0.00023
38.550	NS	0.00002	0.00019
133.330	NS	NS	0.00007

NS = No sample

Appendix 12.6 Concentration (µg/ml) of thromboxane B_2 in the serum of horses. Blood clotting occured in the presence of meclofenamic acid.

Drug Concentration (μg/ml)	Dog	Goat	Horse
0.0005	NS	NA	NA
0.0010	NS	NS	NS
0.0050	NS	NS	<0.05
0.0075	NS	NA	NA
0.0100	NS	NS	NS
0.0200	NS	NA	<0.05
0.0250	NS	NA	NA
0.0500	NS	NA	<0.05
0.0750	NS	NA	NA
0.1000	<0.05	<0.01	<0.05
0.2000	NS	NS	NA
0.2500	NS	<0.01	NS
0.5000	<0.05	NS	NS
0.7500	NS	<0.01	<0.01
1.0000	NS	<0.05	NS
2.5000	<0.01	NS	NS
5.0000	NS	NS	NS
7.5000	NS	NS	NA
10.0000	NS	NS	NS
20.0000	NA	NA	NA
38.5500	NA	NA	NA
133.3300	NA	NA	NA

NS = Not significant
NA = No sample

Appendix 12.7 Level of significant difference in inhibition of <u>in vitro</u> serum TxB_2 which occured in the presence of flunixin when compared with the inhibition which occured in the presence of meclofenamic acid in each of three species.

Drug Concentration (μg/ml)	Dog versus Goat	Dog versus Horse	Goat versus Horse
0.0005	NS	NS	NA
0.0010	NS	NS	NA
0.0050	<0.05	NS	<0.05
0.0075	<0.01	NS	<0.01
0.0100	<0.05	<0.05	NS
0.0200	<0.05	NS	NS
0.0250	<0.05	NS	NS
0.0500	<0.01	<0.05	NS
0.0750	<0.05	NS	NS
0.1000	<0.01	<0.05	NS
0.2000	NS	NA	NA
0.2500	NS	NS	NS
0.5000	NS	NS	NS
0.7500	<0.05	NS	<0.05
1.0000	<0.05	NS	<0.05
2.5000	<0.05	NA	NA
5.0000	<0.05	NS	NS
7.5000	NS	NA	NA
10.0000	NS	NS	NS

NS = Not significant
NA = No sample

Appendix 12.8 Level of significant difference in inhibition of <u>in vitro</u> serum TxB_2 when each species was compared to another species. Blood clotting occured in the presence of flunixin.

Drug Concentration (μg/ml)	Dog versus Goat	Dog versus Horse	Goat versus Horse
0.0005	NA	NA	NA
0.0010	NS	NS	NS
0.0050	NS	NS	NS
0.0075	NA	NA	NA
0.0100	NS	NS	NS
0.0200	NA	NS	NA
0.0250	NA	NA	NA
0.0500	NS	NS	NS
0.0750	NA	NA	NA
0.1000	NS	NS	NS
0.2000	NA	NA	NA
0.2500	NS	NS	<0.01
0.5000	NS	NS	NS
0.7500	NS	NS	<0.05
1.0000	NA	NA	NS
2.5000	<0.01	<0.01	NS
5.0000	NS	NS	NS
7.5000	NS	NS	0.014
10.0000	NS	NS	NS
20.0000	NA	NA	NS
38.5500	NA	NA	NA
133.3300	NA	NA	NA

NS = Not significant

NA = No sample

Appendix 12.9 Level of significant difference in inhibition of <u>in vitro</u> serum TxB_2 when each species was compared to another species. Blood clotting occured in the presence of meclofenamic acid.

REFERENCES

Abbruzzo, J.L., Gordon, G.V. and Myers, A.R. (1982) Double blind study comparing piroxicam and aspirin in the treatment of osteoarthritis. *American Journal of Medicine*, 72, 45-59.

Adams, S.S., Bough, R.G, Cliffe, E.E., Dickenson, W., Lessel, B., McCullough, K.F., Mills, R.F., Nicolson, J.S. and Williams, G.A. (1970) Some aspects of the pharmacology, metabolism and toxicology of ibuprofen I. Pharmacology and metabolism. *Rheumatology and Physical Meicine* 10 (Supplement), 9-26.

Adams, S.S. and Cobb, R (1958) A possible basis for anti-inflammatory activity of salicylates and other non-hormonal anti-rheumatic drugs. *Nature*, 181, 773-774.

Adamska-Dyniewska, H., Kowakzyk, L. and Tkaczewski, W. (1979) Farmakokinetyka kwasu mefenaminowego u chorych z przewleka niewydilnoscia krazenia. *Polski Tygodnik Lekarski,* 34, 177-180

Ahlengard, S., Tufvesson, G., Petterson, H. and Andersson, T. (1978) Treatment of traumatic arthritis in the horse with intra-articular orgotein (Palosein). *Equine Veterinary Journal* 10(2), 122-124.

Alanko, J.(1990) Tolfenamic acid, a wide spectrum anti-inflammatory analgesic. Abstracts *XXIII Scandinavian Congress of Rheumatology*, Tampere, Finland, 7-9.

Alanko, J., Moilanen, E., Opas, J. and Vapaatalo, H. (1989). Tolfenamic acid inhibits leukotriene synthesis in human granulocytes. *Agents Actions*, 26, 254-255.

Allison, A.C. (1971) Lysosomes and toxicity of particulate pollutants. *Archives of International Medicine* 128, 131-139.

Altman, R.D., Dean, D.D., Muniz, O.E. and Howell, D.S. (1988) The Effect of glycosaminoglycan polysulfuric acid ester (GAGPS) in a dog model of osteoarthritis (OA). *Arthritis and Rheumatism* 31 (Supplement), 30S.

Altman, R.D., Dean, D.D., Muniz, O.E. and Howell, D.S. (1989) Prophylactic treatment of canine osteoarthritis with glycosaminoglycan polysulfuric acid ester. *Arthritis and Rheumatism* 32(6), 576-579.

Anderson, K.L., Neff-Davis, C.A., Davis, L.E. and Bass, V.D. (1990) Pharmacokinetics of flunixin meglumine in lactating cattle after single and multiple intramuscular and intravenous administrations. *American Journal of Veterinary Research*, 51, 1464-1467.

Anggard, E. and Jonsson, C.E. (1971) Efflux of prostaglandins in lymph from scalded tissue. *Acta Physiologica Scandinavia* 81, 440-447.

Annegers, J.H., Snapp, F.E., Ivy, A.C. and Atkinson, A.J. (1943) A study of the excretion of cinchophen in bile and urine and the posology of the drug. *Gastroenterology*, 1, 597-614.

Ascheim, E. and Zweifach, B.W. (1962) Quantitative studies of fluid and water shift during inflammation. *American Journal of Physiology* 202, 554-558.

Ashley, S.W., Sonnenschein, L.A. and Cheung, L.Y. (1985) Focal gastric mucosal blood flow at the site of aspirin induced ulceration *American Journal of Surgery* 149, 53-59.

Axelsson, C.K., Christiansen, L.V., Johansen, A. and Ejby Poulsen, P. (1977). Comparative effects of tolfenamic acid and acetylsalicylic acid on human gastric mucosa. *Scandanavian Journal of Rheumatology*, 6, 23-27.

Bachmann, F. (1980) Correlation of plasma and synovial fluid concentrations of piroxicam in patients with rheumatoid arthritis. In *Rheumatology of the Eighties. An Advance in Therapy - Piroxicam.*, Ed. P. Dessain, *Excerpta Medica*, Princeton, New Jersey, USA, 41-44.

Baggot , J.D. (1977) In *Principles of Drug Disposition in Domestic Animals: the Basis of Veterinary Clinical Pharmacology*. W.B. Saunders & Co., Philidelphia, USA.

Baggot, J.D. (1982) Disposition and fate of Drugs in the Body. In *Veterinary Pharmacology and Therapeutics*, 5th edn., Eds N.H. Booth and L.E. McDonald, Iowa State University Press, Ames, USA. 36-69.

Balat, L.P. (1985) Binding of drugs to blood components and renal elimination. In *Protein Binding and Drug Transport,* Ed J. Tillement and E. Lindenlaub, Symposium Medica Hoechst 20. Algarve, Portugal. 231-252.

Barbour, H.G. and Lozinsky, E.J. (1923) Non-toxicity and anti-pyretic effects of tolysin (ethyl ester of paramethylphenylcinchonic acid) *Journal of Laboratory and Cinical Medicine*, 8, 217-226.

Barnardo, D.E., Currey, H.L.F., Mason, R.M., Fox, W.R and Weatherall, M. (1966) Meclofenamic acid and flufenamic acid compared with aspirin and phenylbutazone in rheumatoid arthritis. *British Medical Journal*, 2, 342-363.

Baruth, H., Berger, L., Bradshaw, D., Costin, C., Coffey, J.W., Gupta, N., Konikoff, J.Roberts, N.A. and Wyler-Plant, R. (1986) Carprofen. In *Anti-inflammatory and Anti-rheumatic Drugs,* Vol.II. Newer Anti-inflammatory Drugs. Ed K.D. Rainsford, CRC Press, Inc. Florida, 33-47.

Bass, B.H. (1974) Letter : Jaundice associated with naproxen. *Lancet* 1 (864), 998.

Beermann, B. Hellstrom, K., Lindstrom, B. and Rosen, A. (1975) Binding site interactions of chlorthalidone and axetazolamide, two drugs transported by red blood cells. *Clinical Pharmacology and Therapeutics*, 17, 424-432.

Behrens, P.O. Spiekerman, A.M. and Brown, J.R. (1975) The structure of human serum albumin. *Federation Proceedings*, 34, 591.

Bell, R.L., Kennerly, D.A., Stanford, N. and Majerus, P.W. (1979) Diglyceride lipase: a pathway for arachidonate release from human platelets. *Proceedings of the National Academy of Sciences*. USA, 76, 3238-3241.

Benitz, A.M. (1984) Pharmacology and pharmacokinetics of flunixin meglumine in the bovine. In *Proceedings 13th World Congress on the Diseases of Cattle*, Durban, South Africa, 928-930.

Bennett, A., Jarosik, C., Sanger, G.J. and Wilson, D.E. (1980b). Antagonism of prostanoid-induced contractions of rat gastric fundus muscle by SC-19220, sodium meclofenamate, indomethacin or trimethoquinol. *British Journal of Pharmacology* 71, 169-172

Bennett, A., Pratt, D. and Sanger, G.J. (1980a). Antagonism by fenamates of prostaglandin action in guinea-pig and human alimentary muscle. *British Journal of Pharmacology* 68, 357-358

Bhattacherjee, P., Eakins, K.E., and Hammond, B. (1981) Chemotactic activity of arachidonic acid lypoxygenase products in rabbit eye. *British Journal of Pharmacology* 73, 245-255.

Biberfield, J. (1913) Zur Wirkungsweise des atophan. *Ztschr fur Experimentelle Pathologie und Therapie*, 13, 301-309.

Bills, T.K., Smith, J.B. and Silver, M.J. (1977) Selective release of arachidonic acid from the phospholipids of human platelets in response to thrombin. *Journal of Clinical Investigation* 60, 1-6.

Birkett, D.J and Wanwimolruk, S .(1985) Albumin as a specific binding protein for drugs and endogenous compounds. In *Protein Binding and Drug Transport,* Ed J. Tillement and E. Lindenlaub, Symposium Medica Hoechst 20. Algarve, Portugal. 11-23.

Bjornstad H. and Vik , O (1986) Thrombocytopenic purpura associated with piroxicam. *British Journal of Clinical Practice*, 40, 42.

Boctor, A.M., Eickholt, M. and Pugsley, T.A. (1986) Meclofenamate sodium is an inhibitor of both 5-lipoxygenase and cyclooxygenase pathways of the arachidonic acid cascade in vitro. *Prostaglandins Leukotrienes Med.* 23, 229-238.

Bollman, J.L. Stalker, L.K. and Mann, F.C. (1938) Experimental peptic ulcer produced by cinchophen. *Archives of International Medicine*, 61, 119-128.

Boobis, S. (1977) Alteration of plasma albumin in relation to decreased drug binding in uremic and normal serum. *Clinical Pharmacology and Therapeutics*, 22, 147-153.

Booker, H.E. and Darcey, B. (1973) Serum concentrations of free diphenylhydantoin and their relationship to clinical intoxication. *Epilepsia*, 14, 177-184.

Borg, D.C. (1965) Transient free radicals from salicylate. *Biochemical Pharmacology* 14, 627-631.

Borga, O. (1980), Protein binding and therapeutic drug monitoring. *Acta Pharmaceutica Suecica,* 17, 89.

Borga, O., Azarnoff, D.L. and Sjoqvist, F. (1968) Species differences in the plasma protein binding of desipramine. *Journal Pharmacy Pharmacology,* 20, 571-572.

Borgeat, P. and Samuelsson, B. (1979) Arachidonic acid metabolism in polymorphonuclear leukocytes: effect of the ionophore A23187. *Proceedings of the National Academy of Sciences* U.S.A., 76, 2148-2152.

Bottoms, G.D., Fessler, J.F., Roesel, O.F., Moore, A.B. and Frauenfelder, H.C. (1981). Endotoxic induced hemodynamic changes in ponies: effects of flunixin meglumine. *American Journal of Veterinary Research,* 42, 1514-2518.

Bottoms, G.D., Johnson, M.A. and Roesel, O.F. (1983). Endotoxin-induced hemodynamic changes in dogs: Role of thromboxane and prostaglandin I_2. *American Journal of Veterinary Research,* 44, 1497-1500.

Bowman, W.C. and Rand, M.J. (1984) Local hormones and autocoids: Biogenic amines, purines, kinins and prostaglandins. In *Textbook of Pharmacology.* 2nd Edn , Blackwell Scientific Publications,.12.1-12.41.

Bray. M.A., Cunningham, F.M., Ford-Hutchinson, A.W. and Smith, M.J.H. (1981) Leukotriene B_4: a mediator of vascular permeability. *British Journal of Pharmacology* , 72, 483-486.

Brodie, D.A., Cook, P.G., Bauer, B.J. and Dagle, G.E. (1970) Indomethacin induced intestinal lesions in the rat. *Toxicology and Applied Pharmacology* 17(3), 615-624.

Broekman, M.J. (1986) Stimulated platelets release equivalent amounts of arachidonate from phosphatidylcholine, phosphatidylethanolamine and inositides. *Journal of Lipid Research* 27, 884-891.

Brogden, R.N. Heel, R.C. and Speight, T.M. (1979) Naproxen up to date: A review of its pharmacological properties and therapeutic efficacy in rheumatic diseases and pain states. *Drugs,* 18, 241-277.

Brogden, R.N., Heel, R.C., Speight, T.M. and Avery, G.S. (1981) Piroxicam: A review of its pharmacological properties and therapeutic efficacy. *Drugs*, 22, 165-187.

Brown, J.R. and Shockley, P. (1982) Serum albumin: structure and characterisation of its ligand binding sites. In *Lipid-Protein Interactions.* Vol 1. Ed P.C. Jost and O.H. Griffith, Wiley and Son Inc., New York, USA. 25-68.

Brune, K and Lanz, R. (1985) Pharmacokinetics of non-steroidal anti-inflammatory drugs. In *Handbook of Inflammation Vol 5: The Pharmacology of Inflammation.* Ed I.L. Bonta, M.A. Bray, and M.J. Parnham, Elsevier, Oxford, 413-449.

Brune, K. (1974) How aspirin might work : a pharmacokinetic approach. *Agents Actions* 4, 230-232.

Brune, K., Glatt, M., and Graf, P. (1976) Mechanism of action of anti-inflammatory drugs. *General Pharmacology* 7, 27-33.

Buchanan, W.W., Rooney, P.J. and Rennie, J.A.N. (1983) Aspirin and the Salicylates. In *Anti-Rheumatic Drugs*, Ed. E.C. Huskisson, Praeger Publishers, London, 55-95.

Burch, R.M., Luini, A. and Axelrod, J. (1986) Phospholipase A_2 and phospholipase C are activated by distinct GTP-binding proteins in response to alpha$_1$ adrenergic stimulation in FRTL5 thyroid cells. *Proceedings of the National Academy of Sciences* USA, 83, 7201-7205.

Burgoyne, R., Cheek, T.R. and O'Sullivan, A.J. (1987) Receptor activation of phospholipase A_2 in cellular signalling. *Trends in Biochemical Science* 12, 332-333.

Carty, T.J., Eskra, J.D., Lombardino, J.H. and Hoffman, W.W. (1980b) Piroxicam, a potent inhibitor of prostaglandin production in cell culture. Structure activity study. *Prostaglandins*, 19, 671-682.

Carty, T.J., Stevens, J.S., Lombardino, J.G., Parry, M.J. and Randall, M.J. (1980a) Piroxicam, a structurally novel anti-inflammatory compound. Mode of action of prostaglandin synthesis inhibition. *Prostaglandins*, 19, 671-682.

Chamouard, J.M., Barre, J., Urien, G., Houin, G. and Tillement, J.P. (1985) Diclofenac binding to albumin and lipoproteins in human serum. *Biochemical Pharmacology*, 34, 1695-1700.

Chay, S., Nugent, T. and Tobin, (1982) The pharmacology of non-steroidal anti-inflammatory drugs in the horse: flunixin meglumine (Banamine). *Equine Practice*, 4, 16-23.

Chignell, C.F. (1977) Protein Binding. In *Drug Fate and Metabolism, Methods and Techniques* Vol 1. Ed. E.R. Garret and L. Hirz, Marcel Dekker Inc., New York,USA. 76-84.

Chignell, C.F. and Starkweather, D.K. (1971) Optical studies of drug-protein complexes V. The interactions of phenylbutazone, flufenamic acid and dicoumarol with acetylsalicylic acid-treated human serum albumin. *Molecular Pharmacology*, 7, 229-237.

Churchill, F.H. and Manshardt, D.O. (1933) Experimental production of gastric and duodenal ulcers in dogs in cinchophen poisoning. *Proceedings of the Society of Experimental Biology and Medicine*, 30, 825-827.

Cifone, M.G., Alesse, E., Ianni, G. Continenza, M.A. and Conti, P. (1982) Actions of some salicylates associated with other non-steroidal anti-inflammatory drugs on the release of prostacyclin from gastric mucosa of the rat. *Bollettino Societa Italiana di Biologica Sperimentale*, 58, 1666-1671.

Classen, M., Koch, H., Bickhardt, J., Topf, G. and Demling, L. (1971) The effect of prostaglandin E_1 on the pentagastrin stimulated gastric secretion in man. *Digestion* 4(6), 333-344.

Cohnheim, J. (1867) Uber Entzundung und Eiterung. *Virchows Archive fuer Pathologische Anatomie* 40, 1-79.

Cohnheim, J. (1882) Lectures on general pathology, 2nd Ed. (translated from 2nd German Ed.) Vol. 1, *The New Sydenham Society*, London, 1889.

Collier, H.O.J. and Sweatman, W.J.F. (1968) Antagonism by fenamates of prostaglandin F_{2alpha} and of slow-reacting substance on human bronchial muscle. *Nature*, 219, 864-865.

Cooke, A.R. (1976) The role of the mucosal barrier in drug-induced gastric ulceration and erosion. *American Journal of Digestive Diseases* 21, 155-164.

Counsell, R.E. and Pohland, R.C. (1982) Lipoproteins as a potential site-specific system for diagnostic and therapeutic agents. *Journal de Medicine de Caen*, 25, 1115-1120.

Cowan, D.H. (1973) Thrombokinetic studies in alcohol-related thrombocytopenia. *Journal Laboratory and Clinical Medicine*, 81, 64-76.

Cowan, D.H., and Hines, J.D. (1971) Thrombocytopenia of severe alcoholism. *Annals of Internal Medicine*, 74, 37- 43.

Cushman, S.W. (1970) Structure-function relationships in the adipose cell II pinocytosis and factors influencing its activity in the isolated adipose cell. *Journal of Cell Biology*, 46, 342-353.

D'Arcy, P.F. and McElnay, J.C. (1982) Drug interactions involving the displacement of drugs from plasma protein and tissue binding sites. *Pharmacology and Therapeutics*, 17, 211-220.

D'Arcy, P.F. and McElnay, J.C. (1985) Plasma protein binding and drug interactions. In *Protein Binding and Drug Transport.*, Ed J. Tillement, and E. Lindenlaub, Symposium Medica Hoechst 20. Algarve, Portugal. 199-206.

Daniel, L.W., Waite, M. and Wykle, R.L., (1986) A novel mechanism of diglyceride formation. *Journal of Biological Chemistry* 261, 9128-9132.

Danon, A. and Assouline, G. (1978) Inhibition of prostaglandin biosynthesis by corticosteroids requires RNA and protein synthesis. *Nature*, 273, 552-554.

Davies, D.M. (1985) Blood disorders. In *Textbook of Adverse Drug Reactions*, Ed Davies, D.M, 3rd Edn., Oxford University Press, Oxford, 578-594.

Davies, P. and Allison, A.C. (1976) Secretion of macrophage enzymes in relation to the pathogenesis of chronic inflammation. In *Immunology of the Macrophage*, Ed Nelson, D.S., Academic Press, New York, 427-461.

Dawson, J., Lees, P. and Sedgwick, A.D. (1987). Actions of non-steroidal anti-inflammatory drugs on equine leucocyte movement in vitro. *Journal of Veterinary Pharmacology and Therapeutics*, 10, 150-159.

Dawson, W. and Willoughby, D.A. (1985) Inflammation - mechanisms and mediators. In *Non-steroidal Anti-inflammatory Drugs*, Ed. Lombardino, J.G., Wiley-Interscience Publications, New York, USA, 75-109.

Delbarre, F., Roucayrol, J.C., Amor, B., Ingrand, J., Bourat, G., Fournel, J. and Courjaret, J. (1976) Pharmacokinetic study of ketoprofen in man using the tritiated compound. *Scandanavian Journal of Rheumatology* (Supplement), 14, 45.

Dewey, W.C. (1959) Vascular - extravascular exchange of I^{131} plasma proteins in the rat. *American Journal of Physiology*, 197, 423-431.

Di Rosa, M., Giroud, J.P. and Willoughby, D.A. (1971a) Studies on the mediators of acute inflammatory response induced in rats at different sites by carrageenan and turpentine. *Journal of Pathology*, 104, 15-29.

Di Rosa, M., Papadimitriou, J.M. and Willoughby, D. (1971b) A histopathological and pharmacological analysis of the mode of action of non-steroidal anti-inflammatory drugs. *Journal of Pathology*, 105, 239-256.

Dobner, O. and Gieseke, M. (1887) Uber a phenylcinchonins aure und ihre homologen. *Annali di Chimica*, 242, 290-300.

Domenjoz, R. (1960) The pharmacology of phenylbutazone analogues Annals of New York Academy of Science, 86, 263 - 291.

Doolittle, R.F.(1979) Protein evolution. In *The Proteins*, Vol IV, Ed H. Neurat, R.L. Hill and C. Boeder, Academic Press, London. 2-118.

Duggan, A.W. (1983) Electrophysiology of opioid peptides and sensing systems. *British Medical Bulletin* ,39, 65-70.

Duggan, D.E., Hooke, K.F., Noll, R.M. and Kwan, K.C. (1975) Enterohepatic circulation of indomethacin and its role in intestinal irritation. *Biochemical Pharmacology*, 25, 1749-1754.

Dupont, P.D., Dayer, P., Balant, L. and Fabre, G. (1982) Variations inter-et intraindividuelles du comportment du piroxicam. Pharmacocinetique chez l'homme enbonne sante et chez le malade en insuffisance renale. *Pharmaceutica Acta Helvetiae*, 57(1), 20-26.

Edelson, H.S., Kaplan, H.B., Korchak, H.M., Smolen, J.E. and Weissman, G. (1982) Dissociation by piroxicam of degranulation and superoxide anion generation from decrements in chlortetracycline fluorescence activated human neutriphils. *Biochemical Biophysical Research Communications*, 104, 247-253.

Egan, R.W., Humes, J.L. and Kuehl, F.A. (1978) Differential effects of prostaglandin synthesis stimulators on inhibition of cyclooxygenase. *Biochemistry*, 17, 2230-2234.

Ehrnebo, M (1980) Influence of drug binding to blood cells on pharmacokinetics. *Acta Pharmaceutica Suecica*, 17, 81-82.

Ehrnebo, M (1985) Drug binding to erythrocytes. In *Protein Binding and Drug Transport*. Ed J. Tillement and E. Lindenlaub, Symposium Medica Hoechst 20. Algarve, Portugal. 49-57.

Ehrnebo, M. and Odar-Cederlof, I. (1977) Distribution of pentobarbital and diphenylhydantoin between plasma and cells in blood. Effect of salicylic acid, temperature and total drug concentration. *European Journal of Clinical Pharmacology*, 11, 37-42.

Espinasse, J., Schelcher, F. and Valarcher, J-F. (1992) Flunixin meglumine in bovine therapy. In *International Symposium on the Use of Non-Steroidal Anti-inflammatory Drugs in Domesticated Animals*. Nurnberg, Germany, 37-52.

Erill, S. and Calvo, R. (1985) Qualitative changes of proteins as a cause of altered drug binding in serum. In *Protein Binding and Drug Transport*. Ed J. Tillement and E. Lindenlaub, Symposium Medica Hoechst 20. Algarve, Portugal. 337-348.

Esteve A., Martinez, L., Roser, R. and Sagarra, R. (1986) Pharmacokinetics of droxicam in the rat and dog. *Methods and Findings in Experimental Clinical Pharmacology*, 8, 423-429.

Evans, A.M., Nation, R.L., Sansom, L.N., Bochner, F. and Somogyi, A.A. (1989) Sterioselective plasma protein binding in ibuprofen enantiomers. *European Journal of Clinical Pharmacology*, 36, 283-290.

Evans, G.H., Nies, A.S. and Shand, D.S. (1973) The disposition of propranolol. III Decreased half life and volume of distribution as a result of plasma binding in man, monkey, dog and rat. *Journal of Pharmacology and Experimental Therapeutics*, 186, 114-122.

Farr, R.S., Reid, R.T. and Minden, P. (1966) Spontaneous and induced alteration in the anion-binding properties of human albumin. *Journal of Clinical Investigation*, 45, 1006.

Fehske, K.J., Muller, W.E. and Wollert, U. (1981) The location of binding sites on human serum albumin. *Biochemical Pharmacology*, 30, 687-692.

Ferreira, S.H. (1972) Prostaglandins, aspirin like drugs and analgesia. *Nature* 240, 200-203.

Ferreira, S.H., Moncada, S. and Vane, J.R. (1971) Indomethacin and aspirin abolish prostaglandin release from the spleen. *Nature New Biology* 231(25), 237-239.

Ferreira, S.H., Moncada, S., Parsons, M. and Vane, J.R. (1974) Proceedings : The concomitant release of bradykinin and prostaglandin in the inflammatory response to carrageenin. *British Journal of Pharmacology* 52(1),108-109.

Fessler, J.F., Bottoms, G.D., Roesel, O.F., Moore, A.B., Fravenfelder, H.C. and Boon, G.D. (1982). Endotoxin-induced changes in hemograms, plasma enzymes and blood chemical values in anaesthetised ponies: effects of flunixin meglumine. *American Journal of Veterinary Research*, 43, 140-144.

Fichtl, B. and Schuhmann, G. (1985) Relationships between plasma and tissue-binding of drugs. In *Protein Binding and Drug Transport.* Ed J. Tillement and E. Lindenlaub, Symposium Medica Hoechst 20. Algarve, Portugal. 255-271.

Finstad, R. (1981) A double-blind crossover multicentre study of piroxicam and indomethacin in the treatment of rheumatoid arthritis. *British Journal of Clinical Practice*, 35, 35-39.

Fitzgerald, G.A. Pedersen, A.K. and Patrono, C. (1983) Analysis of prostacyclin and thromboxane biosynthesis in cardiovascular disease. *Circulation*, 67, 1174-1177.

Fitzpatrick, F.A., Gorman, R.R., McGuire, J.C., Kelly, R.C., Wynalda, M.A. and Sun, F.F. (1977) A radioimmunoassay for thromboxane B_2. *Analytical Biochemistry*, 82, 1-7.

Flower, R.J. and Blackwell, G.J. (1979) Anti-inflammatory steroids induce biosynthesis of phospholipase A inhibitor which prevents prostaglandin generation. *Nature* 278, 456-459.

Flower, R.J. and Vane, J.R. (1972) Inhibition of prostaglandin synthetase in brain explains the antipyretic activity of paracetamol (4-acetaminophen) *Nature New Biology*, 240, 410-411.

Flower, R.J. and Vane, J.R. (1974) Some pharmacologic and biochemical aspects of prostaglandin biosynthesis and its inhibition. In *Prostaglandin Synthetase Inhibitors*, Ed H.J. Robinson, and J.R. Vane, Raven Press, New York,USA. 9-31.

Flower, R.J., Gryglewski, R., Herbaczynska-Cedro, K. and Vane, J.R. (1972) The effects of anti-inflammatory drugs on prostaglandin biosynthesis. *Nature New Biology*, 238, 104-106.

Flower, R.J., Moncada, S. and Vane, J.R. (1985) Analgesic-antipyretics and anti-inflammatory agents: drugs employed in the treatment of gout. In *The Pharmacological Basis of Therapeutics*, 7th Edn., Eds. A.G. Goodman, L.S. Gilman, T.W.Rall and F. Murad, MacMillan, New York, 674-715.

Frey, H.H. and Rieh, B. (1981) Pharmacokinetics of naproxen in the dog. *American Journal of Veterinary Research* 42, 1615-1617.

Fruchtmann, R., Krupka, U. and Pelster, B. (1984). Hemmung des lipoxygenase und cyclooxygenaseweges im arachidonsaure -metabolismus durch etofenamat. *Aktuelle Rheumatologie*, 9, 165-168.

Fuerst, K. (1925) Verminderung der entzundungs bereitschaft durch. *Archiv fur Experimentelle Pathologie und Pharmakologie*, 195, 238-248.

Fuller, R.W. (1972) Selective inhibition of monoamine oxidase. In *Monomamine Oxidase- New Vistas*, Ed E. Costa and M. Sandler, Raven Press, New York,USA. 339-354.

Gallus, A.S. (1979) Antiplatelet drugs: clinical pharmacology and therapeutic use. *Drugs* 18, 439-477.

Gana, T.J., Huhlewych, R. and Koo, J. (1987) Focal gastric mucosal blood flow in aspirin induced ulceration. *Annals of Surgery*. 205, 399-403.

Gaut, Z.N. Baruth, H. Randall, L.O., Ashley, C. and Paulsrud, J.R. (1975) Sterioisomeric relationships among anti-inflammatory activity, inhibition of platelet aggregation and inhibition of prostaglandin synthesis. *Prostaglandins* 10, 59-66.

Gibaldi, M. (1991). Compartmental and noncompartmental pharmacokinetics. In *Biopharmaceutics and Clinical Pharmacokinetics*. 4th Edn. Leat Febinger, Philidelphia,USA. 14-23

Gibaldi, M. and Perrier, D. (1982) Multicompartment models. In *Pharmacokinetics*. Marcel Dekker Inc., New York, USA. 45-111

Glazko, A.J. (1967) Pharmacology of the fenamates. III. Medical disposition. *Annals of Physical Medicine*, 9, 23-36.

Glazko, A.J., Chang, T., Borondy, P.E., Dill, W.A., Young, R. and Croskey, L. (1978) Metabolic disposition of meclofenamic acid (Meclomen) in laboratory animals and in man. *Current Therapeutic Research* 23, S22.

Glenn, E.M., Bowman, B.J. and Rohloff, N.A. (1979) Anomalous biological effects of salicylates and prostaglandins. *Agents Actions* 9, 257-264.

Goldie, I. (1981) Piroxicam and naproxen in osteoarthritis: a clinical comparison. *European Journal of Rheumatology and Inflammation,* 4, 348-356.

Goldstein, I.M. and Weissmann, G. (1974) Generation of C5 - derived lysosomal releasing-enzyme activity (C5a) by lysates of leukocyte lysosomes. *Journal of Immunology* 113, 1583-1588.

Gorman, R. (1982) Mechanism of action of prostacyclin and thromboxane A_2. In *Prostaglandins in Clinical Medicine - Cardiovascular and Thrombotic Disorders.* Ed K.K. Wu and E.C. Rossi, Year Book Medical Publishers, Chicago, USA. 21-34.

Gould, S.R. (1976) Assay of prostaglandin like substances in faeces and their measurement in ulcerative colitis. *Prostaglandins* II, 489-497.

Graham, G.G., Chamption, G.D., Day, R.O. and Paull, P.D. (1977) Patterns of Plasma Concentrations and Urinary Excretion of Salicylates in Rheumatoid Arthritis. *Clinical Pharmacology and Therapeutics,* 22, 410-420.

Granger, D.N., Benoit, J.N., Suzuki, M. and Grisham, M.B. (1989) Leukocyte adherence to venular endothelium during ischemia-reperfusion. *American Journal of Physiology* 253, G683-688.

Granger, D.N., Rutili, G. and McCord, J.M. (1981) Superoxide radicals in feline intestinal ischaemia. *Gastroenterology* 81, 22 -29.

Granstrom, E., Kindahl, H. and Samuelsson, B. (1976) Radioimmunoassay for thromboxane B_2. *Analytical Letters,* 9, 611-627.

Greaves, M.W., Sondergaard, J. and McDonald-Gibson, W. (1971) Recovery of prostaglandins in human cutaneous inflammation. *British Medical Journal* , 2, 258-260.

Gryglewski, R.J. (1974) Structure -activity relationship of some prostaglandin synthetase inhibitors. In *Prostaglandin Synthetase Inhibitors.* Ed H.J. Robinson and J.R. Vane, Raven Press New York, USA. 33-52.

Hakkarainen H., Vapaatalo, H., Gothoni, G. and Parantainen, J. (1979). Tolfenamic acid is as effective as ergotamine during migraine attacks. *Lancet*, 2, 326-328.

Hallesy, D.W., Shott, L.D., and Hill, R. (1973) Comparative toxicology of naproxen. *Scandinavian Journal of Rheumatology* 2 (Supplement), 20-28.

Hamberg, M., Svensson, J. and Samuelsson, B. (1975) Thromboxanes: A new group of biologically active compounds derived from prostaglandin endoperoxides. *Proceedings of the National Academy of Science (USA)*, 72, 2994-2998.

Hanke, H. (1934) Uber experimentelle akute atophanylgastritis als erscheinungsform einer vorweigeng toxisch bedingen gastritis. *Beitrage zur Pathologie Anatomischer und zur Pathologie*, 94, 313-326.

Hardee, M.M. and Moore, J.N. (1986) Effects of flunixin meglumine, phenylbutazone and a selective thromboxane synthetase inhibitor (UK-38, 485) on thromboxane and prostacycline production in healthy horses. *Research in Veterinary Science*, 40, 152-156.

Hardee, G.E., Smith, J.A. and Harris, S.J. (1985) Pharmacokinetics of flunixin meglumine in the cow. *Research in Veterinary Science*, 39, 110-112.

Hardie, E.M. Kolata, R.J. and Rawlings, C.A. (1983). Canine septic peritonitis: Treatment with flunixin meglumine. *Circulatory Shock*, 11, 159-173.

Hardie E.M., Rawlings, C.A. and Collins, L.G. (1985b), Canine E. coli peritonitis: treatment with flunixin meglumine. *Journal of the American Animal Hospital Association*, 21, 691-699.

Hardie, E.M., Hardee, G.E. and Rawlings, C.A. (1985a) Pharmacokinetics of flunixin meglumine in dogs. *American Journal of Veterinary Research*, 46(1), 235-237.

Hart, D.F. (1983) Corticosteroid therapy in rheumatic disorders. In *Anti-inflammatory Drugs*, Ed E.C. Huskisson. Praeger Publishers, London. 497-507.

Haut, M.J. and Cowan, D.H. (1974) The effect of ethanol on hemostatic properties of human blood platelets. *American Journal of Medicine*, 56, 22-33.

Hawkins, D. (1971) Biopolymer membrane: a model system for the study of neutrophilic leucocyte response to immune complexes. *Journal of Immunology* 107, 344-352.

Hawkins, D., Pinkard, R.N. and Farr, R.S. (1968) Acetylation of human serum albumin by acetylsalicylic acid. *Science*, 160, 780-781.

Hench, P.K.and Farr, R.S. (1967) The alteration of serum albumin by acetylsalicylic acid. *Arthritis and Rheumatism*, 10, 283-284.

Henson, P.M. (1971a) The immunologic release of constituents from neutrophil leucocytes. I The role of antibody and complement on nonphagocytosable surfaces or phagocytosable particles. *Journal of Immunology* 107, 1535-1546.

Henson, P.M. (1971b) Interaction of cells with immune complexes: adherence, release of constituents and tissue injury. *Journal of Experimental Medicine* 134, 114s.

Henson, P.M. (1972) Pathologic mechanisms in neutrophil mediated injury. *American Journal of Pathology* 68, 593-605.

Hernandez, L.A. Grisham, M.B., Twohig, B., Arfors, K.E., Harlan, J.M. and Granger, D.N., (1987) Role of neutrophils in ischemia-reperfusion-induced microvascular injury. *American Journal of Physiology* 253, H699-703.

Higgins, A.J. (1985) The biology, pathophysiology and control of eicosanoids in inflammation. *Journal of Veterinary Pharmacology and Therapeutics* 8, 1-18.

Higgins, A.J. and Lees, P. (1984b) Arachidonic acid metabolites in carrageenin-induced equine inflammatory exudate. *Journal of Veterinary Pharmacology and Therapeutics*, 7, 65-72.

Higgins, A.J. and Lees, P. (1984a) Detection of LTB_4 in equine inflammatory exudate. *The Veterinary Record* 115, 275.

Higgins, A.J., Lees, P. and Higgs, G.A. (1984b) The detection of prostaglandin like activity in equine inflammatory exudate. *Equine Veterinary Journal*, 16, 71-73.

Higgins, A.J., Lees, P. and Sedgwick, A.D. (1987) Development of equine models of inflammation. *The Veterinary Record* 120 (22) (Supplement). 517-522.

Higgins, A.J., Lees, P. and Wright, J.A. (1984a) A tissue cage model for the collection of inflammatory exudate in ponies *Research in Veterinary Science*, 36, 284-289.

Higgins, A.J., Lees, P. Taylor, J.B.O. and Ewins, C.P.(1986) Flunixin meglumine: quantitative determination in and effects on composition of equine inflammatory exudate. *British Veterinary Journal* 142, 163-169.

Higgs, G.A. and Salmon, J.A. (1979). Cyclooxygenase products in carageenin-induced inflammation. *Prostaglandins*, 17, 737-746.

Higgs, G.A., Bax, C.M. and Moncada, S. (1981b) The inflammatory properties of lipoxygenase products and the effects of indomethacin and BW755C on prostaglandin production, leukocyte migration and plasma exudation in rabbit skin. In *Leukotrienes and Other Lipoxygenase Products*, Eds. B. Samuelsson and R. Paoletti, Raven Press, New York, 331-339.

Higgs, G.A., Bunting, S., Moncada, S. and Vane, J.R. (1976) Polymorphonuclear leukocytes produce thromboxane A_2 like activity during phagocytosis. *Prostaglandins* 12, 749-757.

Higgs, G.A., Copp, F.C., Denyer, C.V., Flower, R.J., Tateson, J.E., Vane, J.R. and Walker, J.M.G. (1978) Reduction of leukocyte migration by cyclooxygenase and lipoxygenase inhibitors. *Proceedings of the International Congress of Pharmacology* (VII), Paris, France, 334, no 843.

Higgs, G.A., Eakins, K.E., Mugridge, K.G., Moncada, S. and Vane, J.R. (1980) The effects of non-steroidal anti-inflammatory drugs on leukocyte migration in carageenin induced inflammation. *European Journal of Pharmacology* 66, 81-86.

Higgs, G.A., Flower, R.J. and Vane, J.R. (1979) A new approach to anti-inflammatory drugs. *Biochemical Pharmacology* 28(2), 1959-1961.

Higgs, G.A., McCall, M.E. and Youlten, L.J.F. (1975) A chemotactic role for prostaglandin released from polymorphonuclear leukocytes during phagocytosis. *Journal of Pharmacology* 53, 539-546.

Higgs, G.A., Moncada, S., Salmon, J.A. and Seager, K. (1983) The source of thromboxane and prostaglandin in experimental inflammation. *British Journal of Pharmacology* 79, 863-868.

Higgs, G.A., Salmon, J.A. and Spayne, J.A. (1981a) The inflammatory effects of hydroperoxy and hydroxy acid products of arachidonate lipoxygenase in rabbit skin. *British Journal of Pharmacology* 74, 429-433.

Hirsch, J., Street, D. Cade, J.F. and Amy, H. (1973) Relation between bleeding time and platelet connective tissue reaction after aspirin. *Blood*, 41, 369-377.

Hla, T. and Neilson, K. (1992) Human cyclooxygenase-2 cDNA. *Proceedings of the National Academy of Sciences*, (USA), 89, 7384-7388.

Hobbs, D.C. and Twomey, T.M. (1979) Piroxicam pharmacokinetics in man: aspirin and antacid interaction studies. *Journal of Clinical Pharmacology*, 19, 270-281.

Hong, S.L. and Deykin, D., (1981) The activation of phosphatidylinositol-hydrolyzing phospholipase A_2 during prostaglandin synthesis in transformed mouse BALB/3T3 cells. *Journal of Biological Chemistry* 256, 5215-5219.

Hong, S.L. and Levine, L. (1976a) Stimulation of prostaglandin synthesis by bradykinin and thrombin and their mechanisms of action on MC5-5 fibroblasts. *Journal of Biological Chemistry* 251, 5814-5816.

Hong, S.L. and Levine, L., (1976b) Inhibition of arachidonic acid release from cells as the biochemical action of anti-inflammatory corticosteroids. *Proceedings of the National Academy of Sciences* USA, 73, 1730-1734.

Hook, J.B. (1971) Fluoride and methoxyfurane nephropathy. *Anesthesiology*, 35, 238-240.

Horovitz, Z.P., Beer, B., Coldy, D.E., Vogel, J.R. and Chasin, M. (1972) Cyclic AMP and anxiety. *Psychosomatics*, 13, 85-92.

Houdeshell, J.W. and Hennessey, P.W. (1977) A new non-steroidal, anti-inflammatory analgesic for horses. *Journal of Equine Medicine and Surgery* 1, 57-63.

Houin, G. (1985) Drug binding and apparent volume of distribution. In *Protein Binding and Drug Transport.* Ed J. Tillement, and E. Lindenlaub, Symposium Medica Hoechst 20. Algarve, Portugal. 213-226.

Hucker, H.B., Zacchei, A.G., Cox, S.V., Brodie, D.A. and Cantwell, N.H.R. (1966) Studies on the absorption, distribution and excretion of indomethacin invarious species. *Journal of Pharmacology and Experimental Therapeutics.* 153, 237-249.

Hueper, W.C. (1948) Cinchophen (Atophan): A critical review. *Medicine,* 27(1), 43-103.

Humes, J.L., Bonney, R.J., Pelus, L. Dahlgren, M.E., Sadowski, S.J., Keuhl, F.A. and Davies, P. (1977) Macrophages synthesise and release prostaglandins in response to inflammatory stimuli. *Nature,* 269, 149-151.

Hurley, J.V. (1978) The sequence of early events in inflammation. In *Handbook of Experimental Pharmacology* Vol. 50/I. Inflammation, Ed. J.R. Vane and S.H. Ferreira, Springer-Verlag, New York, 26-67.

Ishizaka, T., Sasaki, T., Suganuma, T., Horai, Y., Chiba, K., Watanabe, M., Asuke, W. and Hoshi, H. (1980) Pharmacokinetics of ketoprofen following single oral, intramuscular and rectal doses and after repeat oral administration. *European Journal of Clinical Pharmacology,* 18, 407-414.

Ivy, A.C., Nelson, D. and Bucher, G. (1941) The standardisation of certain factors in the cutaneous venostasis bleeding time technique. *Journal Laboratory and Clinical Medicine,* 26, 1812-1822.

Jain, A.K., McMahon, F.G., Ryan, J.R., Raphan, H. and Richard, W. (1978) Piroxicam, a novel analgesic in post partum pain. *European Journal of Rheumatology and Inflammation,* 1, 356-359.

Jamieson J.C. and Ashton, F.E. (1973) Studies on acute phase proteins of rat serum 3: Site of synthesis of albumin and alpha-1-acid glycoprotein and the contents of these

proteins in liver microsome fractions from rats suffering from induced inflammation. *Canadian Journal of Biochemistry*, 51, 1034-1045.

Jaussaud, P., Bellon, C., Besse, S, Courtot, D. and Delatour, P. (1993) Enantioselective pharmacokinetics of ketoprofen in horses. *Journal of Veterinary Pharmacology and Therapeutics*, 16, 373-376.

Jeffries, G.H., Halstead, M.R.C.P., Holman, H.R. and Sleisenger, M.H. (1962) Plasma proteins and the gastrointestinal tract. *New England Journal of Medicine*, 266, 652-660.

Johnson, L.R. (1966) Histamine liberation by gastric mucus of pylorus-ligated rats damaged by acetic or salicylic acid. *Proceedings of the Society for Experimental Biology and Medicine*. 121, 384-386.

Johnson, L.R. and Overholt, B.F. (1967) Release of histamine into gastric venous blood following injury by acetic or salicylic acid. *Gastroenterology* 52, 505-509.

Judson, D.G. and Barton, M.P. (1981) Effect of aspirin on haemostatis in the horse. *Research in Veterinary Science*, 30, 241-242.

Joulou, L., Guyonnet, J.C., Ducrot, R., Fournel, J. and Pasquet, J. (1976) Ketoprofen: main pharmacological properties - outline of toxicological and pharmacokinetic data. *Scandanavian Journal of Rheumatology* (Supplement) 14, 33-44.

Jusko, W.J. and Gretch, M. (1976) Plasma and tissue binding of drugs in pharmacokinetics. *Drug Metabolism Reviews*, 5, 43-140.

Juul, J. (1965) Acute poisoning with butazolidine (phenylbutazone). *Acta Pediatrica Scandanavia*, 54, 503-507.

Kaldestad, E., Hansen, T. and Brath, H.K. (1975) Interaction of indomethacin and acetylsalicylic acid as shown by the serum concentration of indomethacin and salicylate. *European Journal of Clinical Pharmacology*, 9, 199-207.

Kandasamy, B., Giroult, J.M. and Jacob, J. (1975) Central effects of a purified bacterial pyrogen, prostaglandin E_1 and biogenic amines on the awake rabbit. In

Temperature Regulations and Drug Action, Eds. Lomax, J., Schonbaum, E. and Jacob, J., S. Karger, Basel, 124-132

Kandil, A., Gaber, A., Fayez, M. and El-Ashmaury, H. (1980), Study on anti-inflammatory potential of piroxicam. *Journal Drug Research Egypt,* 12, 139-146.

Kaneko, J.J. (1989) Serum proteins and the dysproteinemias. In *Clinical Biochemistry of Domestic Animal Species,* Ed J.J. Kaneko, Academic Press, California, USA 142-165.

Kajander, A, Laine, V. and Gothoni, G. (1972) Effect of tolfenamic acid in rheumatoid arthritis. *Scandanavian Journal of Rheumatology,* 1, 91-96.

Kankaanranta, H., Moilanen, E. and Vapaatalo, H. (1993) Comparison of in vitro effects of flunixin and tolfenamic acid on human leukocyte and platelet function. *Inflammation,* 17(4), 417-425.

Kaplan, H., Edelson, H. Korchak, H., Given, W., Abramson, S. and Weissmann, G. (1984) Effects of non-steroidal anti-inflammatory agents on human neutrophil functions in vitro and in vivo. *Biochemical Pharmacology* 33, 371-378.

Kauppila, A. and Ylikorkala, O (1977) Indomethacin and tolfenamic acid in primary dysmenorrhea. *European Journal of Obstetrics,Gynacology and Reproductive Biology,* 7, 59-64.

Kaya, H., Patten, G.M. and Hong, S.L. (1989) Bradykinin induced activation of phospholipase A_2 is independent of the activation of polyphosphoinositide-hydrolising phospholipase C. *Journal of Biological Chemistry.* 264(9), 4972-4977.

Keller, H.U. and Borel, J.F. (1971) Chemotaxis of phagocytes., *Advances in Experimental Medicine and Biology,* 15, 53-58.

Kelly, M.J. and Benitz, A.M. (1988) The use of flunixin meglumine in dogs: the result of clinical trials. In *Proceedings of the World Small Animal Veterinary Association Congress,* Barcelona, Spain, 21-27.

Kilpatrick, C.J., Wanwimolruk, S. and Wing, L.M.H. (1984) Plasma concentrations of unbound phenytoin in the management of epilepsy. *British Journal of Pharmacology*, 17, 539-546.

Kitahora, T. and Guth, P.H. (1987) Effects of aspirin plus hydrochloric acid on the gastric mucosal microcirculation. *Gastroenterology* 93, 810-817.

Kitchen, E.A., Dawson, W., Rainsford, K.D. and Cawson, T. (1985) Inflammation and possible modes of action of anti-inflammatory drugs. In *Anti-inflammatory and Anti-rheumatic Drugs*, Vol.I. Newer Anti-inflammatory Drugs. Ed K.D. Rainsford, CRC Press, Inc. Florida, 21-87.

Kivilaasko, E and Silen, W. (1979) Pathogenesis of experimental gastric-mucosal injury, *New England Journal of Medicine*, 301, 364-369.

Knadler, M.P., Brater, D.C. and Hall, S.D. (1989) Plasma protein binding of fluribiprofen: enantioselectivity and influence of pathophysiological status. *Journal of Pharmacology and Experimental Therapeutics*, 249, 378-385.

Konturek, S.J., Piastucki, I., Brzozowski, I, Radecki,T. Dembinska-Kiec, A., Zmuda, A. and Gryglewski, R. (1981) Role of prostaglandins in the formation of aspirin - induced gastric ulcers. *Gastroenterology* 80, 4-9.

Kopp, K.J., Moore, J.N., Byars, T.D. and Brooks, P. (1985) Template bleeding time and thromboxane generation in the horse: Effects of three non-steroidal anti-inflammatory drugs. *Equine Veterinary Journal*, 17, 322-324.

Koren, G., Silverman, E., Sundel, R., Edney, P., Newburger, J.W., Klein, J., Robieux, I., Laxer, R., Giesbrecht, E. and Burns, J.C. (1991) Decreased binding of salicylates in Kawasaki disease. *Journal of Pediatrics*, 118(3), 456-459.

Kuninaka, T., Sugai, K., Saito, T. Mori, N., Kimura, R., and Murata, T. (1981). Studies on the metabolism of N-(3-chloroi-2-methylphenyl) anthranilic acid (GEA6414) a new anti-inflammatory agent. I Urinary metabolites of GEA6414 in humans, dogs, rabbits and rats., *Chemical Abstracts* 95, 168X.

Kurz, H. and Fichtl, B. (1983) Binding of drugs to tissues. *Drug Metabolism Reviews*, 14, 467-510.

Lambert, T.L., Kent, R.S. and Whorton, A.R. (1986) Bradykinin stimulation of inositol polyphosphate production in porcine aortic endothelial cells. *Journal of Biological Chemistry* 261, 15288-15293.

Larson, D.L. and Lombardino, J.D. (1980) The topical anti-inflammatory effects of piroxicam in rodents. *Agents Actions*, 10, 246-251.

Lee, P., Anderson, J.A. and Buchanan, W.W. (1976) The state of current non-steroidal anti-rheumatic drugs. *Australian and New Zealand Journal of Medicine*, 6, 173.

Lees, P. and Higgins, A.J. (1984) Flunixin inhibits prostaglandin E_2 production in equine inflammation. *Research in Veterinary Science*, 37, 347-349.

Lees P., and Higgins, A.J. (1985) Clinical pharmacology and therapeutic uses of non-steroidal anti-inflammatory drugs in the horse. *Equine Veterinary Journal* 17, 83-96.

Lees, P. Ewins, C.P., Taylor, J.B. and Sedgwick, A.D. (1987). Serum thromboxane in the horse and its inhibition by aspirin, phenylbutazone and flunixin. *British Veterinary Journal*, 143, 462-476.

Lees, P., May, S.A. and McKellar, Q.A. (1991) Pharmacology and therapeutics of non-steroidal anti-inflammatory drugs in the dog and cat: 1 General pharmacology. *Journal of Small Animal Practice* 32, 183-193.

Lees, P., Matho, T.E., Michell, A.R. and Taylor, J.B. (1983) Pharmacokinetics of phenylbutazone in Welsh mountain ponies. *AVCPT Proceedings*, 7, 32-37.

Lees, P., Sedgwick, A.D., Higgins, A.J. and Taylor, J.B.O. (1986). Role and control of eicosanoid in acute non-immune inflammation in the equine. *New Perspectives in Veterinary Pharmacology and Therapeutics; Fifth Biennial Symposium of the American Academy of Veterinary Pharmacology and Therapeutics*, 43-62.

Lehmann, V.W., Wintzer, H.J. and Frey, H.M. (1981). Kinetik einiger analgetisch-antiinflammatorischer arneimittel in serum und synovia bein pferd. *Deutsch Tierarztliche Wochenschrift*, 88, 218-220.

Lemaire, M. and Tillement, J.P. (1982) Role of lipoproteins and erythrocytes in the in vitro binding and distribution of cyclosporin A in the blood. *Journal of Pharmacy and Pharmacology*, 34, 715-718.

Levy, G. (1980) Clinical implications of inter-individual differences of plasma protein binding of drugs. *Acta Pharmaceutica Sueica*, 17, 92-94.

Lewis, G.P. and Piper, P.J. (1975) Inhibition of release of prostaglandins as an explanation of some of the actions of anti-inflammatory corticosteroids. *Nature* 254, 308-311.

Lewis, T. and Grant, R.T. (1924). Vascular reactions of skin to injury. *Heart Bulletin* 11, 209-265.

Lichtman, S.S. (1931) Cinchophen oxidation test of function of hepatic cells. *Archives of International Medicine*, 48, 98-125

Linden, I.B., Parantainen, J. and Vapaatalo, H. (1975). Inhibitory effects of tolfenamic acid, indomethacin and acetylsalicylic acid on prostaglandin synthetase and phosphodiesterase in vitro. In *Sixth International Congress of Pharmacology,* Helsinki, Finland July 20-25, Abstract 594.

Linden, I.B., Parantainen, J. and Vapaatalo, H. (1976) Inhibition of prostaglandin biosynthesis by tolfenamic acid in vitro. *Scandanavian Journal of Rheumatology*, 5, 129-132.

Lindenbaum J. and Hargrove R.L. (1968) Thrombocytopenia in alcoholics. *Annals of International Medicine*, 68, 526-532.

Lindenbaum, J. and Leiber, C.R. (1969) Hematologic effects of alcohol in man in the absence of nutritional deficiency. *New England Journal of Medicine*, 281, 333-338

Maeda, M., Tanaka, Y., Suzuki, T. and Nakamura, K. (1977) Pharmacological studies on carprofen - a new non-steroidal anti-inflammatory drug in animals. *Folia Pharmacologica Japonica* 73, 757-777.

Mahley, R.W., Weisgraber, K.H. and Innerarity, T.L. (1979) Interactions of plasma lipoproteinscontaining apolipoproteins B and E with heparin and cell surface receptors. *Biochimica Biophysica Acta*, 575, 81-89.

Majno, G. and Palade, G.E. (1961) Studies in inflammation I: The effect of histamine and serotonin on vascular permeability: an electron microscopic study. *Journal of Biophysics Biochemistry and Cytology* 11, 571-605.

Malik F. and Wichramasinge S.N. (1986) Haematological abnormalities in mice exposed to ethanol vapour. *British Journal of Pathology*, 67(6), 831-938.

Malseed, R.T. (1990) Narcotic analgesics and antagonists, In *Pharmacology Drug Therapy and Nursing Considerations*, (3rd Edn) Ed R.T Malseed, R.T and S.E. Girton, Lippencott Co, Philadelphia, 155-169.

Mathews, K.A., Doherty,T., Dyson, D.H., Wilcock, B and Valliant, A. (1990) Nephrotoxicity in dogs associated with methoxyflurane anaesthesia and flunixin meglumine analgesia. *Canadian Veterinary Journal* 31, 766-771.

May, S.A., Hooke, R.E. and Lees, P. (1988) The effect of various drugs used in the treatment of equine degenerative joint disease on equine stromelysin. *British Journal of Pharmacology* 93 (Supplement), 281P.

Maylin, G.A. (1974) Disposition of phenylbutazone in the horse., In *Proceedings of the 20th Annual Convention of American Association of Equine Practitioners*, 243-249.

Mburu, D.N. (1991) Evaluation of anti-inflammatory effects of a low dose of acetaminophen following oral surgery in dogs. *Journal of Veterinary Pharmacology and Therapeutics*. 14, 109-111.

Mburu, D.N., Mbugua, S.W., Skoglund, A. and Lokken, P. (1988) Effects of paracetamol and acetylsalicylic acid on the post-operative course after experimental surgery in dogs. *Journal of Veterinary Pharmacology and Therapeutics* 11, 163-171.

McCann, D.S., Tokarsky, J. and Sorkin, R.P. (1981) Radioimmunoassay for plasma thromboxane B_2. *Clinical Chemistry*, 27, 1417-1420.

McCarty D.J. and Hollander, J.L. (1961) Identification of urate crystals in gouty synovial fluid. *Annals of Internal Medicine* 54, 452-460.

McCord, J.M. (1982) Roles of superoxide in inflammation and ischaemic shock. In *Inflammatory Diseases and Copper.*, Ed. J.R.J. Sorenson, Humana Press, New Jersey, 255-262.

McCord, J.M., Stokes, S.H. and Wong, K. (1979.) Superoxide radical as a phygocyte-produced chemical mediator of inflammation., In *Advances in Inflammatory Research.* Vol. I, Ed. G. Weissman, B. Samuelsson and R. Paoletti, Raven Press, New York, 273-280.

McElnay, J.C. and D'Arcy, P.F. (1983) Protein binding displacement interactions and their importance. *Drugs*, 25, 495-513.

McKay, R.J., French, T.W., Nguyen, H.T. and Mathew, I. G. (1983) Effects of large doses of phenylbutazone administration to horses. *American Journal of Veterinary Research* 44(5),774-780.

McKean, M.L., Smith, J.B. and Silver, M.J., (1981) Formation of lysophosphatidylcholine by human platelets in response to thrombin. *Journal of Biological Chemistry* 256, 1522-1524.

McKeller, Q.A., Nolan, A.M. and Galbraith, E.A., (1990). Serum thromboxane generation by platelets in several domestic animal species. *British Veterinary Journal*, 146, 398-404

McKellar, Q.A., Pearson, T., Galbraith, E.A., Boyle,J. and Bell, G. (1991b) Pharmacokinetics and clinical efficacy of a cinchophen and prednisolone combination in the dog. *Journal of Small Animal Practice* 32, 53-58.

McNeil, P.E. (1992) Acute tubulo-interstitial nephritis in a dog after halothane anaesthesia and administration of flunixin meglumine and trimethoprim-sulphadiazine. *Veterinary Record* 131, 148-151.

Meilke, C.H., Kansieshiro, M.M., Maher, I.A., Weiner, J.M. and Rapaport, S.I. (1969) The satndardised normal ivy bleeding time and its prolongation by aspirin. *Blood*, 34, 204-215.

Meloun, B. Moravek, L. and Kostka, V. (1975) The complete amino acid sequence of human serum albumin. *FEBS Letters*, 58, 134-137.

Menasse, R. Hedwall, P.R., Kraetz, J., Pericin, C., Riesterer, L., Sallmann, A., Ziel, R. and Jaques, R. (1978) Pharmacological properties of diclofenac sodium and its metabolites. *Scandinavian Journal of Rheumatology* (Supplement) 22, 5-16.

Menguy, R. (1969) Gastric mucous and the gastric mucous barrier: A review. *American Journal of Surgery* 117(6), 806-812.

Meschter, C.L., Maylin, G.A. and Krook, L. (1984) Vascular pathology in phenylbutazone intoxicated horses. *Cornell Veterinarian* 74, 282-297.

Miller, L.L., Bly, C.G., Watson, M.L. and Bale, W.F. (1951) Dominant role of liver in plasma protein synthesis; direct study of isolated perfused rat liver with acid lysine C^{14}. *Journal of Experimental Medicine*, 94, 431.

Milne, G.M. and Twomey, T.M. (1980) The analgetic properties of piroxicam in animals and correlation with experimentally determined plasma levels. *Agents Actions*, 10, 1-2.

Mitchell, J.R.A. (1983) Clinical aspects of the arachidonic acid-thromboxane pathway. *British Medical Bulletin*, 39, 289-295.

Moilanen, E., Alanko, J., Seppala, E. and Vapaatalo, H. (1988). Effects of antirheumatic drugs on leukotriene B_4 and prostanoid synthesis in human polymorphonuclear leukocytes in vitro. *Agents Actions*, 24, 387-394.

Moncada, S. (1986). Prospects for inhibition of leukotriene biosynthesis. In *The Leukotrienes: Their Biological Significance*, Ed P.J. Piper, Raven Press, New York, USA. 99-107.

Moore, J.N., Garner, H.E., Shapland, J.E. and Hatfield, D.G. (1981). Prevention of endotoxin-induced arterial hypotaemia and lactic acidosis with flunixin meglumine in the conscious pony. *Equine Veterinary Journal*, 13, 95-98.

Morley, J., Bray, M.A., Jones, R.W., Nugeteren, D.H. and Van Dorp, R.W. (1979) Prostaglandin and thromboxane production by human and guinea pig macrophages and leukocytes. *Prostaglandins* 17, 730-736.

Moroney,M.J. (1963) On the average. In *Facts and Figures.* Penguine Books, Middlesex, U.K.. 34-55.

Movat, H.Z., Steinberg, S.G., Habal, F.M., and Ranadive, N.S. (1972) Demonstration of kinin-generating enzyme in the lysosomes of human polymorphonuclear leukocytes. *Laboratory Investigation.* 29, 669-684

Muller, F.O., Hundt, H.K.L. and Muller, D.G. (1977) Pharmacokinetic and pharmacodynamic implications of long term administration of non-steroidal antinflammatory agents. *International Journal of Clinical Pharmacology*, 15, 317-402.

Muller, W.E. and Wollert, U. (1979) Human serum albumin as a 'silent receptor' for drugs and endogenous substances. *Pharmacology*, 19, 59-67.

Muller, W.E., Rick, S. and Brunner, F. (1985) Drug binding to human alpha-1-acid glycoprotein. Focus on a single binding site. In *Protein Binding and Drug Transport* Ed J. Tillement and E. Lindenlaub, Symposium Medica Hoechst 20. Algarve, Portugal. 29-44.

Nash, A. S., Carmichael, S. McKellar, Q.A. and Bennett, D. (1987), Flurbiprofen warning. *The Veterinary Record* 120, 399.

Nevo, Z. and Dorfman, A. (1972) Stimlation of chondromucoprotein synthesis in chondrocytes by extracellular chondromucoprotein. *Proceedings of the National Academy of Science.* USA, 69, 2069-2072.

Nicolairer, A. and Dohrn, M. (1908) Veber die wirkung von chinolincarbonsaure und ihrer derivate auf die ausscheidung der harnsaure. *Deutsches Archiv fur Klinische Medizin,* 93, 331-355.

Nilsen, O.G. (1976) Serum albumin and lipoproteins as the quinidine binding molecules in normal human sera. *Biochemical Pharmacology*, 25, 1007-1012.

Norheim, G., Hoie, R., Froslie, A. and Bergsjo, S. (1978) Gas chromatographic determination of small amounts of phenylbutazone and oxyphenylbutazone in horse plasma and urine. *Fresenius' Zeitschrift fur Analytische Chemie*, 289, 287-288.

Nuotio, P. and Makisara, P.M. (1978) Pharmacokinetic and clinical study of piroxicam. *Proceedings of the Royal Society of Medicine*, 1, 25-30

O'Brien, J.R. (1986) Effect of anti-inflammatory agents on platelets. *Lancet*, 1, 894-895.

Ohtsuki, I., Ishikawa, J. and Tachibana, M. (1981) Pharmacologic activities of the non-steroidal anti-inflammatory drug piroxicam. *Chemical Abstracts*, 94, 18533q.

Oronsky, A.L., Ignarro, L.J. and Perper, R.J. (1973) Release of cartilage mucopolysaccharide-degenerating neutral protease from human leukocytes. *Journal of Experimental Medicine* 138(2), 461-472.

Otterness, I.G., Larson, D.L. and Lombardino, J.G. (1982) An analysis of piroxicam (Feldene) in rodent models of arthritis. *Agents Actions*, 12, 308-312.

Oyanagui, Y. (1976) Participation of superoxide anions at the prostaglandin phase of carageenen foot oedema. *Biochemical Pharmacology* 25, 1465-1472.

Pardridge, W.M. (1985) Transport of plasma protein bound drugs into tissues in vivo. In *Protein Binding and Drug Transport.* Ed J. Tillement and E. Lindenlaub, Symposium Medica Hoechst 20. Algarve, Portugal. 277-292.

Pardridge, W.M. and Landaw, E. (1984) Tracer kinetic model of blood-brain barrier transport of plasma protein bound ligands. Empiric testing of the free hormone hypothesis. *Journal of Clinical Investigation*, 74, 745-752.

Pardridge, W.M., Eisenberg, J., Cefalu, W.T. (1985) Absence of albumin receptor on brain capillaries in vivo or in vitro. *American Journal of Physiology*, 249, E264-E267.

Parks, D.A., Bulkley, G.B., Granger, D.N., Hamilton, S.R. and McCord, J.M. (1982) Ischaemic injury in the cat small intestine: role of superoxide radicals. *Gastroenterology* 82, 9-15.

Parmar, N.S. and Ghosh, M.N. (1981) Gastric anti-ulcer activity of (+)-cyanidanol-3, a histidine decarboxylase inhibitor. *European Journal of Pharmacology* 69, 25-32.

Paterson, S.J., Robson, L.E. and Kosterlitz, H.W. (1983) Classification of opioid receptors. *British Medical Bulletin* 39, 31-36.

Patrono, C. (1989) Aspirin and human platelets: from clinical trials to acetylation of cyclooxygenase and back. *Trends in Pharmacological Sciences*, 10(11), 453-458.

Patrono, C. (1990) Thromboxane synthesis inhibitors and receptor antagonists. *Thrombosis Research*, (Supplement Xl) 15-23.

Patrono C., Ciabattoni, G, Pugliese, F., Pierucci, A., Blair, I.A. and FitzGerald, G.A. (1986) Estimated rate of thromboxane secretion into the circulation of normal humans. *Journal of Clinical Investigation*, 77, 590-594.

Patrono, C., Ciabattoni, G., Greco, F., Grossi-Belloni, D. (1976) Comparative evaluation of the inhibitory effects of aspirin-like drugs on prostaglandin production by human platelets and synovial tissue. In *Advances in Prostaglandins and Thromboxane Research*, Vol. 1, Ed.B Samuelsson and R. Paoletti, Raven Press, New York, 125-131.

Pearson, T.D. (1989) Non-steroidal anti-inflammatory drugs in the therapy of canine osteoarthritis. MVM Thesis, University of Glasgow.

Peck, H.M. (1968) An appraisal of drug safety and evaluation in animals and the extrapolation of results to man, In *Importance of Fundamental Principles in Drug Evaluation*, Ed. D.H. Tedeschi and R.E. Tedeschi, Raven Press, New York, 449-471.

Pentikainen, P.J., Neuvonen, P.J. and Backman, C. (1981) Human pharmacokinetics of tolfenamic acid; a new anti-inflammatory agent. *European Journal of Clinical Pharmacology*, 19, 359-365.

Pentikainen, P.J., Penttila, A., Neuvonen, P.J., Khalifah, R.G. and Hignite, C.E., (1982). Human metabolism of tolfenamic acid. I Isolation, preliminary characterisation and pharmacokinetics of tolfenamic acid and its metabolites, *European Journal of Drug Metabolism and Pharmacokinetics*, 7(4), 259-267.

Perper, R.J. and Davies, P. (1977) Modulation of the expression of the immune response by anti-inflammatory drugs. In *Immunopharmacology* (Comprehensive Immunology Series), Ed J.W. Hadden, R.G. Coffey, and F. Spreafico, Plenum Medical Book Co., London, 227-244.

Perper, R.J. and Oronsky, A.L. (1974) Enzyme release from human leukocytes and degradation of cartilage matrix. *Arthritis Rheumatism* 17, 47-55.

Perrin, J.H. (1985) Bonds between drugs and plasma components. In *Protein Binding and Drug Transport.* Ed J. Tillement, and E. Lindenlaub, Symposium Medica Hoechst 20. Algarve, Portugal. 115-131.

Peters, T. (1970) Serum albumin. *Advances in Clinical Chemistry*, 13, 37-111.

Peters, T. (1975). Serum albumin, In *The Plasma Proteins.* Vol 1, Ed F.W. Putnam, Academic Press, London, 133-181.

Peters, T., Fleischer, B. and Fleischer, S. (1971) The biosynthesis rate of serum albumin. *Journal of Biological Chemistry*, 246, 240-244.

Pierson, R.N., Holt, P.R., Watson, R.M. and Keating, R.P. (1961) Aspirin and gastrointestinal bleeding. *American Journal of Medicine*, 31, 259-265.

Piper, D.W., McIntosh, J.H., Ariotti, D.E., Fenton, B.H. and MacLenman, R. (1981) Analgesic injestion and chronic peptic ulcer. *Gastroenterology* 80, 427-432.

Piper, P. and Vane, J.R. (1971) The release of prostaglandins from lung and other tissue. *Annals of the New York Academy of Science*, 180, 363-385.

Piperno, E., Ellis, D.J., Getty, S.M. and Brody, T.M. (1968) Plasma and urine phenylbutazone levels in the horse. *Journal of the American Veterinary Medical Association* 153(2), 195-198.

Pitts, N.E. (1982) Efficacy and safety of piroxicam. *American Journal of Medicine*, 72, 77-86

Post R.M. and Desforges J.F. (1968b) Thrombocytopenia and alcoholism. *Annals of International Medicine* , 68, 1230-1236.

Post, R.M. and Desforges, J.F. (1968a) Thrombocytopenic effect of ethanol infusion. *Blood, Journal of Haematology*, 31(3), 344-347.

Powis, G (1974) A study of the interaction of tetracycline with human serum lipoproteins and albumin. *Journal of Pharmacy and Pharmacology*, 26, 113-118.

Rainsford, K.D. (1978) The effect of aspirin and other non-steroidal anti-inflammatory/analgesic drugs on gastrointestinal mucus glycoprotein byosynthesis in vivo: relationship to ulcerogenic actions. *Biochemical Pharmacology* 27, 877-885.

Rainsford, K.D. (1984a) Pharmacological and biochemical actions of the salicylates. In *Aspirin and the Salicylates*, Butterworths, London, 67-147

Rainsford, K.D. (1984b) Side effects and toxicology of the salicylates. In *Aspriin and the Salicylates*, Butterworths, London, 148-271.

Rainsford, K.D. (1984c) Mechanisms of gastrointestinal ulceration by non-steroidal anti-inflammatory drugs/analgesic drug. In *Side Effects of Anti-inflammatory/Analgesic Drugs, Advances in Inflammatory Research*, Vol. 6 Eds K.D. Rainsord and G.P.Velo. Raven Press, New York, 51-61.

Rainsford, K.D. (1985) Salicylates In *Anti-inflammatory and Antirheumatic Drugs* Vol. 1, Ed K.D. Rainsford, CRC Press, Florida, USA. 109-147.

Rainsford, K.D., Schweitzer, A. and Brune, K. (1981) Autoradiographic and biochemical observations on the distribution of non-steroidal anti-inflammatory drugs. *Archives Internationales de Pharmacodynamie et de Therapie* 250, 180-193.

Rainsford, K.D., Schweitzer, A. and Brune, K. (1983) Distribution of acetyl compared with the salicyl moiety of acetylsalicylic acid. *Biochemical Pharmacology* 32, 1301-1308.

Ramwell, P.W., Foegh, M., Loeb, R. and Leovey, E.M.K. (1980) Synthesis and metabolism of prostaglandins, prostacyclin and thromboxanes: the arachidonic acid cascade. *Seminars in Perinatology*, 4, 3-13

Redman, C.M. and Cherian, M.G. (1972) The secretory pathways of rat serum glycoproteins and albumin. Localistation of newly formed proteins within the endoplasmic reticulum. *Journal of Cell Biology*, 52, 231-245.

Rejholec, V., Vapaatalo, H., Tokola, O. and Gothoni, G. (1979b) Tolfenamic acid in the treatment of rheumatoid arthritis. *Scandanavian Journal of Rheumatology*, 24 (Supplement), 9-12.

Rejholec, V., Vapaatalo, H., Tokola, O. and Gothoni, G. (1979a) A comparative, double blind study on tolfenamic acid in the treatment of rheumatoid arthritis. *Scandanavian Journal of Rheumatology*, 24 (Supplement), 13-16.

Resink, T.J., Grigorian, G.Y., Moldabaeva, A.K., Danilow, S. M., and Buhler, P. (1987) Histamine induced phosphoinositide metabolism in cultured human umbilical vein endothelial cells. Association with thromboxane and prostaglandin release. *Biochemical Biophysical Research Communications* 144, 438-446.

Risi, A. (1932) Ricerche istologiche sullazione tossica da acido fenilchinolincarbonico. *Archives Internationales de Pharmacodynamie et de Therapie*, 42, 117-127.

Rivier, J.E. (1988) Veterinary clinical pharmacokinetics. Part 1. Fundamental Concepts. *Compendium on Continuing Educationfor the Practicing Veterinarian* 10(1), 24-30.

Robert, A. (1981) Current history of cytoprotection. International workshop on protective actions of prostaglandins on the gastrointestinal mucosa. *Prostaglandins* 21, (Supplement), 89-96.

Rohde, J., Schwegler, F. and Standel, W. (1980) Treatment of rheumatoid arthritis. Open parallel studies with non-steroidal antirheumatics piroxicam and diclofenac. *Deutsch Medizinische Wochenschrift*, 105, 1614-1617.

Roszkowski, A.P., Rooks, W.H., Tomolonis, A.J. and Miller, L.M. (1971) Anti-inflammatory and analgesic properties of d-2-(6'-methoxy-2'-naphthyl)-propionic acid (naproxen). *Journal of Pharmacology and Experimental Therapeutics* 179, 114-123.

Roth, G.J. and Majerus, P.W. (1975) The mechanism of the effect of aspirin on human platelets. *Journal of Clinical Investigation* 56, 624-632.

Roth, G.J., Stanford, N. and Majerus, P.W. (1975) Acetylation of prostaglandin synthase by aspirin. *Proceedings of the National Academy of Sciences* , USA, 72, 3073-3076.

Roth, G.R. and Siok, C.J. (1978) Acetylation of NH_2 -terminal serine of prostaglandin synthetase by aspirin. *Journal of Biological Chemistry* 253, 3782-3784.

Routledge, P.A. (1985) Respective parts of different plasma proteins and blood cells in the overall binding of drugs. In *Protein Binding and Drug Transport* . Ed J. Tillement, and E. Lindenlaub, Symposium Medica Hoechst 20. Algarve, Portugal. 179-195

Rowland, M. and Tozer, T.N., (1989). In *Clinical Pharmacokinetics: Concepts and Applications*. 2nd Edn, Lea and Febiger, Philadelphia, USA. .

Rubin, S.I. and Papich, D.V.M. (1989) Non-steroidal anti-inflammatory drugs. In *Current Veterinary Therapy* X, Ed R.W. Kirk, W.B. Saunders Co., Philadelphia, USA, 47-54

Runkel, R., Forchielli, E., Boost, G., Chaplin, M., Hill, R., Sevelius, H., Thompson, G. and Segre, E. (1973) Naproxen - metabolism excretion and comparative pharmacokinetics. *Scandanavian Journal of Rheumatology* 2, (Supplement) 24-36.

Salin, M.L. and McCord, J.M. (1975) Free radicals and inflammation, protection of phagocytosing leukocytes by superoxide dismutase. *Journal of Clinical Investigation* 56, 1319-1323.

Salmon, J.A. (1978). A radiomunoassay for 6-ketoprostaglandin F_{1alpha}. *Prostaglandins*, 15, 383-397.

Salmon, J.A. and Flower, R.J. (1979) Prostaglandins and related compounds. In *Hormones in Blood*, Vol. 2, 3rd edn., Ed. G. Gray and V. James, Academic Press, London, 237-319.

Sams, R.A. (1987) Principles of drug disposition in the horse. *Veterinary Clinics of North America: Equine Practice.* 3(1), 221-250.

Samuelsson, B. Goldyne, M. Granstrom, E., Hamberg, M., Hammerstrom, S. and Malmsten, C. (1978) Prostaglandins and thromboxanes. *Annual Review of Biochemistry*, 47, 997-1029.

Samuelsson, B., Hammarstrom, S., Murphy, R.C. and Borgeat, P. (1980) Leukotrienes and slow reacting substance of anaphylaxis (SRS-A). *Allergy*, 35, 375-384.

Scherrer, R.A. (1985) Fenamic acids. In *Anti-inflammatory and Antirheumatic Drugs, Vol. II. Newer Anti-inflammatory Drugs*. Ed. K.D. Rainsford, CRC Press Inc., Florida, 65-85.

Schiantarelli, P. and Cadel, S. (1981) Piroxicam pharmacologic activity and gastrointestinal damage by oral and rectal route. *Arzneimittel Forschung*, 31, 87-92.

Schiantarelli, P., Acerbi, D. and Bovis, G. (1981) Some pharmacokinetic properties and bioavailability by oral and rectal route of piroxicam in rodents and man. *Arzneimittel Forschung*, 31, 92-94.

Schiavinato, A., Lini, E., Guidolin, D., Pezzoli, G., Botti, P., Martelli, M., Cortivo, R., DeGalateo, A. and Abatangelo, G. (1989) Intra-articular sodium hyaluronate injection in the pond-nuki experimental model of osteoarthritis in dogs. *Clinical Orthopaedic and Related Research* 241, 286-299.

Schultze, H.E. and Heremans, J.F. (1966), The principal methods of fractionation of plasma proteins. In *Molecular biology of human proteins*. Vol 1 Pub Elsevier, New York, 236-304.

Schwartz, S.O. and Simonds, J.P. (1935) Peptic ulcers produced by feeding cinchophen to mammals other than the dog. *Proceedings of the Society of Experimental Biology and Medicine*, 32, 1133-1134.

Sedman, A.J. and Wagner, J.G. (1976) CSTRIP, a Fortran IV computer programme for obtaining initial polyexponential parameter estimates. *Journal of Pharmaceutical Sciences*, 65, 1006-1010.

Segre, E.J., Chaplin, M., Forchielli, E., Runkel, R. and Svelius, H. (1974) Naproxen-aspirin interactions in man. *Clinical Pharmacology and Therapeutics*, 15, 374-379.

Sellers, E.M. and Koch-Weser, J. (1971) Kinetics and clinical importance of displacement of warfarin from albumin by acidic drugs. *Annals of the New York Academy of Science*, 179, 213-225.

Semrad, S.D., Hardee, G.E., Hardee, M.M. and Moore, J.N. (1985). Flunixin given in small doses: pharmacokinetics and prostaglandin inhibition in healthy horses. *American Journal of Veterinary Research*, 46, 2474-2479.

Seppala, E., Nissila, M., Isomaki, H., Nuotio, P., Nykanen, E., Laitinen, O. and Vapaatalo, H. (1985) Comparison of the effects of different anti-inflammatory drugs on synovial fluid prostanoid concentrations in patients with rheumatoid arthritis. *Clinical Rheumatology*, 4(3), 315-320.

Shelly, S. (1978) Pharmacological mechanisms of analgesic nephropathy. *Kidney International* 13, 15-16.

Shoji, A. (1933) Ueber den einfluss von Atophan auf die inneren organe von kaninchen und hund mit besonderer berucksichtigung der magenschleimhaut. *Transactiones Societatis Pathologicae Japonicae,* 23, 520-522.

Siegmeth, W. (1980) Serum konzentrationen von piroxicam in beziehung zur klinische wirking bei patienten mit chronischer polyarthritis. *Wiener MedizinischeWochenschrift*, 81 (special issue), 31-36.

Simmons, P.A., Salmon, J.A. and Moncada, S. (1983) The release of leukotriene B_4 during experimental inflammation. *Biochemical Pharmacology* 32, 1353-1359.

Simonds, J.P. (1938) Mode of origin of experimental gastric ulcer induced by cinchophen, *Archives of Pathology*, 26, 44-50.

Sjoholm, I., Kober, A. Odar-Cederlof, I. and Borga, O. (1976) Protein binding of drugs in uremic and normal serum: The role of endogenous binding inhibitors. *Biochemical Pharmacology*, 25, 1205-1213.

Skjelbred, P., Album, B. and Lokken, P. (1977) Acetylsalicylic acid vs paracetamol: effects on post-operative course. *European Journal of Clinical Pharmacology* 12, 257-264.

Smith, C.A. and Hawk, P.B. (1915) Action of atophan and novatophan in gout and iritis. *Archives of International Medicine*, 15, 181-187

Smith, M.J.H. (1966) Metabolic effects of salicylates. In *The Salicylates, a Critical Bibliographic Review*, Ed M.J.H. Smith and P.K. Smith. Wiley-Interscience, New York, 49-105.

Smith, M.J.H. and Dawkins, P.D. (1971) Salicylate and enzyme. *Journal of Pharmacy Pharmacology*, 23(10) 729-744.

Snow, D.H., Baxter, P and Whiting, B. (1981b) The pharmacokinetics of meclofenamic acid in the horse. *Journal of Veterinary Pharmacology and Therapeutics*, 4, 147-156.

Snow, D.H. and Douglas, T.A. (1983) Studies on a new paste preparation of phenylbutazone. *The Veterinary Record*, 112(26), 602-607.

Snow, D.H., Douglas, T.A., Thompson, H., Parkins, J.J. and Holmes, P.H. (1981a) Phenylbutazone toxicosis in equidae: a biochemical and pathophysiologic study. *American Journal of Veterinary Research*, 42 (10),1754-1759.

Snyder, S.H., Pasternak, G.W. and Pert, C. (1973) Opiate receptor mechanisms. In *Synaptic Modulators, Handbook of Psychopharmacology 5*, Eds. L.L. Iversen, S.D. Iversen, and S.H. Snyder, 5, 329-360.

Soma, L.R., Behrend, E., Rudy, J. and Sweeney, R.W. (1988) Disposition and excretion of flunixin meglimine in horses. *American Journal of Veterinary Research*, 49(11), 1894-1898.

Soma, L.R., Uboh, C.E., Rudy, J and Fegely, J. (1992) Plasma concentrations of flunixin in the horse: its relationship to thromboxane B_2 production. *Journal of Veterinary Pharmacology and Therapeutics*, 15, 292-300.

Sorensen, K. and Christiansen, L.V. (1977) Long term therapy with tolfenamic acid. *Scandanavian Journal of Rheumatology*, 20(Supplement), 1-7.

Spector, S.L., Morris, H.G. and Seiner, J.C. (1979) Pulmonary, prostaglandin and clinical responses of asthmatic patients with aspirin idiosyncrasy to a new non-steroidal anti-inflammatory agent compaired to aspirin. *Journal of Allergy and Clinical Immunology*, 63 154-155.

Squires, R.F. (1972) Multiple forms of monoamine oxidase in intact mitochondria as characterised by selective inhibitors and thermal stability: a comparison of eight mammalian species. In *Monomamine Oxidase-New Vistas*, Ed E. Costa, and M. Sandler, Raven Press, New York, 355-370.

Stafanger, G., Carsen, H.W., Hansen, H. and Sorensen, K. (1981) Pharmacokinetics of ketoprofen in patients with chronic renal failure. *Scandanavian Journal of Rheumatology* 10, 189-192.

Stalker, L.K., Bollman, J.L. and Mann, F.C. (1937) Experimental peptic ulcer produced by cinchophen. *Archives of Surgery*, 35, 290-308.

Stenderup, J. Eriksen, J., Bols Pedersen, S. and Christiansen, L.V. (1985). Pharmacokinetics of tolfenamic acid in patients with cirrhosis of the liver. *European Journal of Clinical Pharmacology*, 28, 573-579.

Stoff, J.H. and Clive, D.M. (1983) Role of prostaglandins and thromboxane in acute renal failure. In *Acute Renal Failure*, Eds B.M. Brennar and J.M. Lazarus, W.B. Saunders, Philadelphia, USA. 157-174.

Strom, H. and Krogsgaard Thomsen, M. (1990). Effects of non-steroidal anti-inflammatory drugs on canine neutrophil chemotaxis. *Journal of Veterinary Pharmacology and Therapeutics*, 13, 186-191.

Strub, K.M., Aeppli, L. and Muller, R.K.M. (1982) Pharmacological properties of carprofen. *European Journal of Rheumatology and Inflammation*, 5, 478-487.

Sturman, J.A. and Smith, M.J.H. (1967) The binding of salicylate to plasma proteins in different species. *Journal of Pharmacy and Pharmacology*, 19, 621-622.

Sudlow, G., Birkett, D.J. and Wade, D.N. (1976) Further characterisation of specific binding sites on human serum albumin. *Molecular Pharmacology*, 12, 1052-1061.

Svensson, J., Hamberg, M. and Samuelsson, B. (1975) Prostaglandin endoperoxides IX. Characterisation of rabbit aeorta contracting substance from guinea pig lung and human platelets. *Acta Physiologica Scandinavica*, 94, 222-228.

Swanstrom, O.G. (1978) Hyaluronate (hyaluronic acid) and its use. *Proceedings of the American Association of Equine Practice* , 24th Annual Convention, 345-348.

Sydness, O.A., (1981) Comparison of piroxicam with naproxen in rheumatoid arthritis: a double blind crossover, multicentre study. *European Journal of Rheumatology and Inflammation*, 4, 318-322.

Tausch G. (1982) Placebo controlled study of piroxicam in the treatment of rheumatoid arthritis. *American Journal of Medicine*, 72, 18-22.

Templeton, C.B., Bottoms, G.D. and Fessler, J.F. (1983). Effects of flunixin meglumine on hemodynamics, hematology, prostaglandins and survival during chronic exposure to endotoxin. *Federation Proceedings*, 42, 323.

Tobin, T. (1981) Phenylbutazone and its brothers: The non-steroidal anti-inflammatory drugs. In *Drugs and the Performance Horse*, Springfield, Illinois, USA ,85-88.

Toivanen, J., Ylikorkala, O. and Viinikka, L. (1984) One milligramme of acetylsalacylic acid daily inhibits platelet thromboxane A_2 production. *Thrombosis Research*, 35, 681-687.

Trillo, M.A., Soto, G. and Gunson, D.E. (1984). Flunixin toxicity in a pony. *Equine Practice*, 6, 21-29.

Trujillo, O., Rios, A., Maldonado, R and Rudolph, W. (1981) Effect of oral administration of acetylsalicylic acid on haemostasis in the horse. *Equine Veterinary Journal*, 13, 205-206.

Tsuchia, T.M. (1980) Disposition and enterohepatic circulation of diclofenac in the dog. *Arzneimittel Forschung*, 30, 1650-1653.

Unanue, E.R. (1972) The regulatory role of macrophages in antigenic stimulation. *Advances in Immunology* 15, 95-165.

Urien, S., Riant, P., Albengres, E., Brioude, R. and Tillement, J.P. (1984) In vitro studies on the distribution of probutil among human plasma lipoproteins. *Molecular Pharmacology*, 26, 322-327.

Vaananen, P.M., Meddings, J.B. and Wallace, J.L. (1991) Role of oxygen derived free radicals in indomethacin induced gastric injury. *American Journal of Physiology* 261, G470-475.

Van Wagoner, F. H. and Churchill, F.H. (1932a) Production of gastric and duodenal ulcers in experimental cinchophen poisoning: Prelimainary report. *Journal of the American Medical Association,* 99, 1859-1860.

Van Wagoner, F. H. and Churchill, F.H. (1932b) Production of gastric and duodenal ulcers in experimental cinchophen poisoning of dogs. *Archives of Pathology*, 14, 860-869.

Vane, J.R. (1971) Inhibition of prostaglandin synthesis as a mechanism of action for aspirin like drugs. *Nature New Biology* 231, 232-235.

Vane, J.R. (1976) The mode of action of aspirin and similar compounds. *Journal of Allergy and Clinical Immunology*, 58, 691-712.

Vapaatalo, H., Osterman, T., Tokola, O. and Tokola, R. (1986). Acute effects of tolfenamic acid on hemostatic functions and arachidonic acid metabolism. *Current Therapeutic Research*, 39, 250-259.

Vapaatalo, H., Parantainen, J., Linden, I.B. and Hakkarainen, H. (1977) Prostaglandins and vascular headache . Action of tolfenamic acid, a new anti-inflammatory analgetic, on migraine. In *Headach: New Vistas*, Biomedical Press, Florence (Italy), 287-300.

Velo, G.P., Dunn, C.J., Giroud, J.P., Timsit, J. and Willoughby, D.A. (1973) Distribution of prostaglandins in inflammatory exudate. *Journal of Pathology*, 111, 149-158.

Vernimb, G.D. and Hennessey, P.W. (1977). Clinical studies with flunixin meglumine in the treatment of equine colic. *Journal of Equine Medicine and Surgery*, 1, 111-116.

Villiaumey, J. (1981) Piroxicam in rheumatoid arthritis: a double-blind comparison versus ketoprofen. In *Proceedings XV Congress of International League Against Rheumatism (ILAR), Paris*, Abstract 30.

Vinegar, R., Schreiber, W. and Hugo, R. (1969) Biphasic development of carrageenin edema in rats. *Journal of Pharmacology and Experimental Therapeutics*, 166, 96-103.

Von Dell, H.D.,Fiedler, J, Jacobi, H. and Wasche, B. (1977) Zur biochemie und pharmacokinetic von etofenamat. *Arzneimittel Forschung*, 27, 1322-1325.

Wallace, J.L. and Granger, D.N. (1992) Pathogenesis of NSAID gastropathy: are neutrophils the culprits? *Trends in Pharmacological Sciences*, 13, 129-131.

Ward, P.A., Cohen, S. and Flanagan, T.D. (1972) Leukotactic factors elaborated by virus infected tissues. *Journal of Experimental Medicine* 135, 1095-1103.

Webbon, P.M. and Wooliscroft, G.J. (1984). Cautious use of flunixin advocated. *Veterinary Record*, 115, 45.

Weintraud, W. (1911) Die behandlung der ghicht mit phenylcholine carbonsavre (Atophan) nebst bumerkungen uber die diatetsche therapie der krankheit. *Die Therapie der Gegnwart*, 52, 97-105.

Weintraud, W. (1912) Weitere klinsche erfahrungen mit Atophan nebst benerkungen uber gicht und hornsaure diathese. *Therapeutische Monatschefte*, 26, 21-29.

Weissmann, G., Zurier, B.B. and Hoffstein, S. (1972) Leukocytic proteases and the immunologic release of lysosomal enzymes. *American Journal of Pathology* 68, 593-564.

Welsh, E.M., McKellar, Q.A and Nolan, A. M. (1993) The pharmacokinetics of flunixin meglumine in sheep. *Journal of Veterinary Pharmacology and Therapeutics*, 16, 181-188.

Westfall, G.A. (1926) Toxicity of cinchophen - clinical study. *Journal of the Kansas Medical Society*, 37, 311.

Whitehouse, M.W. (1968) The molecular pharmacology of anti-inflammatory drugs : some possible mechanisms of action at biochemical level. *Biochemical Pharmacology* (Supplement) 293. 293-307.

Whitehouse, M.W. and Haslam, J.M. (1962) Ability of some anti-rheumatic drugs to uncouple oxidative phosphorylation. *Nature*, 196, 1323-1324.

Whittle, B.J.R., and Vane, J.R. (1983) Prostacyclin, thromboxanes and prostaglandins-actions and roles in the gastrointestinal tract. In *Progress in Gastroenterology*. Ed G.B. Jerzyglass and P. Sherlock, Grune and Stratton Inc., New York. 3-30.

Widmark, P. (1978) Safety and efficay of piroxicam in the treatment of acute gout. *European Journal of Rheumatology and Inflammation*, 1, 346-348.

Wigren, A., Wik, O and Falk, J. (1976) Intra-articular injection of high molecular hyaluronic acid: an experimental study on normal rabbit knee joints. *Acta Orthopaedica Scansdinavica*, 47, 480-485.

Wilkinson, P.C. (1978) The adhesion, locomotion and chemotaxis of leukocytes. In *Handbook of Experimental Pharmacology* Vol. 50/I Inflammation, Eds. J.R. Vane and S.H. Ferreira, Springer-Verlag, New York, 109-137

Willis, A.L. (1969) Release of histamine, kinin and prostaglandins during carrageenan-induced inflammation in the rat. In *Prostaglandins, Peptides and Amines*. Ed. P. Mantegazza and E.W. Horton, Academic Press, London. 31-38.

Willkens, R.F., Ward, J.R., Louis, J.S. and McAdam, L.P. (1982) Double-blind study comparing piroxicam and aspirin in treatment of rheumatoid arthritis. *American Journal Medicine*, 72(2A), 23-26.

Wilson, D.E., Phillips, C., and Levine, R.A. (1971) Inhibition of gastrointestinal secretion in man by prostaglandin A_1. *Gastroenterology* 61(2), 201-206.

Wind, M., Berliner, A. and Stern, S. (1973) The binding of phenothiazines to oxyhaemoglobin A and S., *Research Communication in Chemical Pathology and Pharmacology*, 5, 759-766.

Wiseman, E.H. (1978) Review of preclinical studies with piroxicam: pharmacology, pharmacokinetics and toxicology. *Royal Society of Medicine International Congress Symposium Series*, 1, 11-23.

Wiseman, E.H. (1985) Piroxicam and related oxicams. In *Anti-inflammatory and Antirheumatic Drugs*. Ed. K.D. Rainsford, CRC Press,Boca Raton, 209-248.

Wiseman, E.H. and Boyle, J.A. (1980) Piroxicam (Feldene). *Clinics in Rheumatic Diseases*, 6, 585-613.

Wiseman, E.H. and Chiaini, J. (1972) Anti-inflammatory and pharmacokinetic properties of sudoxicam, N-(2-thiazolyl)-4-hydrxy-29methyl-2H-1,2-bnzothiazine-3-carboxamide-1,1-dioxide. *Biochemical Pharmacology*, 21, 2323-2334.

Wiseman, E.H. and Hobbs, D.C. (1982) Review of pharmacokinetic studies with piroxicam. *American Journal of Medicine*, 72, 9-17.

Wiseman, E.H., Chang, Y.H. and Lombardino, J.G. (1976) Piroxicam, a novel anti-inflammatory agent, *Arzneimittel Forschung*, 26, 1300-1303.

Yamaoka, K., Terumichi, N and Uno, T. (1978). Application of Akaike's criterion (AIC) in the evaluation of linear pharmacokinetic equations. *Journal of Pharmacokinetics and Biopharmaceutics*, 6(2), 165-172.

Yamashita, S., Numoto, T., Yamaura, T., Namikata, S., Oba, M, Aoyama, M., Noguchie, K. and Tosaka, K. (1981a). Anti-inflammatory activity of N-(2-methyl-3-chlorophenyl)-anthranilic acid, *Journal Medical Society of Toho University*, 28, 76-98.

Yamashita, S., Numoto, T., Takenaga, K., Shimizu, K., Yamaura, T and Tosaka, K. (1981b). A new anti-inflammatory agent N-(2-methyl-3-chlorophenyl)-anthranilic Acid (GEA6414). Analgesic, antipyretic action and safety (including ulcerogenicity). *Journal Medical Society of Toho University*, 28, 99-105.

Yesair, D.W., Callahan, M., Remington, L. and Kensler, C.J. (1970) Comparative effects of salicylic acid, phenylbutazone, probenecid and other anions on the metabolism, distribution and excretion of indomethacin by rats. *Biochemical Pharmacology* 19(5), 1579-1600.

Youdim, M.B.H. (1972) Multiple forms of monoamine oxidase amd their properties. In *Monomamine Oxidase-New Vistas*, Ed E. Costa, and M. Sandler, Raven Press, New York. 67-77.

Youlten, L.J.F. (1978) Inflammatory mediators and vascular events. In *Handbook of Experimental Pharmacology*. Vol 50/I. Inflammation Ed. J.R. Vane and S.H. Ferreira, Springer-Verlag, New York. 571-587.

Zachariae, E. and Sylvest, J. (1972). Osteoarthritis of the knee treated with tolfenamic acid. *Scandanavian Journal of Rheumatology*, 1, 97-99

Zigmond, S.H. (1974) Mechanisms of sensing chemical gradients by polymorphonuclear leukocytes. *Nature* 249, 450-452.

Zizic, T.M., Sutton, J.D. and Stevens, M.B. (1978) Piroxicam and osteoarthritis: a controlled study. In *Procedings of Royal Society of Medicine International Congress Symposium Series*, 1, 53-58.

Zor, U, Koch, R. and Naor, Z. (1976) Differential effects of prostaglandin synthetase inhibitors on prostaglandin E_2 binding and on prostaglandin - or cholera toxin induced cyclic AMP accumulation in the rabbit uterus. In *Advances in Prostaglandin and Thromboxane Research*, Vol 1, Ed B. Samuelsson and R. Paoletti, Raven Press, New York. 331-336.