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Absorption Properties of Non-steroidal Anti-inflammatory Drugs and their Amino Acid Derivatives

by

Karl Levis, B.Sc. (Pharm.), M.P.S.I.

being a thesis submitted for the degree of
Doctor of Philosophy in Pharmaceutics

at

University of Dublin, Trinity College.



under the direction and supervision of

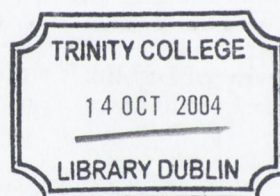
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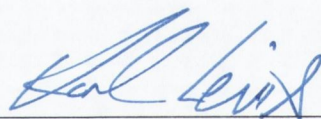
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DECLARATION

This thesis is submitted by the undersigned to the University of Dublin, Trinity College, for examination for the degree of Doctor of Philosophy. It has not been submitted as an exercise for a degree at any other university. I myself carried out all the practical work except where duly acknowledged. This manuscript was completely written by me with the aid of editorial advice from Dr. Majella Lane and Professor O.I. Corrigan.

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To my parents

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PRESENTATIONS AND PUBLICATIONS ASSOCIATED WITH THIS THESIS

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‘Mechanistic studies of NSAIDs and their derivatives’, in *Proceedings of the twenty-fourth TCD/QUB annual joint research seminar*, The Queen’s University of Belfast, March 2002, 11 [K.A. Levis, M.E. Lane, M. Hanlon, J.F. Gilmer and O.I. Corrigan].

Poster Presentations

‘Influence of perfusion medium on the absorption characteristics of nonsteroidal anti-inflammatory drugs’, in *Proceedings of the 8th United Kingdom and Ireland Controlled Release Society Symposium 2002: Oral Administration of Difficult Drugs*, Loughborough, January 2002, 15 (abstract) [K.A. Levis, M.E. Lane and O.I. Corrigan].

‘Mechanistic studies of the absorption characteristics of NSAIDs and their novel L-arginine derivatives’, in *Proceedings of the 4th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology*, Florence, April 2002, 529 (Abstract) [K. Levis, M.E. Lane, M. Hanlon, J. Gilmer and O.I. Corrigan].

‘Effect of buffer media on the solubility and effective permeability coefficient of ibuprofen’, in *Proceedings of the International Symposium on Scientific and Regulatory Aspects of Dissolution and Bioequivalence*, Athens, April 2002, 301 (Abstract) [K. Levis, M.E. Lane and O.I. Corrigan].

‘Synthesis and characterization of arginine derivatives of Nonsteroidal Anti-Inflammatory Drugs’, *2002 AAPS Annual Meeting and Exposition*, Toronto, Canada, November 2002, R6124 [M. Hanlon, K.A. Levis, J.F. Gilmer, M.E. Lane and O.I. Corrigan].

Publications

'Effect of buffer media composition on the solubility and effective permeability coefficient of ibuprofen', International Journal of Pharmaceutics, 253 (2003) 49-59 [K.A. Levis, M.E. Lane and O.I. Corrigan].

ABBREVIATIONS AND SYMBOLS

<	less than
>	greater than
±	plus or minus
≈	approximately equal to
≤	less than or equal to
≥	greater than or equal to
°C	degree centigrade
AA	arachidonic acid
AD	Alzheimer's disease
ANOVA	analysis of variance
ATP	adenosine triphosphate
BCS	biopharmaceutics classification scheme
β	buffer capacity
β _{max}	maximum buffer capacity
C	concentration
C ₀	intrinsic solubility
CI	confidence interval
cLog P	logarithm of the calculated partition coefficient
COX	cyclooxygenase
C _s	saturated solubility
dpm	disintegrations per minute
FA	fraction absorbed
FaSSIF	fasted state simulated intestinal fluid
FeSSIF	fed state simulated intestinal fluid
G	force of gravity
GI	gastrointestinal
Gly-Sar	glycylsarcosine
H ⁺	proton
HBSS	hanks balanced salt solution
I	ionic strength
Ibu	ibuprofen
IL	interleukin

IVIVC	<i>in vitro in vivo</i> correlation
k	rate constant
k_0	zero order rate constant
$k_{0(\text{gut})}$	zero order input rate constant calculated from perfusate data
$k_{0(\text{plasma})}$	zero order input rate constant calculated from plasma data
k_{12}	rate constant for transfer from the central to the peripheral compartment
k_{21}	rate constant for transfer from the peripheral to the central compartment
k_{elim}	elimination rate constant
K_w	dissociation constant of water
Log D	logarithm of the distribution coefficient
Log P	logarithm of the partition coefficient
LOQ	limit of quantitation
LOX	lipxygenase
LT	leukotriene
M	molar
MCT	monocarboxylate transporter
MP or mp	melting point
MSC	model selection criterion
n	sample size or number of data points
Na^+	sodium ion
NO	nitric oxide
NOS	nitric oxide synthase
NSAID	non-steroidal anti-inflammatory drug
OC	organic cation
P_{app} or P_e	apparent permeability coefficient
$P_{\text{app.man}}$	apparent permeability coefficient in man
$P_{\text{app.rat}}$	apparent permeability coefficient in the rat
P_{aq}	aqueous boundary layer coefficient
PBS	phosphate buffer system
PEG	polyethylene glycol
PepT1	dipeptide transporter
PG	prostaglandin

pH	negative logarithm of the hydrogen ion concentration
pK _a	dissociation constant
P _m	membrane permeability
PSA	polar surface area
Q	flow rate
r ²	r-squared
rpm	revolutions per minute
RSD	relative standard deviation
s.d.	standard deviation
s.e.	standard error
T or t	time
t _{iv}	duration of infusion/perfusion
TPSA	topological polar surface area
UWL	unstirred water layer
V	volume of distribution
Å	angstrom
nm	nanometre
µm	micrometre
mm	millimetre
cm	centimetre
µg	microgram
mg	milligram
kg	kilogram
min	minute
µL	microlitre
ml	millilitre
L or l	litre
mM	millimolar
µCi	microcurie
mCi	millicurie
π	Pi
% w/v	percentage weight per volume
% w/w	percentage weight per weight
®	registered trademark

SUMMARY

The principal objectives of this work were: i) to examine the absorption properties of ibuprofen in a range of different buffer solutions; ii) to study the absorption properties and mechanisms of ibuprofen, ketoprofen and naproxen; and iii) to determine the effect that coupling the ibuprofen molecule to a range of simple amino acids has on its absorption properties and gastrointestinal irritation.

Preliminary studies involved the examination of eight different buffers that had been previously used in absorption/perfusion studies as reported in the literature. The solubility of ibuprofen acid in each system varied six-fold over the range of buffers and was related to the buffer pH. Ibuprofen formed poorly soluble salts with calcium ions in Krebs' buffer and became solubilised in fed state simulated intestinal fluid (FeSSIF). Buffer capacity varied significantly over the eight systems.

Buffer composition was found to significantly affect the absorption properties of ibuprofen. This appeared to be primarily due to differences in net water flux/solvent drag across the intestinal mucosa as a consequence of different buffer osmolarities.

No significant differences were seen between the absorption properties of ibuprofen, ketoprofen and naproxen. The absorption properties of ketoprofen were examined at different concentrations and no changes in permeability coefficient with increasing concentration were observed. Comparison of NSAID permeability coefficients with that of the more hydrophilic compound, Gly-Sar, suggested that the passive absorption of ibuprofen, ketoprofen and naproxen occurs principally by transcellular diffusion, with transport via the paracellular route occurring simultaneously but to a much lesser extent.

The alanine, glycine and phenylalanine derivatives of ibuprofen, showed significantly lower intestinal absorption in the rat. This could be attributed to differences in their polar surface areas. In turn, the L-arginine derivative was significantly less well absorbed than the other three amino acid derivatives of ibuprofen as well as ibuprofen itself. This could be explained by its relatively large polar surface area and relatively low Log P value. When their permeability coefficients in rats were converted to fraction absorbed values in humans, no significant differences were observed between

the alanine, glycine and phenylalanine derivatives, and ibuprofen. These were all significantly larger than the L-arginine derivative.

Ibuprofen and the four amino acid derivatives of ibuprofen appeared to bind non-specifically to peptide transporters involved in the uptake of the dipeptide, Gly-Sar, *in situ* and in cell culture. The extent of this binding varied with the compound. In addition to the passive absorption of these compounds, active transport processes by peptide transporters may also be involved in their absorption.

A two compartment pharmacokinetic model with constant input and first order output was shown to best describe the plasma data for ketoprofen, naproxen, ibuprofen and its amino acid derivatives. There was a good correlation between disappearance from the gut and appearance in the plasma. The rate of elimination of ketoprofen from the blood appeared to decrease with increasing dose. Both the rate of elimination from the blood and the volume of distribution of the phenylalanine derivative of ibuprofen were significantly different from ibuprofen and the other amino acid derivatives.

The parent compound, ibuprofen, was not released by these amino acid derivatives, suggesting that they were not being metabolised by the rat. It is possible they are excreted unchanged.

The amino acid derivatives of ibuprofen were significantly less toxic to the gastrointestinal mucosa than ibuprofen, ketoprofen and naproxen. Elimination or reduction of the direct contact effect of the acidic carbonyl group appeared to be the reason for this reduced toxicity. Increasing the concentration of ketoprofen in the perfusion solution significantly increased the level of gastrointestinal irritation within the concentration range of 0.5–2.0 mg ml⁻¹.

The pH values of the perfusate samples shifted over the course of the perfusion experiments. The direction and extent of this pH change was dependent on the initial pH of the perfusion solution and indicated that the intestinal microclimate buffering system attempts to maintain the intestinal contents at pH 6 and reaches a steady-state after 40 minutes.

ORIGIN AND SCOPE

In addition to their traditional role in the treatment of pain and inflammation, as newer clinical applications of NSAIDs begin to emerge, such as their potential roles in the treatment/prevention of Alzheimer's disease and colon cancer (Katori and Majima, 2000; Aisen, 2002; Kaza et al., 2002), the need to develop safer forms of NSAIDs is becoming more important. In this regard, an understanding of the mechanisms of NSAID absorption and toxicity in the gastrointestinal tract would be useful. This information could serve as a starting point and a reference for newly synthesised amino acid derivatives of ibuprofen, with different physicochemical and absorption properties to the parent compound. Additionally, the ability of these compounds to bind to peptide transporters is of interest as this could potentially provide a means of selective delivery of the compounds to particular tissues, such as the intestinal and tumour selective delivery via H^+ /oligopeptide transporters for peptide mimetic drugs as reported by Tsuji (1999).

In situ intestinal absorption studies in the rat and *in vitro* cell culture studies were carried out using alanine, glycine, phenylalanine and L-arginine derivatives of ibuprofen. Fagerholm et al. (1996) reported a linear correlation of intestinal permeability and fraction of dose absorbed between humans and rats. Consequently, rats, as an animal model for drug absorption, appear to reasonably predict the extent of drug absorption in humans and, accordingly, were used in all *in situ* drug absorption studies. When carrying out all *in situ* absorption studies, a 33.3 cm length of proximal jejunum was used, beginning 1 cm distal to the stomach. This section of intestine was chosen as the ratio of surface area to length of intestine is greatest in this region (DeSesso and Jacobson, 2001) and the small intestine is considered to be the major site for drug and nutrient absorption. However, for some drugs (*e.g.* metoprolol and theophylline) the large intestine is also an important absorption site, in spite of the lower surface area available for absorption (Abrahamsson et al., 1996; Yuen et al., 1993). Passive transcellular diffusion, instead of paracellular and carrier mediated mechanisms, appears to be the principal means of drug absorption in the large intestine due to the small paracellular pore size and low activity of carrier mediated transport (Hardy et al., 1985). Considering these factors, together with

fact that the intestinal peptide transporters, such as PepT1, are expressed predominantly in the jejunum and duodenum (Walter et al., 1996; Gonzalez et al., 1998), the proximal jejunum was selected as the area of the intestine to be perfused. Capan-2 cells were used for cell culture studies as they express a range of transport proteins at high levels (Gonzalez et al., 1998).

Essentially, the rationale for studying the alanine, glycine, phenylalanine and L-arginine derivatives of ibuprofen was that by attaching an amino acid moiety to the ibuprofen molecule it would reduce the gastrointestinal toxicity and release the parent ibuprofen in the plasma by the action of esterases in the blood. One of the reasons why these simple amino acids were chosen as a derivatisation approach is because they are safe, and innocuous and offer opportunities for covalent drug attachment due to the presence of different functional reactive groups (Franssen et al., 1992). The polar nature of these same functional groups might also serve to increase the solubility of the derivative relative to the parent compound. Finally, amino acids might utilise specific carrier mechanisms for their uptake, and by attaching them to a drug molecule, the resulting compound might also be a substrate for these carrier mechanisms.

INTRODUCTION

Chapter 1

Non-steroidal anti-inflammatory drugs

1.1 INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are the principal form of treatment of inflammatory diseases, and despite their renal (Schiff and Whelton, 2000) and gastric (MacDonald et al., 1997) side effects they are one of the most widely used groups of drugs worldwide (Garner, 1992). The first NSAID with clinical benefits was aspirin, which has now been used for more than 100 years. Even today this substance is one of the most commonly used drugs. It has been estimated that 1-2% of the world population consumes at least one aspirin tablet per day (Lichtenberger, 2001), with an average yearly consumption of 30g per person in industrialised countries (Roth and Calverley, 1994).

1.2 THE INFLAMMATORY PROCESS

Inflammation is a normal and essential response to any noxious stimulus that threatens the host and may vary from a localized response to a more generalized one (Hamor, 1989). The inflammation sequence can be summarised as follows:

- (i) initial injury causing release of inflammatory mediators (e.g. histamine, serotonin, leukokinins, lysosomal enzymes, lymphokinins, and prostaglandins).
- (ii) vasodilation.
- (iii) increased vascular permeability and exudation.
- (iv) leukocyte migration, chemotaxis and phagocytosis.
- (v) proliferation of connective tissue cells.

The most common sources of chemical mediators include neutrophils, basophils, mast cells, platelets, macrophages and lymphocytes (Hamor, 1989). The etiology of inflammatory and arthritic diseases has received a great deal of attention and the currently accepted pathogenesis of these disorders can be summarised as follows: an unknown antigen gains access to the patient's tissues and combines with an antibody in the joint, thus activating the complement sequence. An antigen-complement-antibody immune complex then precipitates in the synovial and joint fluid, generating the release of chemical mediators which subsequently cause the migration of

numerous polymorphonuclear leukocytes phagocytising the immune complexes. Lysosomal membranes become unstable and discharge hydrolytic enzymes from the leukocytes and synovial cells. This results in tissue damage with continuing inflammation, tissue destruction and the loss of physical properties of the connective tissue and joints. Anti-inflammatory agents may therefore act at any one of these stages.

1.2.1 Biosynthesis of eicosanoids

Prostaglandins (PGs) contribute to a variety of physiological and pathophysiological functions and, along with thromboxanes, prostacyclin and leukotrienes, belong to a class of compounds called eicosanoids. The eicosanoids are derived from membrane phospholipids and are found in almost every area of the body and their biosynthesis is summarised in Figure 1.1.

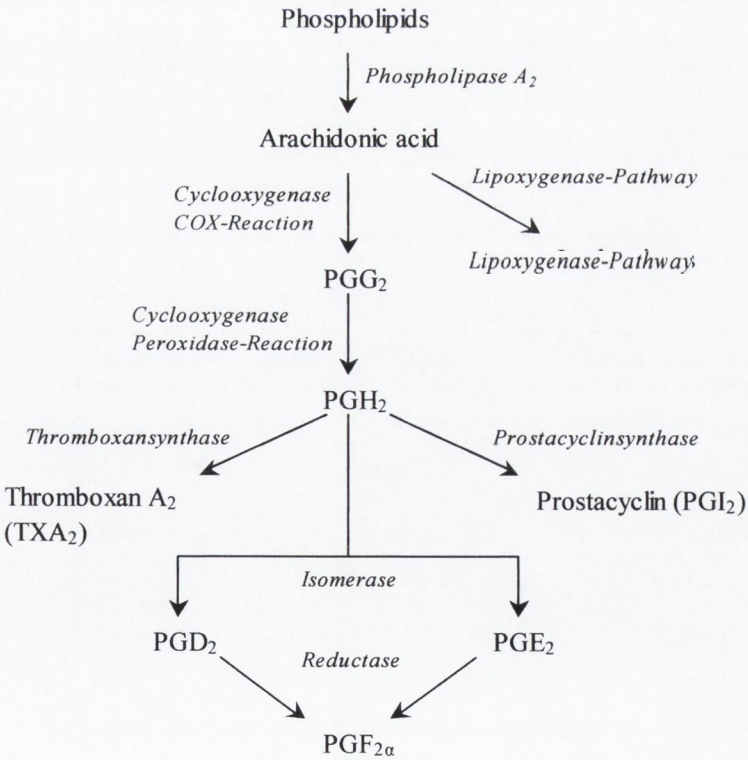


Figure 1.1 Arachidonic acid cascade.

The initial step is the phospholipase A₂ catalysed liberation of arachidonic acid (AA) from the phospholipids of the cell membrane. The next step is the biotransformation of AA by cyclooxygenase (COX) into the unstable PGG₂ (or into leukotrienes via the 5-lipoxygenase (5-LOX) pathway) which is then immediately converted into PGH₂ by the same enzyme in a peroxidase reaction. PGH₂ is then converted into thromboxanes, prostacyclin (PGI₂) or the prostaglandins PGD₂, PGE₂ and PGF_{2α} (Vane, 1971; Vane and Botting, 1992).

1.2.2 Pathophysiological roles of prostaglandins and other eicosanoids

PGs are produced by most cells around the body and result in a broad spectrum of physiological effects. Under basal conditions, PGs are produced in nearly all tissues including the colon, kidney, spleen, stomach, liver, lung, heart and brain. The first eicosanoids produced in the arachidonic acid cascade are PGG₂ and PGH₂. Both are chemically unstable but may themselves be able to mediate pain and act as vasoconstrictors, as well as acting as precursors for the other eicosanoids. PGE₂ is a stimulator of smooth muscle in the gastrointestinal (GI) tract, can elevate body temperature and is a potent vasodilator while also possessing vasoconstrictor effects at certain sites. Many of these properties are shared by PGF_{2α}. PGE₂ also causes relaxation of bronchial and tracheal smooth muscle, while PGF_{2α} and PGD₂ cause its contraction. Alternatively, non-prostanoids can be formed from PGH₂ as it is also a substrate for thromboxane synthase which converts it into thromboxane A₂ (TxA₂). Similarly, prostacyclin synthase converts PGH₂ into prostacyclin (PGI₂). TxA₂ acts as a vasoconstrictor and stimulates platelet aggregation. In contrast, PGI₂ acts as a vasodilator and inhibitor of platelet aggregation. Thromboxane synthase is principally found in platelets, while prostacyclin synthase is mainly expressed in endothelial cells.

Despite their widespread effects and distribution around the body, their most important physiological roles are their cytoprotective properties in the GI tract and the control of renal functions in the kidney. Under physiological conditions, they act as vasodilators at both of these sites. In the kidney they help to maintain renal plasma flow and glomerular filtration during periods of systemic vasoconstriction. Similarly, in the gastric antrum, local vasodilation appears to be critical in maintaining mucosal

defences. Also, as part of the AA cascade, COX in platelets generates thromboxane which plays a key role in mediating platelet aggregation (Crofford, 1997).

PGs also mediate a number of characteristic features of the body's response to tissue injury or inflammation (rubor, calor, tumor, dolor and functio laesa), and in this respect, PGE₂ is the most important. Dilation of small blood vessels due to the action of PGE₂ initiates the development of redness and heat, and the increase in vascular permeability causes the characteristic swelling of tissues. It also sensitises peripheral nerve endings and nociceptors to transmit pain signals to the brain and the spinal cord.

1.2.3 Mechanism of action of the NSAIDs

NSAIDs had been used for decades to treat pain and inflammation without their mechanism of action being fully understood. It was not until the 1970s that Vane made a scientific breakthrough by elucidating that NSAIDs produce their therapeutic and unwanted effects by blocking the biosynthesis of PGs by inhibiting the COX enzyme (Vane, 1971). This was a major advancement in the understanding of the inflammatory process. However, it also became apparent that administration of NSAIDs leads to a lack of the PGs required for physiological functions.

As a consequence, long-term NSAID users suffer from a high incidence of GI irritation or, in the worst case, from the development of life threatening GI ulcers and bleeding. These lesions can lead to increased morbidity in patients (Beck et al., 1990; MacDonald et al., 1997).

Administration of NSAIDs may also lead to renal disorders and have hypertensive effects due to reduced production of PGs, such as PGI₂ and PGE₂, the regulation of renal blood circulation and the rate of glomerular filtration is reduced. This is of importance in patients with reduced renal function, as it leads to retention of water, hypertension and, in some cases, to renal failure (Clive and Stoff, 1984).

The inhibition of COX in thrombocytes results in decreased production of TxA₂. This phenomenon prolongs bleeding time and leads to inhibition of platelet aggregation. Additionally, a severe side-effect of NSAIDs is bronchoconstriction, leading to asthmatic events. This is caused by a reduced amount of bronchodilating PGE₂ and a shift in the metabolic pathway from the COX pathway to the 5-LOX pathway (Kuel et al., 1984). The latter pathway metabolises 'overflow' AA which cannot be

transformed by the blocked COX pathway. The resulting leukotrienes act as bronchoconstrictors (Samuelsson et al., 1987).

1.2.4 *The discovery of COX-2 and its distribution*

The identification of two isoforms of COX, termed COX-1 and COX-2 (Vane et al., 1994; Xie et al., 1999) resulted in a major conceptual advance with the initial theory that COX-1 was a constitutive ‘house-keeping’ enzyme that produced protective PGs, whereas COX-2 was an enzyme upregulated by inflammatory mediators that produced harmful PGs at the site of inflammation and damage (Needleman and Isakson, 1997; Geiss et al., 1998). This theory is now thought to be rather simplistic as it subsequently became apparent that the functions of COX-1 and COX-2 are more complex. Recent findings have suggested a broader spectrum of biological activity of COX-2 (Katori and Majima, 2000) and apart from its induction in inflammatory cells, COX-2 is known to be induced in the kidney in response to sodium depletion or in hyperfiltration states, and in colon adenoma and carcinoma cells. This opens a new spectrum for therapy with COX-2 inhibitors (Lipsky, 1999).

1.2.5 *Characterisation of COX-1 and COX-2*

The COX isoenzymes are membrane-bound enzymes in the endoplasmic reticulum. The main difference between the two seems to be the much larger binding site in COX-2 to NSAIDs (Kurumbail, et al., 1996). Lipophilicity has been shown to be an important physicochemical parameter for the efficacy of NSAIDs. The different binding sites of the two isoenzymes and the interaction of drugs with the protein structure of COX-1 and COX-2 are responsible for the drug specificity.

From X-Ray crystal structure analysis of COX-1 and COX-2 as well as complexes formed with NSAIDs such as flurbiprofen, the COX active site has been characterised (Picot et al., 1994; Kurumbail et al., 1996). The active site (also the site of NSAID binding) is created by a long hydrophobic channel which contains areas of high electron density that interact with the aromatic system of flurbiprofen. The carboxylic group is directed towards the mouth of the channel and lies in a favourable position for interacting with the guanidinium group of arginine 120 (ARG 120). Glutamic acid

524 (GLU 524) is located near ARG 120, and these two residues may form a salt bridge thus blocking the mouth of the enzyme.

The differences between COX-1 and COX-2 have been elucidated. COX-2 contains a valine at position 523, which is exchanged for a relatively bulky isoleucine residue in COX-1 at the same position. This structural modification in the COX-2 enzyme allows access to an additional side pocket, which is a pre-requisite for COX-2 drug selectivity. A further exchange of valine/isoleucine at position 434 is responsible for the formation of a 'gate'. In COX-2 the less bulky valine is able to 'swing' like a gate because of less steric hindrance and offers enough space for the entry of compounds with room-filling substituents.

Studies of the static 3D structure of the enzyme have been very helpful in the process of understanding the enzyme inhibition mechanisms and in the design of selective compounds, but dynamic factors such as enzyme flexibility and rearrangement of the hydrogen-bonding network at the entrance of the active site must also be considered.

1.3 HISTORY OF NSAID DEVELOPMENT

The development of NSAIDs is very closely linked to advancements in the understanding of the inflammatory process and the enzymes involved. Like many therapeutic agents, the NSAIDs have their origin in the historical recognition that certain plants were observed to produce therapeutic effects in certain conditions. Salicylate-containing plants, such as the bark of the willow, were used by ancient Egyptians and Romans to relieve pain in childbirth and gout, and in the middle ages there are written records of these plants being used to treat wounds, inflammation and pain. Pure salicylic acid was obtained from plants in the early 19th century and in the 1890s Felix Hoffman while working for Bayer synthesised acetylsalicylic acid in commercial quantities. It was subsequently marketed as aspirin (Vane et al., 1990). This marked the beginning of the development of NSAIDs. Aspirin was initially used in the treatment of headaches and fever associated with colds and influenza, and was eventually recognised as the standard for the treatment of pain and inflammation in rheumatoid arthritis up until the mid 1970s. In the decades following the discovery of aspirin there was very little development in the treatment of rheumatic diseases until the 1950s, partly because the mechanisms underlying the disease development were

little understood. In the 1950s-1960s, the drugs available for the treatment of pain and inflammation in rheumatic diseases included aspirin (and the other salicylates), aminophenols (phenacetin) and pyrazolones (discovered in the early 1900s), phenylbutazone (originally used to solubilize aminopyrine and accidentally discovered to have anti-inflammatory properties), and the corticosteroids (discovered in the 1950s). Gold salts had been discovered in the 1930s to have disease-modifying activity in rheumatic diseases, but by the 1950s they were regarded as very toxic. Consequently, the need was identified for a more potent drug than aspirin that would not produce the potentially fatal side-effect of agranulocytosis seen with phenylbutazone or the serious side-effects seen with corticosteroids (Rainsford, 1999).

The 1960s saw greater understanding of the inflammatory process and the involvement of PGs and other lipid soluble mediators. This provided a basis for developing NSAIDs to control the reactions involved, and was facilitated by the development of animal models that could be used to screen compounds for analgesic and anti-inflammatory activity. Therefore, during the years 1955-1970 the earlier NSAIDs such as ibuprofen and indomethacin were developed. By the beginning of the 1990s, a large range of NSAIDs were available forming a chemically diverse group of compounds that share a variety of pharmacological actions and adverse effects. They can be grouped chemically into the salicylates, pyrazolones, fenamic acids, arylalkanoic acids, and oxicams. The salicylates and pyrazolones have a long history of clinical use but during the years 1955-1970 they began to fall out of favour due to the development of the early NSAIDs such as ibuprofen and indomethacin.

1.3.1 Prodrugs of NSAIDs

The introduction of indomethacin in the mid-1960's (Shen et al., 1963) had a significant impact on arthritis therapy and its success led to the proliferation of NSAID development. Ibuprofen and naproxen were introduced in the 1970's along with sulindac, a prodrug of indomethacin (Shen and Winter, 1977). Sulindac was developed as an agent with less side-effects than indomethacin and reduced GI toxicity. It was the first NSAID prodrug and is itself an inactive compound that undergoes two major biotransformations in the lumen of the GI tract. It can be irreversibly oxidised to an inactive sulfone metabolite, or it can be reversibly reduced

to an active sulfide metabolite which inhibits COX activity (Figure 1.2). However, GI toxicity with sulindac was shown to be similar to those of the other NSAIDs (Henry et al., 1996).

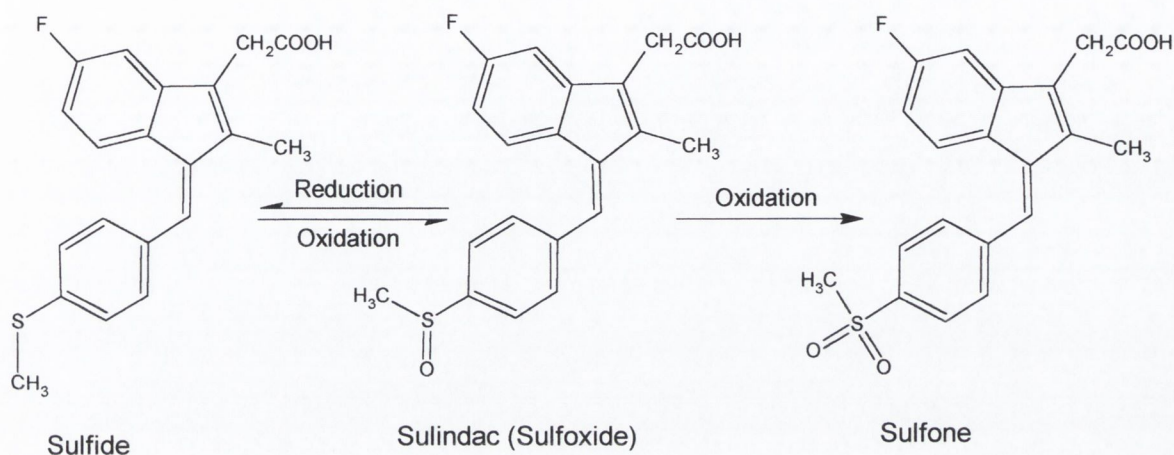


Figure 1.2 Chemical structures of sulindac and its sulfide and sulfone metabolites.

Nabumetone is another NSAID prodrug that is used clinically. It was developed as a non-acidic prodrug in an attempt to reduce the GI toxicity seen with traditional NSAIDs like indomethacin and ibuprofen. Its active metabolite, 6-methoxy-naphthyl acetic acid (6-MNA) is formed by side-chain oxidation of nabumetone and shows greater anti-inflammatory activity than the parent compound (Figure 1.3) (Nunn and Chamberlain, 1982). It is a weak inhibitor of PG synthesis and this is presumed to be the reason for its low GI toxicity (Mangan, 1987).

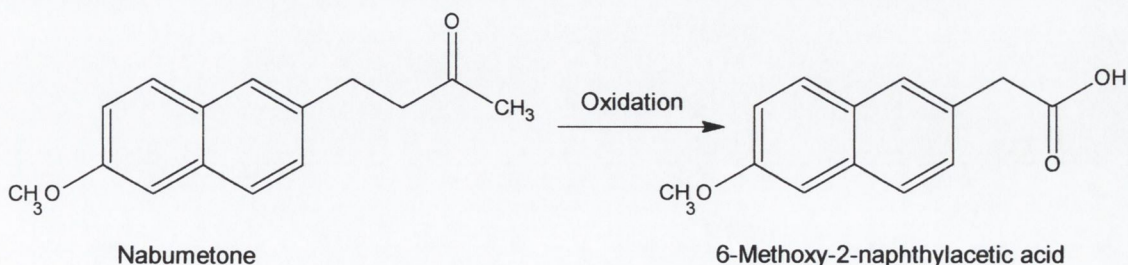


Figure 1.3 Chemical structures of nabumetone and its active metabolite, 6-MNA.

Fenbufen is another NSAID prodrug which is metabolised in the liver to the active metabolites, 4-biphenylacetic acid and 3-(4-biphenylhydroxymethyl)propionic acid (Figure 1.4) (Child et al., 1977).

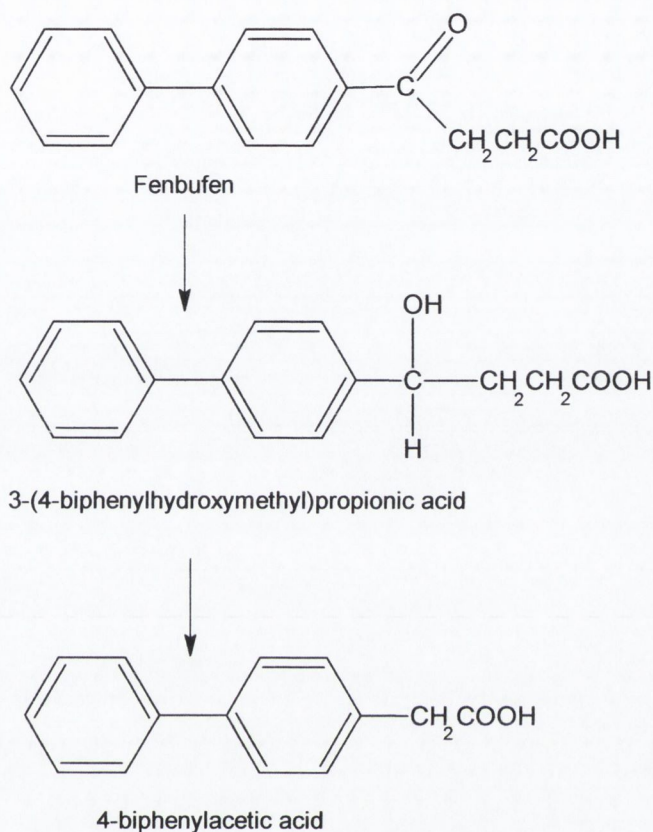


Figure 1.4 Chemical structures of fenbufen and its active metabolites.

1.3.2 Arylalkanoic acids

These all belong to the largest group of NSAIDs, the arylalkanoic acids, of which there are two subgroups: the heterocyclic acetic acids (e.g. indomethacin and sulindac) and the propionic acids (e.g. fenbufen, ibuprofen, ketoprofen and naproxen). The structures of these compounds are given in Figure 1.5.

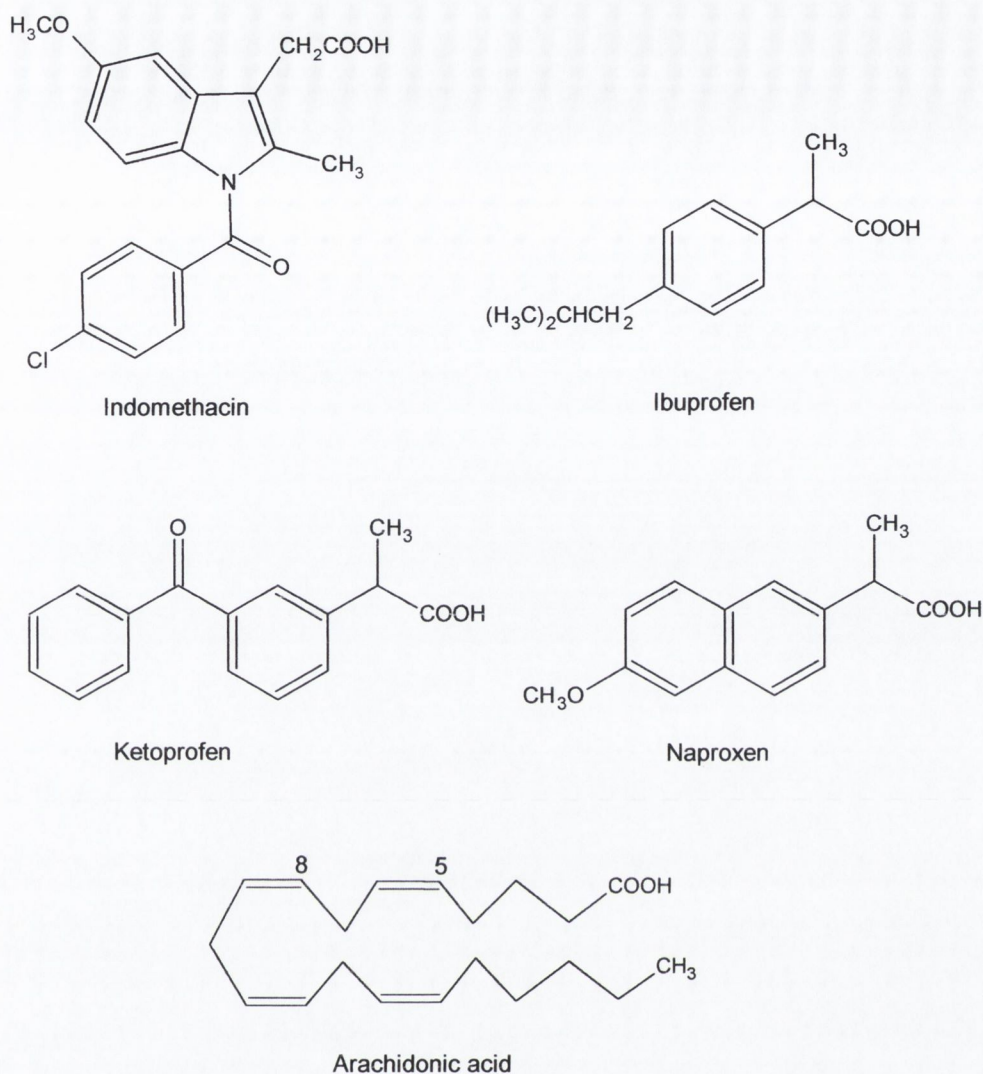


Figure 1.5 Chemical structures of some commonly used arylalkanoic acids.

They all possess a centre of acidity that is generally located one carbon atom adjacent to a flat surface such as an aromatic or heteroaromatic ring. The distance between these centres is critical for activity, with an increase to two or three carbon atoms generally causing a decrease in activity. The aromatic ring system appears to correlate with the double bonds at the 5- and 8- positions of arachidonic acid (Figure 1.5) thereby mimicking the natural COX substrate (Borne, 1995).

1.4 NEW DEVELOPMENTS IN NSAID THERAPY

Many of the traditional NSAIDs described above cause GI side-effects (Rainsford and Quadir, 1995). The most common of these are NSAID-induced GI ulceration and bleeding which have been reported to be present in 15-30% of chronic NSAID users (Gabriel et al., 1991). Several strategies have been introduced to reduce their GI toxicity: COX-2 selective inhibitors, nitric oxide (NO)-releasing NSAIDs, dual inhibitors of COX and LOX enzymes, NSAIDs coupled to zwitterionic phospholipids, chiral NSAIDs and trefoil peptides.

1.4.1 Selective COX-2 Inhibitors

As described in section 1.2.2, PGs have a dual role in physiology and pathogenesis and are important components of the gastroprotective process: they stimulate mucus and bicarbonate secretion, increase mucosal blood flow, and inhibit acid secretion (Feldman, 1990). The discovery that there are two isoforms of the COX enzyme- a constitutive isoform (COX-1) that performs housekeeping functions including gastroprotection, and an inducible form (COX-2) which is involved in the inflammatory response- suggested that differential inhibition of these isoforms could account for the variable toxicity of NSAIDs (Vane, 1994). It is generally accepted that inhibition of COX-2 contributes to NSAID efficacy whereas inhibition of COX-1, combined with the corrosive effect of its acidic moiety while in the GI tract, contributes to its GI toxicity.

The development of various in vitro assays to test for COX-specific inhibition (Mitchell et al., 1993; Pairet et al., 1998), as well as information on the structural differences between the two isoforms, have resulted in more than 500 COX-2 inhibitors being described over the past few years, of which the two most successful are celecoxib (Celebrex[®]) and rofecoxib (Vioxx[®]) (Figure 1.6). These belong to a group of compounds called vicinal diaryl heterocycles and this class of COX-2 inhibitors has seen the greatest amount of research. Another approach has been the structural modification of known NSAIDs. Indomethacin, aspirin and flurbiprofen have been successfully converted into selective COX-2 inhibitors by introducing larger substituents to fit into the active site volume of COX-2 (Bayly et al., 1999).

One feature that is generally common to them all is that, unlike the classic NSAIDs they lack a carboxylic group, thereby leading to COX-2 affinity by a different orientation within the enzyme without formation of a salt bridge in the hydrophobic channel of the enzyme.

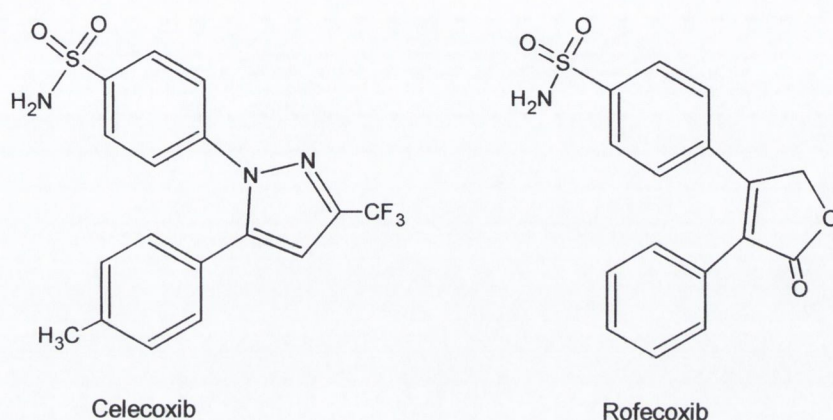


Figure 1.6 Chemical structures of selective COX-2 inhibitors, celecoxib and rofecoxib.

1.4.1.1 Side-effects of selective COX-2 inhibitors

The clinical results of selective COX-2 inhibitors such as celecoxib and rofecoxib are promising. However, the tendency to search for more specific inhibitors has provoked critical reaction. Certainly selective COX-2 inhibitors reduce the risk of GI side-effects, but COX-2 is not only a proinflammatory inducible enzyme, it also has a number of physiological functions which means that it is constitutively expressed to a high degree in the human body (Katori and Majima, 2000).

COX-2 is constitutively expressed in the developing kidney (playing a role in its maturation and function) and seems to be involved in the regulation of the renin-angiotensin system (Morham et al., 1995; Harris et al., 1994). It also possesses vasoactive and anti-atherogenic properties and is constitutively expressed in the developing brain (Yamagata et al., 1993). Moreover, the hormonal induction is important for ovulation and, at the end of pregnancy, high uterine levels of COX-2 are necessary for the onset of labour (Morham et al., 1995). It stands to reason that the use of COX-2 inhibitors will block these physiological functions and lead to side-effects. There is evidence that the use of COX-2 inhibitors can lead to thrombotic cardiovascular events by tipping the balance of PG/thromboxane in favour of TxA₂,

which promotes platelet aggregation and acts as a vasoconstrictor (Mukherjee et al., 2001). Another major issue is the concern for their potential to delay ulcer healing (Halter et al., 1997) as well as their ability to exacerbate the effects of irritants on the gastric mucosa (Gretzer et al., 1998) and the inhibition of other features of COX-2 that are important in GI physiological and inflammatory functions (Wallace, 1999). In light of this, the prospects for highly selective COX-2 inhibitors having the benefits of sparing the GI mucosa from injury in the long term may not be as striking as originally thought. This may be a basis for developing new NSAIDs, such as dual COX-LOX inhibitors. Balanced COX-1/COX-2 inhibitors could also be further investigated.

1.4.1.2 COX-2 and new therapeutic targets

Apart from its role in inflammatory sites, COX-2 is involved in other pathophysiological roles and there is a growing body of evidence that COX-2 may be involved in the development of colon cancer (Lipsky, 1999).

The formation of new blood vessels (angiogenesis) to provide a blood supply is a major requirement for the growth of many tumours. While mature blood vessels express COX-1, new angiogenic cells express inducible COX-2 (Hanahan and Folkman, 1996). It has been hypothesized from this that tumour-derived growth factor promotes angiogenesis by inducing the production of COX-2-derived PGE₂. This is supported by the fact that PGs are known to be pro-angiogenic molecules and contribute to tumour growth by inducing the newly formed blood vessels that sustain tumour cell viability and growth.

COX-2 expression is normally strictly regulated but may be upregulated at certain sites including colon tumours and this seems to be important in colon carcinogenesis. In cultured human colonic fibroblasts it was shown that growth factors such as hepatocyte growth factor are involved in the progression of tumours and that COX-2 mediated PG synthesis is responsible for hepatocyte growth factor expression (Reddy et al., 2000).

In addition to the well known peripheral role of COX-2 in inflammation and the growing body of evidence of its role in the development of colon cancer, recent results indicate an important role in the central nervous system (CNS).

COX-2 in the CNS may have a function in the brain as the expression of COX-2 is associated with brain development (Halliday et al., 2000). COX-2 is constitutively

expressed in neurons and is upregulated in degenerative brain regions in Alzheimer's disease (AD) such as the microglia of the cognitive centres within the hippocampus and cortex. Enhanced COX-2 expression in the brain may be associated with beta-amyloid protein (a protein deposited in senile plaques) deposition in the neuritic plaques of AD. This protein and its peptide precursors are thought to be produced as part of an inflammatory cascade in which microglia (a rich source of prostanoids) probably participate. The role of activated microglia, which express COX-2 in cerebral inflammatory processes has been demonstrated in the rat (Bauer et al., 1997). The fact that COX-2 mRNA is elevated in areas related to memory (hippocampus, cortex) and that the amount of COX-2 correlates with the deposition of beta-amyloid protein represents a possible therapeutic benefit and a hopeful new strategy in the prevention or treatment of AD (Tocco et al., 1997).

This emerging evidence suggests that COX-2 has a variety of physiological and pathophysiological functions. In addition to its physiological roles described in section 1.4.1.1 and its role in inflammation, evidence is emerging of its roles in AD and colon cancer development. These conditions may become important new therapeutic targets for selective COX-2 inhibitors.

1.4.2 Nitric oxide-releasing NSAIDs (NO-NSAIDs)

Like PGs, nitric oxide (NO) plays a dual role in physiology and pathogenesis. NO is a free radical produced constitutively or induced by a family of isoenzymes known as nitric oxide synthases (Palmer et al., 1988). Three main types have been identified: two are expressed in the endothelium of blood vessels (eNOS or type-3 NOS) and neurons in the brain (nNOS or type-1 NOS). These two isoforms synthesise small amounts of NO in response to various agonists that increase Ca^{2+} concentration. The Ca^{2+} -independent isoform (iNOS or type-2 NOS) is induced by lipopolysaccharides and a number of cytokines in different cell types, such as macrophages, neutrophils, endothelial and smooth muscle cells. Once expressed this isoform generates nanomolar amounts of NO for hours or days (Knowles and Moncada, 1994).

1.4.2.1 Role of nitric oxide in the body

The role of NO in the body is still not completely understood but it has been proposed that small fluxes of NO, generated by eNOS and nNOS, cause regulatory/anti-

inflammatory effects, whereas high concentrations of NO, like that generated by iNOS, are mainly involved in pro-inflammatory effects (Grisham et al., 1999). It has also been suggested that the pro-inflammatory effects are mediated by reactive nitrogen species (such as nitrites). These are derived from the interaction between NO and the superoxide anion (O_2^-) and act as potent oxidizing agents (Beckman and Koppenol, 1996). This hypothesis is supported by the anti-inflammatory action of antioxidants which decrease nitrite levels without affecting NO levels (Sandoval et al., 1997).

1.4.2.1.1 Regulatory / anti-inflammatory effects of NO

Some of the regulatory/anti-inflammatory effects of NO include an important role in gastric cytoprotection, possibly by increasing mucosal blood flow, and mucous/fluid secretion by the gastric epithelial cells. It has been suggested that NO and PGs act synergistically to protect the mucosa (Wallace, 1996). In addition, NO counteracts the thrombotic effects of thromboxane by inhibiting platelet aggregation (Bing et al., 1999).

1.4.2.1.2 Pathophysiological effects of NO

NO may be involved in the pathogenesis of colon cancer where its effect is thought to be two-fold:

- a) NO suppresses tumour growth and metastasis (Pipili-Synetos et al., 1995)
- b) NO can also mediate DNA damage (Wink et al., 1998). The expression and activity of iNOS is increased in human colon adenomas, and consequently excessive NO production in 'bursts' by iNOS is thought to be genotoxic in such high local concentrations and also to contribute to the transition of colon adenoma to carcinoma (Ambs et al., 1998).

NO may also be implicated in AD as microglia (the resident macrophages in the CNS) can express iNOS in response to bacterial toxins and cytokines. The generation of NO from these activated cells has been demonstrated to produce neuronal cell injury in culture (Chao et al., 1992) and there is a theory that this may be linked to the development of AD.

1.4.2.2 Effect of NSAIDs on NO production and its therapeutic relevance

Aeberhard et al. (1995) have shown that NSAIDs inhibit the activity of iNOS. More recently, Stratman et al. (1997) have shown that the potency of ibuprofen for inhibiting PGE₂ formation is comparable to its potency of inhibition of iNOS activity in cell culture, suggesting it is likely that ibuprofen would decrease iNOS activity *in vivo*.

The daily administration of NSAIDs for the treatment of arthritic disorders has been shown to delay the onset and/or decrease the risk of AD (Anderson et al., 1995). It was originally thought that the therapeutic effect of NSAIDs was due to inhibition of COX enzymes and, subsequently, reducing synthesis of PGs (Vane, 1971). However, the recent evidence of their ability to inhibit iNOS and the role of NO in neuronal injury in brain disorders associated with inflammation (*e.g.* AD) suggest that this may be an additional mechanism of action.

Also, the role of iNOS and NO in the progression of colon cancer helps to further explain the beneficial effects seen from the use of these agents in this disease.

1.4.2.3 Rationale for NO-NSAID development

The development of NO-NSAIDs was based on the observation that NO produced by eNOS and nNOS possesses some of the same cytoprotective effects as PGs within the gastric mucosa. Coupling a NO-releasing moiety to an NSAID might deliver NO to the site of NSAID-induced damage, thereby compensating for gastric PG reduction induced by NSAIDs, and decreasing gastric toxicity. In this respect, NO-NSAIDs may become a group of safer and perhaps more effective alternatives to traditional NSAIDs (Wallace et al., 1994).

1.4.2.4 Structure and metabolism of NO-NSAIDs

NO-NSAIDs consist of a traditional NSAIDs linked to a NO-releasing group via a chemical spacer. The three key structural components of any NO-NSAID are: i) the traditional NSAID moiety; ii) the spacer molecule; iii) the NO-releasing group.

They are rapidly metabolised *in vivo* in the liver, and hydrolysis of the ester bond linking the –NO₂ group to the spacer is catalysed by various esterases (Wallace et al., 1999; Cuzzolin et al., 1996).

The intestinal wall also possesses esterases, which are responsible for some of the metabolism. Such hydrolysis occurs in the gastrointestinal fluid or the gastrointestinal

wall. This dissociation is metabolic and not a spontaneous process. This allows discrete amounts of NO to be produced that do not interfere with other processes such as regulation of blood pressure. This would explain the effectiveness of these agents in maintaining mucosal integrity by modulating local blood flow and mucous secretion.

The NO-releasing derivative of aspirin, NCX-4016, is one of the best-studied NO-NSAIDs. It is a nitroxybutylated derivative of acetylsalicylic acid and its structure is given in Figure 1.7. Several studies suggest that it is more effective than traditional aspirin: it has more pronounced antithrombotic activity than aspirin, likely due to the NO-releasing moiety in inhibiting neutrophil adherence (Momi et al., 2000); significant neuroprotective effects against ischaemic brain injury in rats (Fredduzzi et al., 2001); and greater cardiac protection in rabbit hearts subjected to low flow ischaemia reperfusion, also likely mediated by NO donation (Rossoni et al., 2000). *In vitro* studies on its effects on platelet aggregation and vascular tone demonstrated that it acts through a double mechanism: by releasing NO in platelets and vascular smooth muscle cells and by inhibiting COX (Minuz et al., 1995).

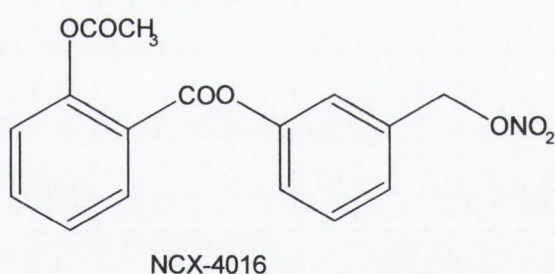


Figure 1.7 Chemical structure of NCX-4016.

A major drawback of the use of traditional NSAIDs is their side-effects. NO-NSAIDs appear to have satisfied the expectation of higher safety although, so far, most of the data come from animal studies. Fiorucci et al. (1999) have demonstrated the GI safety of NO-aspirin in rats.

1.4.2.5 Therapeutic targets of NO-NSAIDs

Like the traditional and COX-2 selective NSAIDs, the NO-NSAIDs also have a role in colon cancer prevention. The chemopreventive effect of traditional NSAIDs against colon cancer has been documented by nearly twenty studies and this has provided the impetus to develop safer and/or more efficacious compounds, including NO-NSAIDs. To date, findings on the chemopreventive potential of NO-NSAIDs, although limited to preclinical studies, appear quite promising. Both cell culture (Williams et al., 2001; Qiao, et al., 2001) and animal studies (Bak et al., 1998) have been reported.

The beneficial effect of NO-NSAIDs in colon cancer prevention is two-fold. Firstly, they produce a low flux of NO to tissues (Wallace et al., 1999; Cuzzolin et al., 1996) and there is no evidence of high concentrations in any tissue compartments including the colon. This sustained low level of NO is thought to suppress tumour growth and metastasis (Pipili-Synetos et al., 1995). This is in contrast to the high levels of NO that are generated in bursts by iNOS and are thought to be genotoxic and contribute to the disease progression (Ambs et al., 1998; Wink et al., 1998). Secondly, the NO-NSAIDs also have an ability to strongly inhibit the induction and expression of iNOS which is responsible for the NO ‘burst’.

1.4.2.6 Appropriate use of NO-NSAIDs and NOS inhibitors

The appropriate use of NOS inhibitors or NO donors seems to be dependent on the time of their administration and the type of disease. NOS inhibitors appear to be beneficial if administered when there is a sustained release of NO due to iNOS expression. For example in chronic inflammation (such as arthritis), the inhibition of NO release is appropriate (Grisham et al., 1994; Miller et al., 1993). On the other hand, in other situations, where there is a functional loss of NO, such as acute intestinal inflammation due to NSAID therapy, the appropriate therapy is the administration of NO donors (Lefer and Lefer, 1999).

1.4.3 Dual inhibitors of COX and 5-lipoxygenase (5-LOX)

In addition to PGs, arachidonic acid can also be converted into leukotrienes (LTs). The LTs are formed from AA by the action of 5-LOX as shown in the AA cascade in Figure 1.1. There is a whole family of LTs, of which LTC₄, LTD₄ and LTE₄ are potent bronchoconstrictors (Bisgaard, 1984). LTB₄ is a potent chemotactic agent for leukocytes and it has been reported to play an important physiological role in the development of gastrointestinal ulcers (Asako et al., 1992) and other inflammatory diseases (Lewis et al., 1990). COX inhibitors are known to induce adverse reactions in patients with asthma either due to shunting of LT synthesis due to increased availability of AA to the 5-LOX pathway (Kuel et al., 1984) or because of a reduction in the vasodilatory PGs such as PGE₂ and PGI₂ that have been shown to protect the airways (Birnbaum et al., 1981). For this reason, developing compounds that will inhibit COX and 5-LOX at the same time could lead to an enhanced anti-inflammatory effect and reduce undesirable side-effects.

An example of one of these is a new compound called S-4274 which shows potent inhibitory effects on both COX-2 and 5-LOX as well as production of IL-1 in *in-vitro* assays (Figure 1.8). It has been selected as an anti-arthritic drug candidate and is now under clinical investigation (Inagaki et al., 2000).

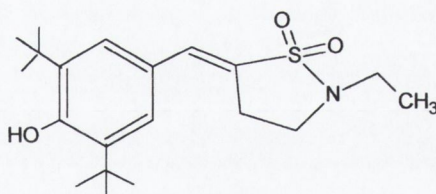


Figure 1.8 Chemical structure of the novel COX-2 and 5-LOX inhibitor, S-2472.

1.4.4 Zwitterionic phospholipids

Zwitterionic phospholipids are phospholipids that have both a positive and a negative charge and the association of an NSAID with one of these molecules is another approach to improving their safety. The rationale of this approach is based on the ability of NSAIDs to reduce the protective effect of the gastric mucosa. By preassociating the NSAID with a phospholipid it reduces or prevents the interaction

between the NSAID and the mucosa (Lichtenberger et al., 1995). Studies so far have been promising showing reduced GI injury compared to the parent compounds (Lichtenberger et al., 1996). They represent a unique approach to producing safer NSAIDs.

1.4.5 Chiral NSAIDs

Many of the commonly used NSAIDs, such as ibuprofen and ketoprofen, are racemic mixtures of two enantiomers, designated R and S. Both have identical physical and chemical properties but differ in their activities as the S-enantiomer inhibits PG synthesis to a greater extent than the R-enantiomer (Carabaza et al., 1996). Chiral conversion from R to S can occur in vivo, and consequently it is possible that a stereochemically pure R-enantiomer could act as a prodrug for the S-enantiomer and reduce GI side-effects. However, Davies et al. (1996) have shown that both enantiomers increase intestinal permeability suggesting no difference in toxicity between the forms.

1.4.6 Trefoil peptides

Trefoil peptides are a family of proteins expressed in the GI tract that show stability in a wide range of pH values, resistance to protease degradation, and induction and localisation during gastric damage. This suggests they have a role in gastroprotection (Hanby et al., 1993). Babyatsky et al. (1996) have shown that oral administration of trefoil peptides protected rats against indomethacin-induced gastric damage. The exact mechanisms involved are still not fully understood, but a PG independent process appears to be involved (Babyatsky et al., 1996).

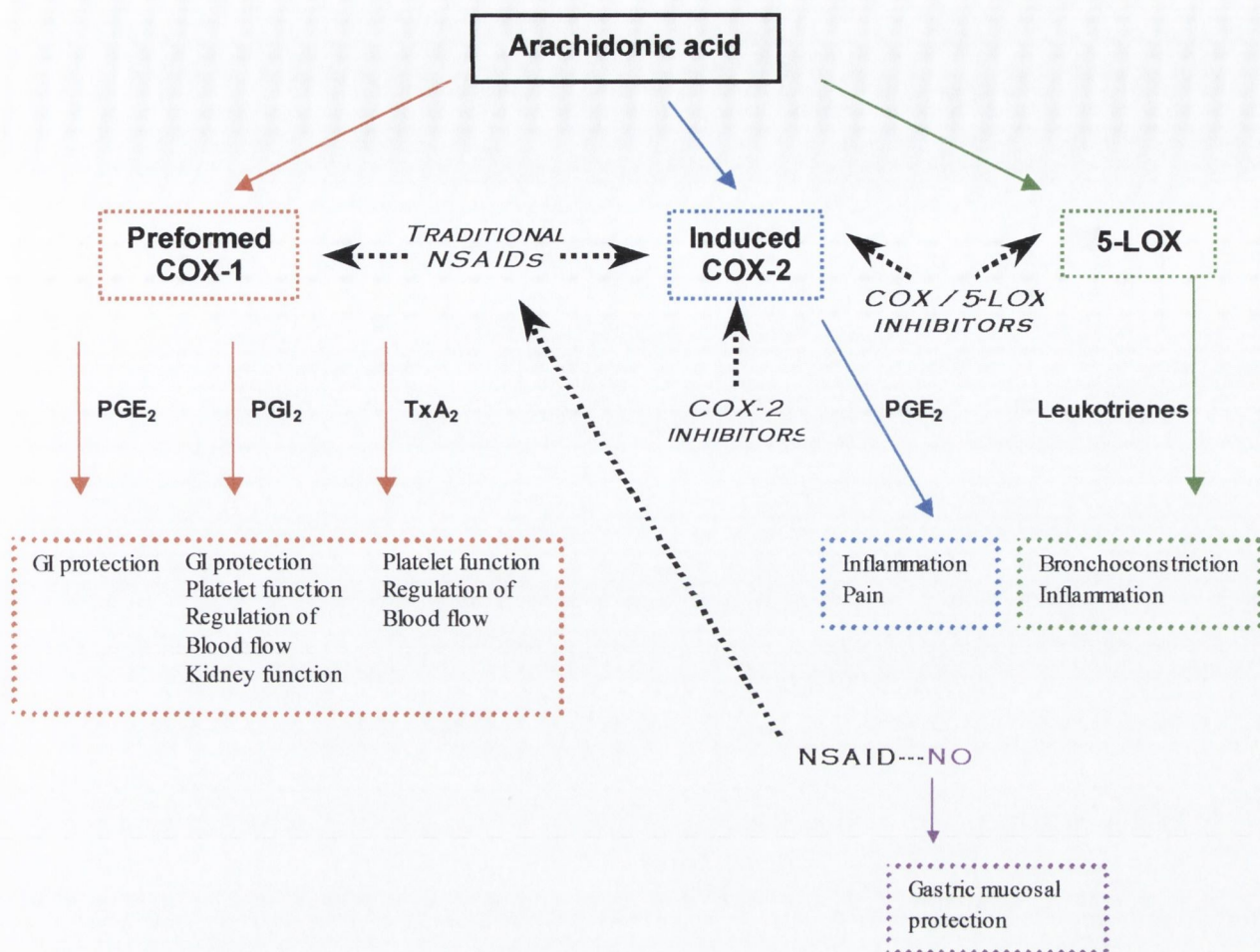


Figure 1.9 Flow chart showing some of the recent developments in NSAID therapy.

1.4.7 Future developments

The use of prodrugs to temporarily mask the acidic group of NSAIDs has been postulated as an approach to decrease the gastrointestinal toxicity due to the direct contact effect (Bundgaard and Nielsen, 1988; Rainsford and Whitehouse, 1976; Whitehouse and Rainsford, 1980). Ester and amide prodrugs of ibuprofen and naproxen have been assessed for anti-inflammatory activity and gastrointestinal toxicity (Shanbhag et al., 1992) with promising results. This may be a promising approach to reducing the gastrointestinal side-effects seen with the traditional NSAIDs.

Chapter 2

The prodrug approach and derivatisation of NSAIDs

2.1 INTRODUCTION

A prodrug is an inactive pharmacological derivative of an active parent compound which undergoes a spontaneous or enzymatic transformation within the body resulting in the release of the free drug, and which has improved delivery properties over the parent drug compound (Albert, 1958). A compound with optimal physico-chemical properties for eliciting the desired therapeutic response at its target site does not necessarily possess the best properties for delivery to its point of action. The oral bioavailability of many drugs is limited by their rapid degradation/metabolism and/or poor intestinal permeability and consequently, only a minor fraction of doses administered may reach the target area (Steffansen et al., 1999). Also, since most drugs interact with non-target sites as well, this may result in side-effects. Bioreversible, chemical derivatisation (i.e. prodrug formation) is a means to improve the physicochemical properties of a drug and optimise its delivery to the site of action. A schematic illustration of the prodrug approach is shown in Figure 2.1.

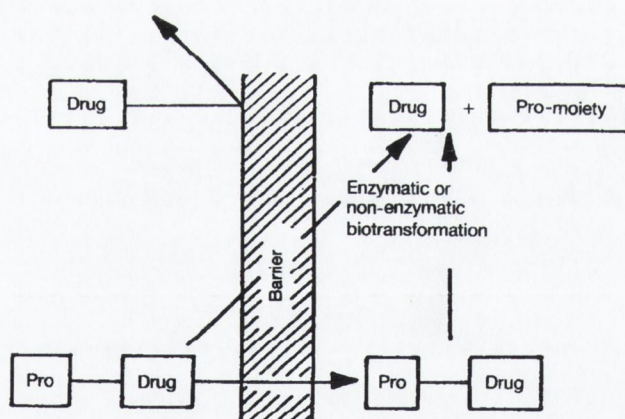
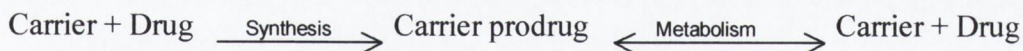


Figure 2.1 Diagram of the prodrug approach in drug delivery.

2.2 TYPES OF PRODRUGS

Prodrugs can be classified in several ways. In some cases, drugs are not designed as prodrugs but after clinical testing are discovered to undergo a metabolic activation and consequently act as prodrugs. However, in most cases it is a drug design strategy, as a specific modification is made to the drug and is designed so that it will be appropriately metabolized after administration. This approach to prodrug development is termed ‘drug latention’ (Harper, 1959). This term has been further refined into two classes of prodrugs called (a) *carrier* and (b) *bioprecursor* prodrugs (Wermuth, 1983).

- a) Bioprecursor prodrugs are compounds that are similar in structure to the active compound and are converted to the active species on metabolism. Unlike carrier prodrugs, a bioprecursor contains a different structure to the active species which cannot be converted simply by cleaving a group from the prodrug.
- b) A carrier prodrug contains an active drug that is linked to a nontoxic, biologically inactive carrier group via a bond which is sufficiently labile (such as an ester or an amide linkage) to allow the active drug to be released efficiently *in vivo*. The overall process is summarised by:



Carrier prodrugs can be further subdivided into (i) bipartate, (ii) tripartate and (iii) mutual prodrugs.

- i Bipartate prodrugs consist of the drug being linked to the carrier directly through a functional group.
- ii Tripartate prodrugs are those in which the carrier is linked to the drug by a separate linker arm. In these prodrugs the carrier is removed by an enzyme-controlled metabolic process and the linker arm is removed by either an enzyme system or a chemical reaction.

- iii A mutual prodrug is a bipartate or tripartate prodrug in which the carrier is a synergistic drug of the drug to which it is linked. Essentially it consists of two drugs attached to each other and each drug acts as the carrier for the other.

2.3 PROPERTIES OF AN IDEAL PRODRUG

The characteristics of an ideal prodrug have been described by Wermuth et al. (1996) and Thomas (2000) and although these are the ideal properties, a particular prodrug seldom meets them all. The ideal characteristics are:

- a) The drug and the transport moiety should be linked via a covalent bond.
- b) In vivo cleavage of this bond with optimal kinetics of release should ensure effective drug levels at the site of drug action that are maintained within the therapeutic window.
- c) The prodrug should be biologically inactive or be significantly less active than the parent drug.
- d) The prodrug should be less toxic than the parent drug and the metabolites from the carrier should also be non-toxic or have a low degree of toxicity.
- e) The prodrug should have an improved bioavailability if administered orally.
- f) The prodrug should be site-specific.

2.4 REASONS FOR DEVELOPING PRODRUGS

Prodrugs are designed to overcome pharmaceutical and/or pharmacokinetic problems associated with the parent molecule that would otherwise limit the clinical usefulness of the drug (Bundgaard, 1992). Examples of these problems include incomplete absorption, poor systemic bioavailability, too rapid absorption or too rapid excretion, toxicity, poor site-specificity, and formulation problems (Testa, 1995; Friis and Bundgaard, 1996; Testa and Caldwell, 1996).

2.4.1 Solubility

If a drug has such poor aqueous solubility to the extent that it will not dissolve sufficiently in the intestinal contents to allow absorption of a therapeutic dose then a water soluble group could be attached which could be metabolically released after absorption.

2.4.2 Absorption and distribution

The ability of a drug to pass through a membrane is dependent on its relative solubilities in water and lipid. If the drug is too water soluble it will not enter the lipid and if it is too lipid soluble it will enter but not leave the membrane. Consequently, if a drug is not absorbed and transported to the target site in sufficient concentration, it can be made more hydrophilic or lipophilic, depending on the desired site of action. Once absorption has occurred or when the drug is at the appropriate site of action, the hydrophilic or hydrophobic group is removed enzymatically.

The most common prodrug strategy to improve oral drug absorption is to increase lipophilicity by masking hydrophilic groups such as carboxylic acids, alcohols, phosphates and other charged groups by forming esters. The reason this approach is widely used is due to the ease of synthesis of esters, the wide range of carboxylic acids and alcohols available to combine to form esters and the numerous esterases present in vivo that convert the ester prodrugs to the parent compounds (Sinkula and Yalkowsky, 1975). The variety of esters that can be synthesised allows precise control of the degree of lipophilicity of the prodrug (Ghosh and Mitra, 1991) as well as regulation of the degree of hydrolysis.

The opposite can also apply as prodrugs have been employed to increase the water solubility of the parent compound. This has traditionally been used in the development of parenteral products of poorly soluble drugs but has recently become a strategy to improve the oral absorption of poorly soluble drugs (Fleisher et al., 1996). The rationale for this approach is that the prodrug's high solubility produces a high intestinal lumen concentration that provides the driving force for absorption. The prodrug then gets reconverted to the parent compound at the mucosal membrane and the parent compound's higher lipophilicity facilitates absorption across the membrane.

2.4.3 Site specificity

When a drug is absorbed it is not just transported to its site of action but is distributed to all the available body compartments. Consequently it is necessary to use a higher concentration than necessary to achieve a therapeutic effect. The result of this is an increased possibility of side-effects. Specificity for a particular organ or tissue can be achieved if there are high concentrations of, or a uniqueness of enzymes present at that site which can cleave the prodrug moiety and unmask the biologically active drug.

2.4.4 Instability

A drug may be rapidly metabolized and inactivated prior to when it reaches the site of action. A prodrug moiety can be attached to block the metabolism until the drug is at the desired site.

2.4.5 Prolonged release

It may be desirable to have a low, constant concentration of drug released over a long period of time. Prodrugs may be used to prolong the duration of action by providing a prolonged release mechanism for the drug which is often facilitated by the slow hydrolysis of amide- and ester-linked fatty acid carriers. Hydrolysis of these groups can release the drug over several hours to several weeks. An example of this is the use of glycine as a carrier for the anti-inflammatory, tolmetin sodium. This increases the duration of its peak concentration from about one hour to nine hours because of the slow hydrolysis of the prodrug amide linkage (Persico et al., 1988).

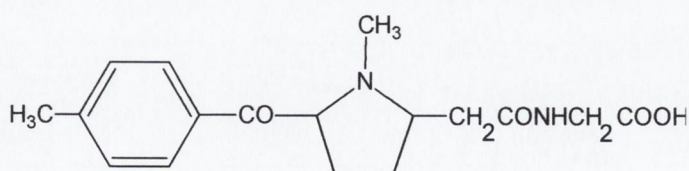


Figure 2.2 Chemical structure of tolmetin-glycine prodrug.

2.4.6 Toxicity

A drug may be toxic in its active form and may have a greater therapeutic index when administered in a nontoxic, inactive form that is converted to the active form only at the site of action. An example of this is aspirin, which is perhaps the earliest prodrug (Sinkula and Yalkowsky, 1975). It was developed by Bayer in order to reduce the gastric irritation and bleeding seen with salicylic acid. The conversion of salicylic acid to its prodrug aspirin by acetylation of the phenolic hydroxy group of salicylic acid reduces the degree of stomach irritation as aspirin is mainly converted to salicylic acid by esterases after absorption from the GI tract.

2.4.7 Poor patient acceptability

An active drug may have an unpleasant taste or odour or produce gastric irritation after oral administration. The structure of the drug can be modified to alleviate these problems, but once administered, the altered drug can be metabolized to the active drug. Palmitic acid and other long-chain fatty acids are often used as carriers as they usually form prodrugs with a bland taste (Sinkula et al., 1973).

2.4.8 Formulation problems

If the drug is a volatile liquid, it would be more desirable to prepare it in a solid form so that it may be formulated as a tablet. An inactive solid derivative could be prepared which would be converted in the body to the active drug.

2.5 MECHANISMS OF PRODRUG ACTIVATION

The mechanisms by which prodrugs are converted to the active drug principle vary with the type of prodrug depending on whether it is a carrier linked or a bioprecursor prodrug.

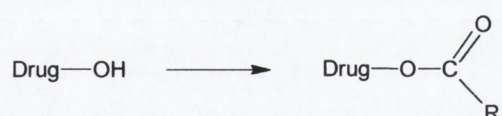
2.5.1 Carrier prodrugs

The most common reaction involved in the activation of carrier prodrugs is hydrolysis and the type of bond involved depends on the functional groups involved.

2.5.1.1 Alcohols and carboxylic acids

Drugs containing alcohol or carboxylic acid functional groups are usually converted to esters if a prodrug is needed. This is because it is possible to prepare an ester with almost any degree of lipophilicity or hydrophilicity and a range of stabilities in the ester can be obtained by altering electronic and steric factors. Additionally, esterases are widespread around the body which facilitates release of the active moiety.

Alcohol-containing drugs can be acylated with aliphatic or aromatic carboxylic acids to decrease water solubility or with carboxylic acids containing amino or additional carboxylate groups to increase water solubility (Bundgaard, 1985). Conversion to sulphate or phosphate esters also increases water solubility. By using these approaches a wide range of solubilities can be achieved that will influence the absorption and distribution properties of the drug.



Formation of ester derivatives of alcohols.

One problem with the use of this prodrug approach is that in some cases the esters are not very good substrates for the endogenous esterases and so may not be hydrolysed at a rapid enough rate. When this occurs, a different ester can be investigated or the rate of hydrolysis can be accelerated by attaching electron withdrawing groups (for basic

hydrolysis) or electron donating groups (for acid hydrolysis) to the carboxylate side of the ester (Reynolds, 1983).

When carboxylic acids are converted to prodrugs, they are often esterified and the reactivity of the prodrug can be altered by appropriate structural manipulations. If a slower rate of ester hydrolysis is desired, long-chain aliphatic or sterically hindered esters can be used. If hydrolysis is too slow, addition of electron withdrawing groups on the alcohol part of the ester can increase the rate.

2.5.1.2 Amines

N-acylation of amines to give amide prodrugs is not widely used due to the stability of amides towards metabolic hydrolysis. However, activated amides or amides of amino acids are more susceptible to enzymatic cleavage. Although carbamates in general are too stable, phenyl carbamates are rapidly cleaved by plasma enzymes and can be used as prodrugs (Bundgaard, 1987).



Derivatisation of amines.

2.5.1.3 Carbonyl compounds

The principal forms of prodrugs of aldehydes and ketones are oximes, acetals, enol esters, oxazolidines, thiazolidines and Schiff bases (Silverman, 1992).



Derivatisation of carbonyl compounds.

2.5.2 Bioprecursor prodrugs

Bioprecursor prodrugs generally use oxidative or reductive activation reactions which alter the chemical structure of the compound and convert it into the active species (Silverman, 1992).

2.6 PRODRUGS OF NSAIDs

The toxicity of NSAIDs to the mucosa of the gastrointestinal tract is a significant problem associated with their clinical use and is one of the most challenging problems in medicinal chemistry. As described in Section 1.3.1, sulindac was the first NSAID prodrug to be used clinically and was developed in an attempt to produce an NSAID with fewer GI side-effects, and was followed by nabumetone and fenbufen. This GI toxicity is attributed to direct and/or indirect effects (Cioli et al., 1979; Bundgaard and Nielsen, 1988). The direct effect is due to a local irritation produced by the acidic group of the NSAID and local inhibition of PG synthesis in the GI tract. The indirect mechanism is due to a generalised systemic action occurring after absorption. A possible approach to solve these delivery problems may be derivatisation of the carboxylic function of the NSAIDs to produce prodrug forms with adequate stability at the acidic pH of the stomach. This derivatisation should ideally prevent the local irritation on the stomach mucosa and be capable of releasing the parent drug spontaneously or enzymatically in the blood following absorption. Several prodrugs and structural analogues of NSAIDs have been synthesised and tested for their anti-inflammatory activity and gastrointestinal toxicity.

2.6.1 *Macromolecular prodrugs*

A considerable amount of research has been focused on the use of macromolecular prodrugs in order to improve the delivery of a wide range of drug compounds (Azori, 1987; Friend and Pangburn, 1987; Ghose and Blair, 1987). A number of polysaccharides have been tested as possible transport moieties for drug molecules, of which dextran is probably the most prominent due to its physicochemical properties and lack of toxicity (Larsen and Johansen, 1985).

A range of polysaccharides have been tested as carriers for naproxen (Larsen, 1989) including dextran, soluble starch and hydroxyethyl starch through ester linkages. Hydrolysis of the prodrugs was found to be base catalysed and slow, with the half-lives for these reactions being 183 hours for the dextran, 800 hours for the soluble starch and 1600 hours for the hydroxyethyl starch derivative. These relatively long half-lives made

them ideal candidates for sustained release prodrugs of naproxen but were unlikely to be used as such due to their low aqueous solubilities.

Jakšić et al. (1996) carried out studies on a macromolecular ketoprofen prodrug and reported that the stability of these types of prodrugs towards different enzymatic systems was probably caused by steric hindrance preventing the enzymes from hydrolysing the covalent link. This encouraged the development of prodrugs with a prolonged distance between the main chain and the NSAID that would make enzyme-substrate interaction more favourable (Zovko et al., 2001). Examples of these include the attachment of fenoprofen to polyhydroxy aspartamide-type polymers such as poly[α,β -(*N*-2-hydroxyethyl-DL-aspartamide)] (*i.e.* PHEA) and poly[α,β -(*N*-3-hydroxypropyl-DL-aspartamide)] (*i.e.* PHPA) by amide and ester bonds through different non-toxic spacers (Figure 2.3).

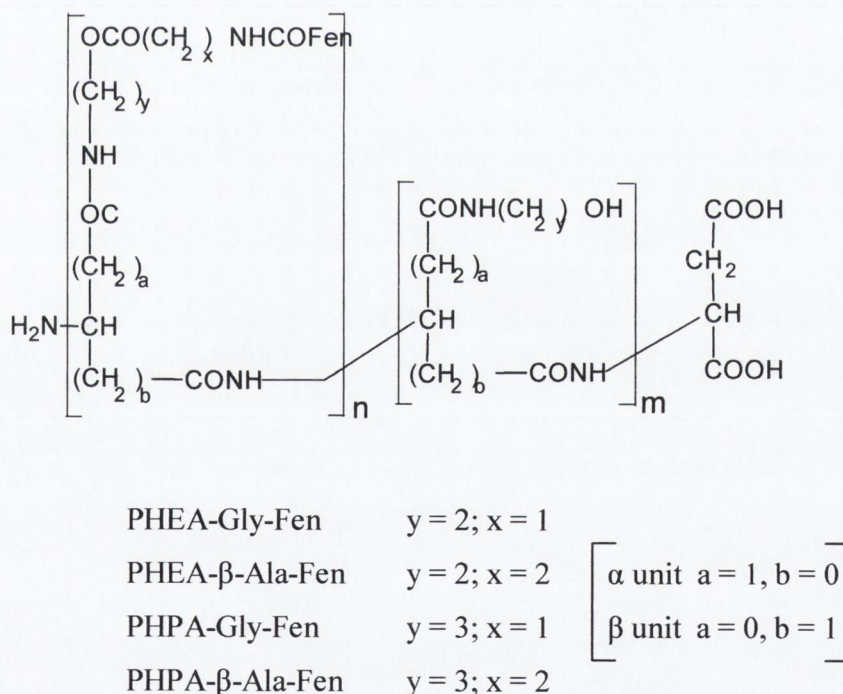


Figure 2.3 Chemical structures of some PHEA- and PHPA-fenoprofen conjugates.

Zovko et al (2001) synthesised these conjugates to enable the easier approach of enzymes which would theoretically facilitate cleavage of the covalent bonds between drug and spacer. However, this assumption still remains to be confirmed.

2.6.2 Glycolamide and other esters of aspirin

Nielsen and Bundgaard (1989) evaluated the ability of glycolamide and other esters of aspirin to act as true prodrugs. It emerged that a significant problem with some of these compounds was the lability of other groups in the molecule. For example, aspirin ester prodrugs could convert into aspirin, as intended, or alternatively could convert into the salicylic acid ester, with both finally forming salicylic acid (Figure 2.4).

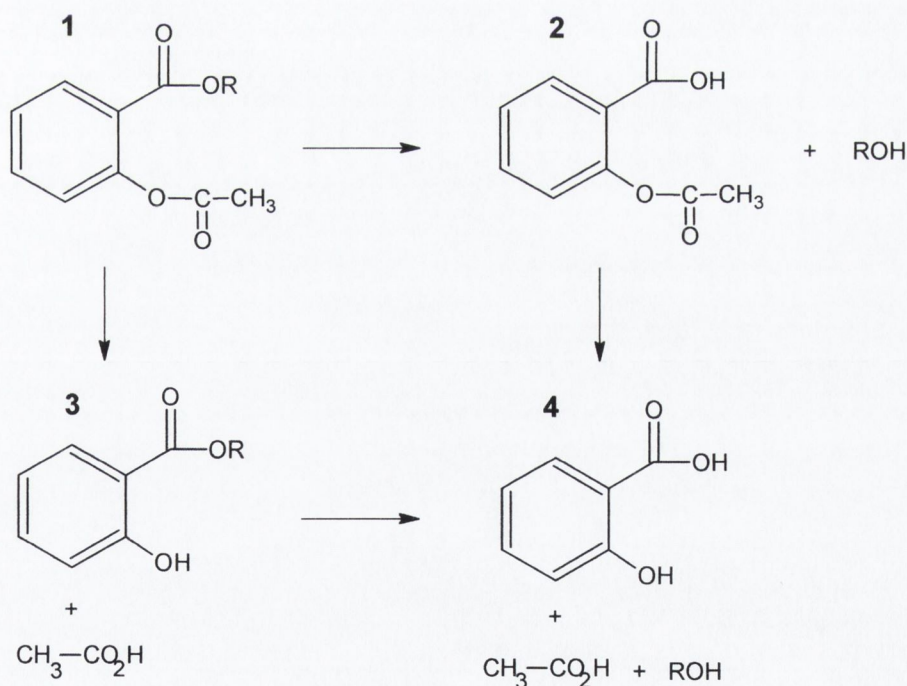


Figure 2.4 Chemical structures of the aspirin ester prodrug, 2-acetoxybenzoic acid (1) and its various metabolites: aspirin (2), the salicylic acid ester (3), and salicylic acid (4).

2.6.3 2-formylphenyl ester derivatives

Abordo et al. (1998) succeeded in synthesising 2-formylphenyl esters of indomethacin, ketoprofen and ibuprofen (Figure 2.5) which acted as true prodrugs by yielding the parent NSAID and acylphenol on hydrolysis, while maintaining anti-inflammatory activity.

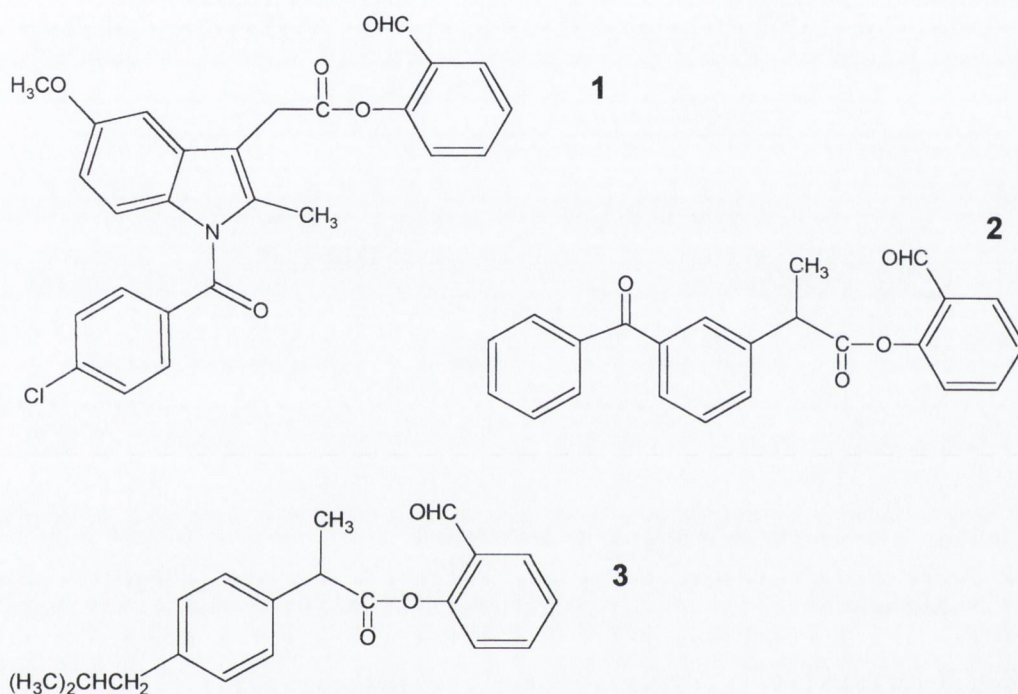


Figure 2.5 Chemical structures of the 2-formylphenyl esters of (1) indomethacin, (2) ketoprofen and (3) ibuprofen.

2.6.4 Aspirin derivatives

Work carried out by Banerjee and Amidon (1981a, 1981b, 1981c) reported attempts to synthesise aspirin derivatives based on enzyme substrate specificities, that would reduce the local gastric irritation and bleeding associated with aspirin therapy. This culminated in the development of three derivatives: (1) aspirin phenylalanine ethyl ester; (2) aspirin phenylalanine amide; and (3) aspirin phenylacetic ethyl ester (Figure 2.6).

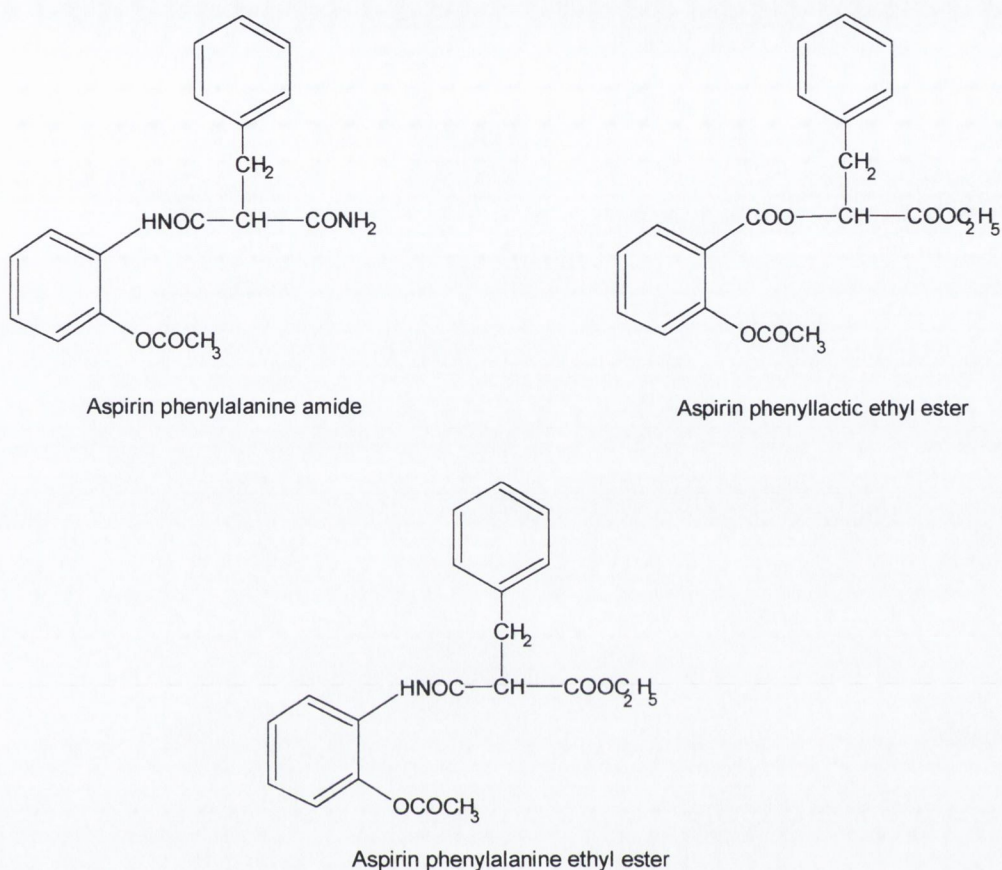


Figure 2.6 Chemical structures of the aspirin esters studied by Banjee et al. (1981).

The target reconversion sites for the prodrugs were the proteolytic enzymes α -chymotrypsin and carboxypeptidase A. The aspirin derivatives were designed so that after an initial cleavage of the terminal ethyl ester or amide linkage by α -chymotrypsin, carboxypeptidase A would liberate aspirin in vivo.

Their results showed that aspirin phenylalanine ethyl ester was the best substrate for α -chymotrypsin as it showed the highest rate of hydrolysis. The best substrate for carboxypeptidase A was aspirin phenyllactic acid (produced from the action of α -chymotrypsin on aspirin phenyllactic ethyl ester).

From these studies the authors were able to show that the aspirin derivatives could be successfully converted into aspirin *in vivo* but did not report if they offered any real advantage over the parent compound in terms of their side-effect profiles.

2.6.5 Ester and amide prodrugs of ibuprofen and naproxen

Shanbhag et al. (1992) synthesised and evaluated the anti-inflammatory activity and gastrointestinal toxicity of ester and amide prodrugs of ibuprofen and naproxen. Their results supported the existence of a direct contact effect and a systemic action of gastrointestinal irritation. Additionally, they showed that the direct contact effect was more important than the systemic effect in gastrointestinal irritation, and within the direct contact effect itself, inhibition of PG synthesis was more significant than the effect of the free carboxylic acid group of the NSAID.

2.6.6 Mutual prodrugs

Sheda et al. (2002) produced a mutual prodrug of naproxen by coupling it to propyphenazone through an ester linkage (Figure 2.7). Propyphenazone is a non-acidic pyrazole drug that has good analgesic and antipyretic activity with no anti-inflammatory activity (Burne, 1986). The rationale of the mutual prodrug approach was to have a compound with a synergistic analgesic effect from the two moieties with reduced GI irritation. They showed that the prodrug had improved GI toxicity compared to the parent compounds while maintaining anti-inflammatory and analgesic activity.

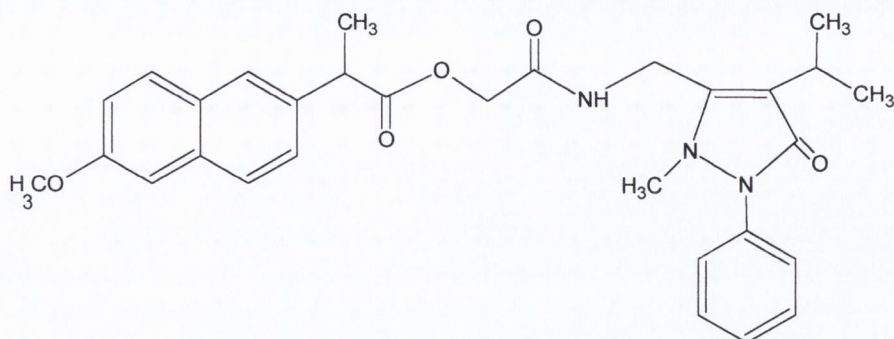


Figure 2.7 Chemical structure of naproxen-hydroxyacetamidopropylphenazone.

2.6.7 *Oligoethylene ester derivatives*

In a study of esters of indomethacin (De Caprariis et al., 1994) the parent acid was conjugated to oligoethylene groups, producing ester prodrugs employing oligoethylene glycols of molecular weight 106-282. All were cleaved rapidly in human plasma, were stable at pH 7.4 and 2.0 and exhibited anti-inflammatory activity equivalent to the parent drug, but were much less ulcerogenic, even at higher doses.

2.6.8 *Morpholinoalkyl ester derivatives*

The widely used NSAIDs indomethacin, naproxen (Tamarra et al., 1993) and diclofenac (Tammara et al., 1994) were derivatised to produce morpholinoalkyl esters (ethyl, propyl, butyl) as their HCl salts (Figure 2.8).

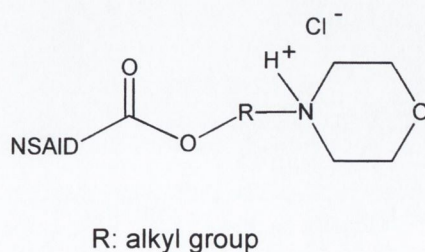


Figure 2.8 General chemical structure of the morpholinoalkyl ester derivatives.

The morpholino esters were chosen because of their balance of improved water solubility and lipophilicity relative to the parent carboxylic acid. All the prodrugs were highly water-soluble and partition coefficients (as free base) increased markedly. Hydrolysis was slow in simulated gastric juice or phosphate buffer (pH 7.4), but rapid in rat plasma and *in vivo*. The ethyl prodrug was not as irritating as the parent drug and increased oral bioavailability by approximately 30%.

2.6.9 Flurbiprofen derivatives

Tsunematsu et al. (1995) synthesised a range of flurbiprofen-basic amino acid ethyl esters (Figure 2.9) and examined the release of the optically active flurbiprofen enantiomers from the racemic flurbiprofen prodrugs by trypsin and two carboxypeptidases. The rationale for this was that flurbiprofen is used clinically as a racemate even though its therapeutic activity is due mainly to the S-enantiomer (Caldwell et al., 1988). To minimise the side-effects associated with flurbiprofen therapy it was postulated that it might be beneficial to develop water-soluble prodrugs of flurbiprofen from which the active S-enantiomer can be selectively released by enzymes. Their results showed stereoselective enzymatic hydrolysis of the flurbiprofen derivatives. They were not utilised as a flurbiprofen prodrug however, as the active S-enantiomer was not predominantly released by the carboxypeptidases.

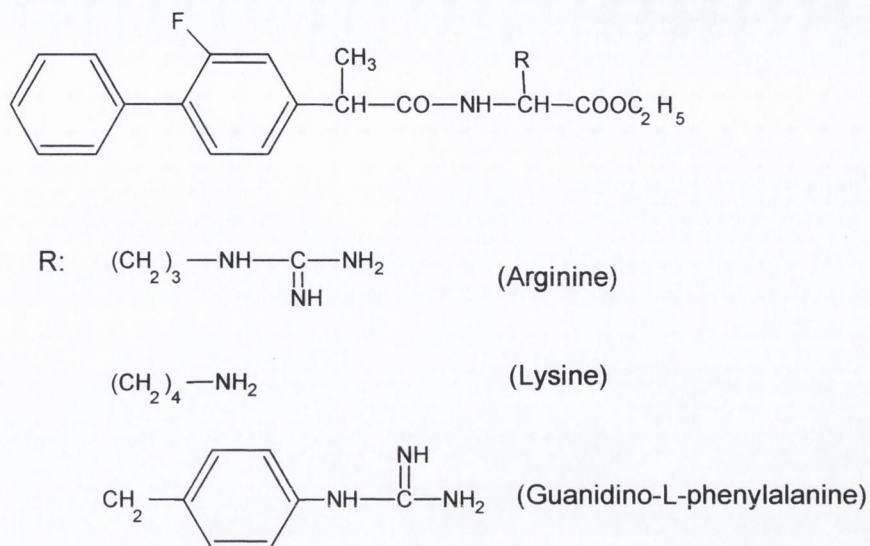


Figure 2.9 Chemical structures of ethyl esters of flurbiprofen L-arginine, flurbiprofen L-lysine and flurbiprofen *p*-guanidino-L-phenylalanine.

Another approach to developing a flurbiprofen prodrug has been reported by Ohmukai (1996). In this approach flurbiprofen axetil (Figure 2.10) was prepared by esterification of flurbiprofen. This makes the compound more lipophilic and soluble in lipid microspheres. The flurbiprofen-axetil was incorporated into lipid microspheres which could be administered intravenously without causing irritation at the site of injection. Once administered the prodrug was rapidly hydrolysed to the active parent compound which then exerted a therapeutic effect.

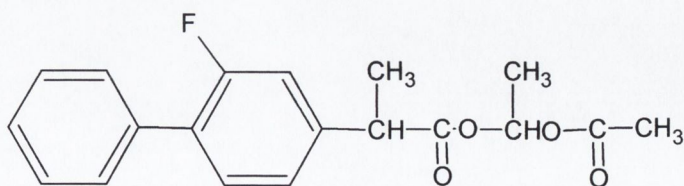
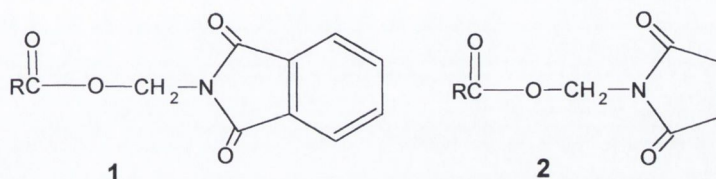


Figure 2.10 Chemical structure of flurbiprofen axetil.

2.6.10 Cyclic amide derivatives

Omar (1998) and Mahfouz et al. (1999) developed cyclic amide derivatives of aspirin, ibuprofen, naproxen and indomethacin (Figure 2.11) as potential prodrugs and reported rapid conversion to the parent NSAIDs in rabbit plasma and significantly less irritation to the gastric mucosa than the parent NSAIDs.



R = aspirin, ibuprofen, naproxen or indomethacin.

Figure 2.11 Chemical structures of N-hydroxymethylphthalimide (1) and N-hydroxymethylsuccinimide (2) derivatives of aspirin, ibuprofen, naproxen and indomethacin.

2.6.11 Nitric oxide (NO)-releasing NSAIDs

Another approach to counteracting the GI side-effects of NSAIDs, as mentioned in Section 1.4.2, has been to attach a NO-releasing moiety to standard NSAIDs (Wallace et al., 1994; del Soltado et al., 1997), the rationale being that NO plays a similar role to prostaglandins in gastric mucosal defence. A wide variety of NO-NSAIDs have been developed including nitroxybutyl esters of flurbiprofen (Arena and del Soltado, 1997) and naproxen (del Soltado, 1998) with the nitroxy groups providing NO by metabolic transformation (Artz et al., 1996). S-nitrosothiol derivatives of NSAIDs have also been reported (Garvey et al., 2000) in which NO is released by S-N bond cleavage. Another approach by Saavedra et al. (1999) involved the use of a molecular linking unit, with ibuprofen linked to a NO-releasing group via a piperazine unit. Similarly, Ingram et al. (1998) investigated the use of glycerol as a molecular linking unit to join the NO and NSAID moieties as glycerol allowed variation in the relative number of NSAID and NO groups incorporated into the prodrugs. More recently, Ingram et al. (2001) reported the

development of a diester of ibuprofen and glycerol-1-nitrate (glycerol-1,2-diibuprofenate-3-nitrate) (Figure 2.12).

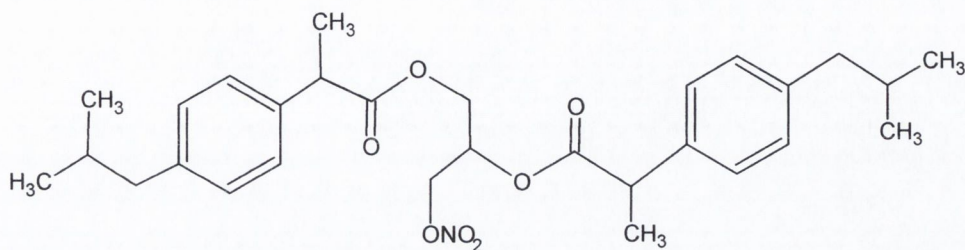


Figure 2.12 Chemical structure of glycerol-1,2-diibuprofenate-3-nitrate.

Ingram et al. (2001) showed that this ibuprofen derivative underwent hydrolysis to the parent compounds in a simulated gastric model, although their results suggested that there may be only partial hydrolysis of the compound over a normal 2-hour gastric emptying period and they did not examine the metabolism of the nitrate ester to NO.

A problem with all of these NO-NSAIDs is the concept that they actually donate NO *in vivo* following absorption from the GI tract is unproven (Rainsford, 2001). Also, it has been reported that the GI toxicity profiles of these derivatives are no better than for other carboxyl ester derivatives of NSAIDs and that their anti-inflammatory activity is similar to their parent compounds (Rainsford, 2001). Consequently, despite the number of NO-NSAIDs that have been developed, none are currently in clinical use and further investigations are warranted to determine if chemical modifications of the NO-donor group would achieve more selective delivery of the NO moiety.

Chapter 3

Drug absorption and gastrointestinal physiology

3.1 INTRODUCTION

One of the most important aims of the pharmaceutical industry, during the development of systemically acting drugs intended for oral administration, is the optimisation of bioavailability (Fagerholm et al., 1996). Bioavailability indicates both the quantity of the active component absorbed from the pharmaceutical form reaching the blood circulation, and the rate of this process. This implies that the molecule crosses one or several biological membranes before reaching the blood circulation. Many factors influence a drug's extent and rate of absorption after oral dosing (Aungst and Shen, 1986; Rozman and Klaassen, 1996). These can be categorised into properties relating to either the drug substance or the biological membranes. The physicochemical properties of the drug substance are considered to be the most important of these as they have been well established to influence passive drug absorption across the gastrointestinal (GI) wall. The most important of these are the drug's lipophilicity, degree of ionisation, and molecular size (Chan and Stewart, 1996; Navia and Chaturvedi, 1996; Krämer, 1999). However, it is rather simplistic to consider only these properties, as for a number of molecules there is no correlation between lipophilicity and oral bioavailability (Rubas and Cromwell, 1997; Lee et al., 1997) due to other factors which relate to the physiology of the GI tract.

3.2 PHYSICOCHEMICAL FACTORS AND THEIR IMPACT ON BIOAVAILABILITY

Physicochemical properties of a drug provide a good insight into how it will behave in the environment of the GI tract and for this reason they are often used as predictors of intestinal absorption. The most significant of these are lipophilicity, pK_a and molecular size.

3.2.1 *Lipophilicity*

A measure of a drug's lipophilicity is generally one of the first approximations in assessing its ability to cross biological membranes, particularly by passive diffusion (Stewart et al., 1997). Lipophilicity is not a single defined parameter, but the sum of different physicochemical characteristics and is traditionally expressed as the *n*-octanol-water partition coefficient (*P*). This is the concentration ratio of the

compound between *n*-octanol and an aqueous phase at equilibrium as shown in Equation 3.1. It describes the ability of a drug molecule to partition into the lipophilic phase, octanol, which is assumed to have a lipophilicity comparable to the cell membrane.

$$P = \frac{A_o \cdot V_w}{A_w \cdot V_o} \quad \text{Equation 3.1}$$

where *P* is the partition coefficient, *A_o* and *A_w* are the amounts of drug in the *n*-octanol and aqueous phases respectively, *V_w* is the volume of aqueous phase and *V_o* is the volume of *n*-octanol phase.

P is the true partition coefficient of one molecular species, such as of one ionisation species. The presence of more than one species results in an average partition coefficient called the apparent partition coefficient or distribution coefficient *D*, which is pH dependent in the case of ionisable compounds (Hogben et al., 1959). This is called the pH-partition hypothesis which suggests that partitioning of a molecule into a lipid environment or membrane is not only dependent on the so-called intrinsic partition coefficient of the uncharged compounds (*P*), but also on the degree of ionisation. The apparent distribution coefficient (*D*) is defined in Equation 3.2 (Leahy et al., 1989).

$$\text{Log } D = \text{Log } P + \text{Log } f_u = \text{Log } P + \text{Log } (1 - f_i) \quad \text{Equation 3.2}$$

where *f_u* is the fraction unionised and *f_i* is the fraction ionised. The correction terms *f_u* and (1-*f_i*) describe the pH dependence of Log *D* and are different for mono acids, mono bases, diacids, dibases, etc. (Avdeef, 1996).

Different correction terms were derived, such as Equations 3.3 and 3.4 for mono acids and mono bases respectively (Scherrer and Howard, 1977; Van de Waterbeemd and Testa, 1987), by neglecting partitioning of ionic forms into the organic phase for the different substance groups.

$$\text{acid : } \text{Log } D = \text{Log } P - \text{Log } (1 + 10^{(pH - pK_a)}) \quad \text{Equation 3.3}$$

$$\text{base : } \log D = \log P - \log (1 + 10^{(pK_a - pH)}) \quad \text{Equation 3.4}$$

As well as the shake flask method for the determination of partition coefficients, they can also be determined by potentiometric titration (Avdeef, 1993).

A study conducted by Kramer et al. (1999) compared the potentiometrically determined $\log D$ of a range of compounds with their apparent permeability coefficient across Caco-2 cell monolayers and with the fraction absorbed in humans after oral administration. The study confirmed that compounds with a $\log D$ values <-1.5 or >4.5 are poorly absorbed (permeability coefficient less than 20 nm sec^{-1}), whereas compounds with $\log D$ between 0-3 are highly permeable (permeability coefficient greater than 100 nm sec^{-1}). This supported the findings of Komiya et al. (1980) who demonstrated that for a group of steroids in the rat, a sigmoidal relationship exists between the permeability of the GI barrier to the steroid and the *n*-octanol-water partition coefficient of the drug. The permeability of the intestinal epithelium increases roughly with the lipophilicity of the drug molecule until it reaches a plateau at a $\log P$ value of approximately 2 (Martin, 1981). Drugs with $\log P$ values close to 2 are generally predicted to be completely absorbed in humans. However, for $\log P > 4$, the permeability starts to decrease (Wils et al., 1994) as very lipophilic drugs generally have low aqueous solubilities and, as a result will partition at slower rates from the cell membranes to the aqueous extracellular fluids (Raub et al., 1993).

Studies have shown that in general $\log P$ is a rough predictor of transcellular absorption of drugs *in vivo* and *in vitro* (Taylor et al., 1985; Buur et al., 1996). Considering that octanol is a relatively simple organic solvent and the cell membrane is a complex lipid bilayer, it is unusual that there is such agreement in the behaviour of drug molecules between the two different systems. However, Franks et al. (1993) have shown that hydrated octanol molecules are arranged in roughly spherical aggregates with a polar centre and a non-polar outer region, with a polar/non-polar interface between them. This suggests that hydrated octanol consists of a range of localised environments mimicking the more complex lipid bilayer and provides a possible explanation as to how $\log P$ values can be used to predict passive transcellular absorption.

The transcellular route is the main route for drug absorption. Transcellular diffusion requires dehydration of the compound and entry into the lipid bilayer of the cell membrane. From the outer lipid layer the compound has to switch to the inner layer and then travel from the apical side of the cell to the basolateral side, either through the cytoplasmic aqueous phase or along the lipid membranes of the cell, such as the inner plasma membrane leaflet or the endoplasmic reticulum and Golgi vesicles. At the basolateral side of the cell, the compound needs to cross the cell membrane again to exit. It is clear that transcellular diffusion must depend on the lipophilicity of the compound as the solute requires a certain affinity for lipid structures in order to enter the cell membrane.

3.2.2 Dissociation constant, pK_a

The dissociation constant (pK_a) of a drug and the pH at the absorption site often determine the absorption characteristics of a drug throughout the gastrointestinal tract. From the pH-partition hypothesis described above, the absorption of a weak electrolyte drug will be determined by the extent to which the drug exists in its unionised form at the site of absorption. The extent to which a weakly acidic or weakly basic drug with a single ionisable group ionises is described by the Henderson-Hasselbach equation (Equation 3.5)

$$\text{Log} \frac{[Base]}{[Acid]} = pH - pK_a \quad \text{Equation 3.5}$$

where pK_a is the negative logarithm of the acid dissociation constant of the drug and $[Base]$ and $[Acid]$ are the respective concentrations of the base and acid forms of the drug, which are in equilibrium and in solution in either the gastric fluid or the blood.

According to the pH partition hypothesis, drugs such as weak acids will be well absorbed from the stomach as they will be predominantly unionised in the acidic environment of the stomach. Conversely, weakly basic drugs will be poorly absorbed from the stomach as they will be predominantly ionised. The pH of the small intestinal fluids is less acidic than that of the stomach and thus the absorption of weak bases generally tends to be favoured over weak acids. However, weak acids are still

quite well absorbed from the small intestine. This is due to the much larger surface area of the small intestine when compared to the stomach, as well as the microclimate pH, which is lower than the bulk pH in the small intestine.

3.2.3 **Molecular size**

Diffusion of molecules through biological membranes has been shown to be dependent on molecular size (Xiang and Andersson, 1994). There are two aspects to this. Firstly, a relationship has been established between Log P and molecular size (Testa and Seiler, 1981) indicating that, in general, as molecular size increases so too does lipophilicity which is a determinant of membrane permeability. Secondly, despite passive transcellular diffusion through the membrane lipids, some compounds may go through the paracellular pathway. This route is limited by molecular size in a process called the 'sieving effect' (Leahy et al., 1989). Sietsema (1989) has shown that molecular weight reasonably substitutes molar volume or molecular size. The upper limit of molecular weight for paracellular transport varies in the literature from 200 (Lennernas, 1995) to 400-500 (Artursson et al., 1993) and is dependent on the flexibility and shape of the drug molecule.

An alternative to molecular weight is the molecular polar surface area (PSA), which is defined as the area occupied by nitrogen and oxygen atoms and the hydrogen atoms attached to these heteroatoms (Palm et al., 1997). PSA has been shown to be an excellent predictor of drug absorption and can be used to identify drugs that may be poorly absorbed at an early stage of the drug discovery process. A study carried out by Palm et al. (1997) showed that there is a sigmoidal relationship between PSA and fraction absorbed (FA). The sigmoidal relationship indicates that drugs with a PSA < 63 Å² will be completely absorbed (FA > 90%) while drugs with a PSA > 139 Å² will be < 10% absorbed. A more recent development in this area is the topological polar surface area (TPSA) which has been shown to agree closely with PSA absorption predictions, though requiring significantly less time to calculate (Ertl et al., 2003).

3.2.4 Biopharmaceutics Classification Scheme

The Biopharmaceutics Classification Scheme (BCS) (Amidon et al., 1995) is a useful way of dividing drug molecules into four classes (Table 3.1) on the basis of their aqueous solubility and their ability to permeate the mucosa of the gut from the apical to the basolateral side.

Table 3.1 *Definitions of the four Biopharmaceutic Drug Classes.*

Class	Aqueous solubility	Membrane Permeability
I	High	High
II	Low	High
III	High	Low
IV	Low	Low

It is essentially a classification of drugs based indirectly on their physicochemical properties, as these properties impact on the criteria that are most important to oral drug delivery, namely membrane permeability and aqueous solubility.

3.3 ANATOMY AND PHYSIOLOGY OF THE SMALL INTESTINE AND ITS IMPACT ON DRUG ABSORPTION

The small intestine is the principal site for digestion and the absorption of nutrients, water and electrolytes. It is divided lengthways into three unequally sized sections: the duodenum, the jejunum and the ileum. These comprise approximately 5, 50 and 45% of the length respectively (Ganong, 1995). Most absorption occurs in the duodenum and the proximal half of the jejunum and approximately 90% of all absorption in the GI tract occurs over the length of the small intestine (Ganong, 1995). The major mechanisms of absorption are passive diffusion, facilitated diffusion, active transport, pinocytosis, and solvent drag (as water moves through membrane pores in response to an osmotic gradient, small solutes are ‘dragged’ through with the flow of water) (Chhabra and Eastin, 1984; Hoensch and Schwenk, 1984; Granger et al., 1985; Pappenheimer and Reiss, 1987). The absorption of water and electrolytes also occurs in the large intestine. Substances that are candidates for

absorption but fail to do so in the ileum, and substances formed by bacterial metabolism are absorbed in the large intestine. Most nutrient processing that occurs here is due to bacteria and not intestinal mucosal/secretory activity as in the small intestine.

3.3.1 *Structure of the intestinal wall*

The GI tract is lined along its length with a mucous membrane (mucosa) the primary function of which is to act as a barrier to the entry of materials into the body. The mucosa can be divided into three distinct layers which are shown in Figure 3.1:

- i Muscularis mucosa
- ii Lamina propria
- iii Layer of epithelial cells

The lamina propria and the layer of epithelial cells are the most important with regard to absorption. The lamina propria is a thin layer of loose connective tissue that contains both blood and lymphatic vessels and it is contained by a continuous sheet of single layered epithelial cells. The lamina propria has a number of important functions including its immunological function and structural support for the intestinal epithelial cells. It also contains the blood and lymphatic vascular channels for the initial transport of material absorbed by the epithelium.

The muscularis mucosa is a continuous thin sheet of smooth muscle three to ten cells thick that separates the mucosa from the submucosa. Its function appears to be related to the rhythmic movements of the villi that agitate the layer of intestinal secretions and chyme in contact with the epithelium, thereby helping to promote absorption.

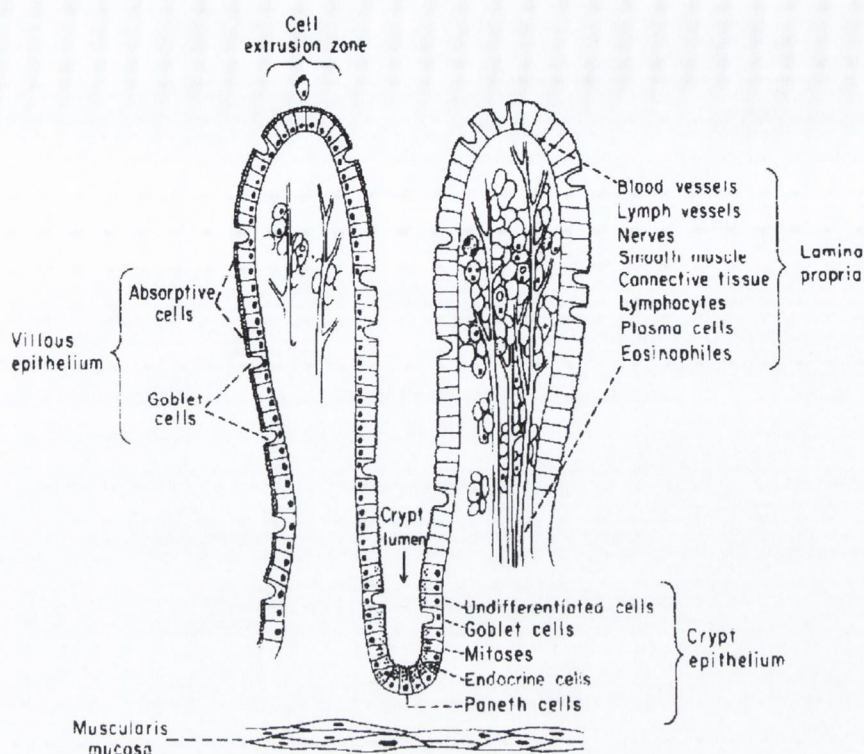


Figure 3.1 Schematic diagram of two sectioned villi and a crypt illustrating the histologic organisation of the mucosa of the small intestine.

Although the mucosa is present along the entire length of the GI tract, there are macroscopic differences between regions which may affect the ability of drug substances to reach the blood or lymph vessels.

In the stomach, the epithelial layer consists of mainly mucus secreting cells. There is little augmentation of the surface area and this limits drug uptake. This reflects its digestive rather than absorptive function and, despite the fact that from a physicochemical point of view there may be ideal conditions for drug absorption, transmucosal movement consists mainly of water and ion secretion (Macheras et al., 1995).

In contrast to the stomach, the small intestinal mucosa has a large surface area compared to the volume that it occupies. In humans, the surface area of the stomach is 0.053 m^2 and the surface area of the small intestine is 200 m^2 (Snyder et al., 1975). This large increase in surface area in the small intestine is produced by three levels of surface area enhancement, each one adding to the effect of the other: circular folds, villi and microvilli.

The circular folds triple the surface area of the small intestine, and protruding from these folds are the villi (Figure 3.1). Villi are finger-like projections approximately 0.5-1.5 mm in height that contain a core of lamina propria which is contained by a layer of epithelial cells. This epithelial layer can be split into two parts: the villous tip epithelium and the crypt epithelium. The tips of the villi are primarily concerned with nutrient absorption and consequently epithelial cells in this area are mainly absorptive cells called enterocytes, with a small number of goblet cells. The crypts of the villi contain undifferentiated cells, goblet cells and endocrine cells, reflecting their role in epithelial cell renewal and ion, water and endocrine secretion. Enterocytes, perhaps the most important cells in the small intestine, are columnar epithelial cells that are bound to their neighbours at the epithelial surface by tight junctions which limit direct contact between the contents of the lumen and the intercellular space. The luminal side of their cell membrane possesses numerous microvilli (estimated at 3000-7000 per cell in the small intestine). This further increases the surface area available for absorption by approximately 14-40 fold (Brown, 1962). Situated within the cell membrane of the microvilli are glycoprotein molecules which consist of a protein and a carbohydrate chain. The protein is embedded in the membrane while the carbohydrate chain protrudes from the cell membrane into the lumen. Collectively these carbohydrate chains form the glycocalyx (Trier, 1969) and in transmission electron micrographs this glycocalyx appears to consist of numerous fine filaments extending perpendicularly from, and attached directly to, the membrane of the microvilli. These glycoproteins mainly function as enzymes but may also act as carriers for the transfer of molecules across the membrane. They are thought to form passages called 'aqueous pores' as they behave as microscopic water-filled channels which allow small ions to selectively pass through.

3.3.2 Transport of substances across the intestinal epithelium

Absorption of a drug molecule from the lumen involves passage through several barriers: the epithelium, a portion of the lamina propria and the walls of the blood or lymph vessels. To pass through the epithelium, the substance can either pass through the epithelial cells by a number of mechanisms, or through the junctions between them (Pappenheimer and Reiss, 1987). These are represented diagrammatically in Figure 3.2.

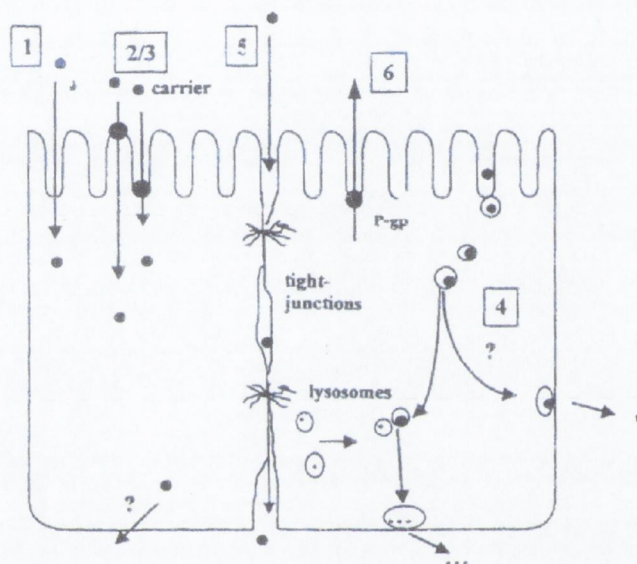


Figure 3.2 Potential modes of transport of molecules across the intestinal epithelium: (1) passive transcellular transport; (2/3) carrier-mediated transport; (4) endocytosis; (5) paracellular transport; (6) P-glycoprotein.

3.3.2.1 Passive Transcellular Transport

The process of passive transcellular transport is essentially a passive diffusion process. Initially it involves partition of a drug between the aqueous fluids in the GI tract and the apical cell membrane of the epithelial cells. The drug in solution in the membrane then diffuses across the membrane followed by a second partition of drug between the membrane and the aqueous fluids within the epithelial cells. The drug then traverses the basal cell membranes in a similar fashion and enters the blood of the capillary network in the lamina propria. In order for a molecule to diffuse through the membrane, it must be sufficiently hydrophobic to interact with the lipid bilayer of the cell membrane. The transcellular route is considered to be the main route of drug transport from the GI fluids to the blood and lymphatic systems as the cell membrane

surface area makes up more than 99% of the total surface area of the intestine. Also, it is an efficient route for a compound to traverse the intestinal epithelium along its entire length, unlike some carrier transport mechanisms that are localised within specific regions of the intestine (e.g. bile acid transporter in the ileum). For highly efficient absorption, the passive transcellular route is the preferred pathway because it is not as likely to be influenced by inter-population/genetic differences as carrier-mediated transport pathways, and it is not a saturable pathway. This permeation process, being passive in nature is mainly controlled by the concentration of drug remaining to be transported from one side of the membrane to the other. Most compounds obey this principle with the exception of highly branched molecules (Macheras et al., 1995) which are generally less permeable than theoretically expected. This is due to their permeation into the lipid layer being physically hindered as they produce a greater disturbance in the arrangement of the lipid bilayers. Additionally, passive transcellular transport of a compound is affected by its degree of ionisation, as the pH-partition hypothesis claims that only the non-ionised form of the compound permeates the cell membrane. Consequently, the ability of a compound to use the passive transcellular route depends on its pK_a , the pH on the luminal side of the membrane, and the lipophilicity of the non-ionised form of the compound.

3.3.2.2 Passive Paracellular Transport

When epithelial cells in the small intestine are viewed by electron microscopy they appear to be in such tight contact that the membrane seems continuous. However, the sites of contact, known as tight junctions, are permeable to water, electrolytes and small molecules. These junctions behave as aqueous channels and access to them is restricted by the presence of tight junctional elements at the apical pole of the epithelial cells. These are controlled within individual cells by a wide variety of physiologically relevant signals (Anderson and Van Itallie, 1995). The barrier varies in tightness by several orders of magnitude between so-called tight and leaky epithelia, shows a slight cation selectivity and the estimated pore radius is in the range 8-20 Å (Powell, 1981; Reuss, 1991).

By defining the characteristics of paracellular transport, tight junctions help define the overall transport characteristics of each epithelia. There have been attempts to enhance the permeability of the intestinal epithelium by regulation of the tight junctions, but this has been met with limited success (Swenson and Curatolo, 1992),

particularly as this pathway accounts for less than 1% of the surface area of the intestinal epithelium. In general, delivery of a compound through the paracellular pathway will only be successful if the compound has a low therapeutic dose and has a size appropriate to allow passage through the junctional complex.

3.3.2.3 Carrier-Mediated Transport

As previously mentioned, most drugs are absorbed from the GI tract by passive diffusion. However, carrier-mediated transport has been the focus of much recent research and it is now thought this may play a more important role in drug transport than was previously envisaged (Tsuji and Tamai, 1996). For example, benzoic acid was originally thought to be absorbed exclusively by transcellular passive diffusion in accordance with the pH-partition hypothesis, but it is now known that it relies mainly on the H^+ -monocarboxylate transporter (MCT) for absorption (Tsuji et al., 1994).

These carrier-mediated transporters may be dependent (active) or independent (passive) on adenosine triphosphate (ATP) for optimal function and involve participation of a carrier in the cell membrane, which may be an enzyme or some other component of the cell membrane. Most of these carriers are coupled to either Na^+ or H^+ ions and are driven by transmembrane ion gradients. The ions are cotransported with substrates and the downhill movement of the ions is energetically coupled with the uphill movement of the substrates. The ion-coupled transport systems are activated by their respective coupling ions due, in most cases, to the ability of the ions to increase the affinity of the transport systems for their substrates by producing conformational changes in the transporters (Brandsch et al., 1997). A whole range of these transporters have been identified in the intestine and include monocarboxylate transporters (Takanaga et al., 1994), organic cation transporters (Lauterbach, 1987), nucleoside transporters (Hung et al., 1993), and peptide transporters (Tsuji and Tamai, 1996).

i The monocarboxylate transporter (MCT) has been reported to transport monocarboxylate drugs such as salicylic acid by a H^+ -dependent carrier-mediated mechanism in the intestine and Caco-2 cells (Takanaga et al., 1994).

ii The organic cation transporters are known to transport cationic compounds across epithelia at several sites in the body including the intestine (Lauterbach, 1987)

and the liver (Klaassen and Watkins, 1984) by carrier-mediated organic cation (OC) transport processes. Many endogenous amines (e.g. dopamine) and some drug substances exist as cations at physiological pH and act as substrates for these transport processes. Two types of these OC transport processes have been identified: (a) a facilitative carrier-mediated system that is driven by an inside-negative membrane potential difference (Moseley et al., 1996); and (b) an energy-dependent OC^+/H^+ exchange mechanism that is driven by an inwardly directed proton gradient generated by H^+ efflux via Na^+/H^+ antiport and/or H^+ -ATPase (Ott et al., 1991).

iii Nucleosides are known to be transported by Na^+ -dependent systems in intestinal (Hung et al., 1993) and other epithelia around the body. The Na^+ -dependent systems consist of four major subtypes: N1, N2, N3 and N4. They vary in their location and nucleoside specificity and both N1 and N2 have been found in intestinal epithelia. N1 is found in bovine and rat renal (Williams and Jarvis, 1991) and intestinal epithelial cells (Hung et al., 1993) and exhibits selectivity for purine nucleosides and uridine. N2 is found in the brush border of bovine and murine renal (Williams and Jarvis, 1991) and intestinal epithelial cells (Vijayalakshmi and Belt, 1988) and is selective for pyrimidine nucleosides and adenosine.

iv The dipeptide transporter, PepT1, is a H^+ -coupled transporter protein and has been the subject of much recent research in intestinal drug delivery. Its density increases from the duodenum to the ileum (Tanaka et al., 1998), and at the villus level it is most abundant at the tip, decreasing towards its base (Ogihara et al., 1999). It has a relatively wide substrate specificity (Dantzig and Bergin, 1990; Liang et al., 1995) including di- and tri-peptides, β -lactam antibiotics, angiotensin converting enzyme inhibitors (Smith et al., 1993), and even compounds without an obvious peptide bond, such as δ -amino-levulinic acid (Temple et al., 1998). Compared to other intestinal transporters it has a robustness to adverse environmental conditions in the intestine (Tanaka, et al., 1998) and this raises the possibility that drugs targeted towards PepT1 may be less susceptible to wide variations in intestinal absorption than those drugs targeted towards other drug transporters.

v Carrier-mediated transport processes can also be involved in efflux mechanisms. An example of this is P-glycoprotein which is an active transporter that is expressed in a broad range of tissues, some of which include the oesophagus, stomach, jejunum and colon (Fojo et al., 1987). It was first characterised as the transporter responsible for the efflux of chemotherapeutic agents from resistant cancer cells (Gottesman and Pastan, 1993). Substrates for P-glycoprotein cover a broad range of structures with diverse therapeutic indications. There are no clear structural features that define P-glycoprotein substrates, but they tend to be large and contain one or more aromatic rings (Benet et al., 1999). The expression of this transporter at the apical membrane of enterocytes in the small intestine has been demonstrated to be an important mechanism for limiting the absorption of a variety of therapeutic agents (Hunter et al., 1993). The implications of this for drug absorption are that the transepithelial permeability of a drug-substrate for the transporter will be dependent not only on its passive permeability, but also on its affinity for P-glycoprotein contained in the apical membrane.

3.3.2.4 Endocytosis

Endocytosis involves a folding of the cell membrane and inclusion of the molecule in the resulting vesicle. This absorption mechanism is mainly associated with M cells which are specialised epithelial cells found at restricted sites throughout the GI tract. They over-lie mucosal lymphoid follicles and their function is to absorb antigens from the mucosa by endocytosis and transport them to the lymphoid tissue (Ermak et al., 1998).

Endocytosis can be further subdivided into fluid-phase endocytosis, receptor-mediated endocytosis and transcytosis. Fluid-phase endocytosis involves the uptake of small droplets of extracellular fluid by membrane vesicles. The efficiency of this process is low (Steinman et al., 1976). Receptor-mediated endocytosis involves the binding of ligands to receptors on the plasma membrane. The resulting complexes cluster in clathrin-coated pits that subsequently invaginate and pinch off from the membrane to form coated vesicles (Ermak et al., 1998). Once inside the cytoplasm the clathrin-coated vesicles lose their coat and release their contents to endosomes (prelysosomal vesicles). In transcytosis the endosomes carrying a ligand or ligand-receptor complex bypass the lysosomes and migrate towards the basolateral membrane and release the ligand to the extracellular space.

Generally, this mechanism of transport is rare and is only important in the case of macromolecular absorption (Daugherty and Mersny, 1999).

3.3.2.5 Aqueous pore transport

In situations where the permeation of small hydrophilic molecules does not correlate with the pH and the partition coefficient, it is believed that the transport of the drug molecule to the interior of the enterocyte mainly occurs through the aqueous pores or channels formed by proteins located in the cell membrane (glycoproteins) (Figure 3.3). The radii of the pores in the human small intestine are estimated to be approximately 0.7 nm in the jejunum and 0.3 nm in the ileum. It is generally considered that for a compound to be a candidate for uptake through the aqueous pores, it must have a molecular weight below about 300 (Macheras et al., 1995).

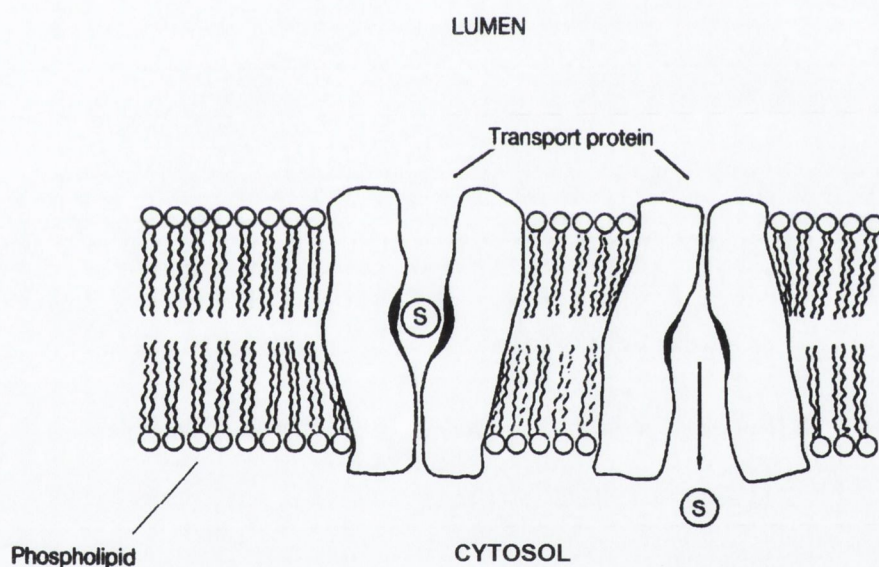


Figure 3.3 Transfer process of a small water-soluble molecule (S) to the interior of the cell through the aqueous pores.

3.3.3 The unstirred water layer

The unstirred water layer (UWL) is a stagnant layer of water, mucus and glycocalyx that lies adjacent to the intestinal wall. It is formed as it is almost impossible to agitate the contents of the lumen so that complete mixing occurs right up to the apical surface of the intestinal epithelial cells (Thomson and Dietschy, 1984). In order for a compound to be absorbed from the intestinal lumen and enter the blood or lymphatic

system, it must first diffuse through this unstirred water layer. The effect of this layer on the absorption of a compound is considered to be related to its ability to permeate the cell membrane (Thomson and Dietschy, 1984). For a compound with low membrane permeability, the rate limiting step in its transmucosal movement is the transport across the apical membrane and not diffusion through the UWL. Consequently, the UWL can be considered as a negligible barrier to the uptake of slowly absorbed drugs (Thomson and Dietschy, 1984). Conversely, if the compound has a high membrane permeability (apparent intestinal permeability coefficient, $P_{app} \geq 2 \times 10^{-4} \text{ cm s}^{-1}$), (Lennernas, 1994), then the UWL is thought to provide the main resistance to intestinal absorption (Levitt et al., 1984; Winne, 1987; Thomas and Dietschy, 1984). Since the compound is rapidly absorbed across the membrane, and it is only slowly replaced by molecules from the luminal contents due to the slow diffusion across the UWL, this creates a concentration gradient between the luminal side of the UWL and the intestinal wall. The effective thickness of the UWL (δ) is described by this concentration difference (Winne, 1984). The δ value is not a true value of the layer thickness, but is a representative value of the distance from the apical membrane to the point where the compound's concentration gradient reaches the homogenous concentration of the well-stirred bulk phase. It follows that compounds that have high membrane permeabilities will have the same P_{app} values, if their diffusion coefficients across the UWL are the same.

A number of studies have been carried out to evaluate the thickness and impact of the UWL on drug absorption, and the most common way to do this has been to determine the P_{app} or intestinal absorption rates at different perfusion flow rates in laboratory animals for highly permeable compounds (Winne, 1979; Levitt et al., 1987). Values of 300-800 μm have been reported. More recently, this figure has been re-evaluated to about 30-100 μm based on absorption data in humans and *in vivo* studies in unanaesthetised animals. These revised values are lower due to consideration of the increased gut motility/luminal stirring in the conscious *in vivo* animal compared to the anaesthetised animal (Andersson et al., 1988; Levitt et al., 1990; Levitt et al., 1992a; Levitt et al., 1992b).

Even more recently, it has been suggested by Fagerholm and Lennernas (1995) that the resistance of the UWL to the intestinal absorption of highly permeable compounds has been previously overestimated. They have proposed that intestinal

absorption in humans is membrane controlled for both low and high permeability compounds, irrespective of the transport mechanism. These proposals have been based on experimental results using two rapidly absorbed compounds, D-glucose and antipyrine, that showed no significant differences in either P_{app} or δ between the lowest and highest flow rates. They estimated the thickness of the UWL in humans to be 83-188 μm . This contradicts the previously reported and widely accepted values where an increased perfusion flow rate significantly reduced the δ value, and increased P_{app} for rapidly absorbed solutes (Winne, 1979 and Levitt et al., 1987).

3.3.4 Intestinal fluid flux

Water flux across intestinal membranes is a passive process that is probably driven by an osmotic gradient across the intestinal mucosa. This osmotic gradient might be a consequence of either active transport mechanisms (Tripathi and Boulpaep, 1989) and/or a Na^+ countercurrent multiplier (Jodal and Lundgren, 1986). The exact mechanism(s) involved in transmucosal fluid movement are still unclear, even though it is well known to occur by either the transcellular and/or paracellular routes. The paracellular route has been reported to be of major importance in the transmucosal flow of water and electrolytes. In animal studies it has been suggested that up to 85% of the transmucosal electrolyte flow is by this route (Madara and Pappenheimer, 1987). However, other research groups have suggested that the transport (in the mucosal to serosal direction) might occur through specialised channels present in the cell membrane (aqueous pores) (Tripathi and Boulpaep, 1989). This has been supported by the elucidation of the molecular structure of this transport channel (van Os et al., 1994).

It has been suggested that the upper part of the villus is the region of fluid absorption and the crypt is the region of fluid secretion (Chang and Rao, 1994). The major purpose of fluid secretion is to provide a suitable aqueous medium for proper digestion in the intestinal lumen. Additionally, it assists in the delivery of secretory immunoglobulins and helps flushing the crypts of infectious agents and noxious stimuli (Chang and Rao, 1994).

This movement of fluid across the intestinal mucosa has been shown to influence the absorption of drugs and other compounds both *in vitro* and *in situ* (Kitazawa et al.,

1975; Madara and Pappenheimer, 1987). The effect of transmucosal flow on membrane transport, a phenomenon called solvent drag, was first reported in 1957 by Andersson and Ussing. The effect has been quantitatively estimated (Kedem and Katchalsky, 1958) but the contribution of this effect to quantitative drug absorption *in vivo* in humans is controversial (Nilsson et al., 1994). Another hypothesis for explaining increased intestinal permeability values during fluid absorption status, relates to the increased concentration gradient close to the intestinal wall. This is a consequence of water absorption and might work to increase the net passive diffusion across the cell membrane (Fagerholm and Lennernas, 1995). This concentration gradient might also be reinforced by the washout flow of drug molecules within the enterocyte. This should then decrease the drug concentration in the cell cytosol, especially at the interior side of the apical cell membrane.

3.3.5 Intestinal pH

Fluid in the lumen of the small intestine is considered to have a pH in the range of 5-8, and in general this will vary from a pH of 5-7 in the duodenum to about pH 7-8 in the lower ileum (Florence and Atwood, 1988; Gruber et al., 1987). When discussing intestinal pH, a distinction is made between the pH of the environment at the membrane surface (microclimate pH) and the pH of the luminal contents.

Lucas et al. (1975) first demonstrated the presence of an acidic microclimate layer in the close vicinity of the cell surface of rat jejunum *in vitro*. Subsequently, Lucas' group and other researchers disclosed important properties of this pH layer. From these studies it has become clear that H^+ secretion by the intestinal epithelial cells and the presence of the surface mucus are of significant importance in the formation and maintenance of the acid microclimate, although there are many other factors affecting it. Studies have shown that there is a gradient of more than one pH unit in the upper jejunum of the rat, the gradient becomes smaller along the lower intestine. This gradient is augmented by glucose in the lumen and maintained by the mucus layer over the surface of the intestinal epithelium (Lucas et al., 1980; Shiau et al., 1985).

Rapid reactions occur at the membrane surface resulting in a dynamic surface pH which is constantly changing, and among the mechanisms of H^+ secretion by the intestinal epithelial cells, the Na^+/H^+ antiport has been shown to be the most important. Lucas and Blair (1978) first showed that the microclimate pH was

sensitive to the presence of sodium in the luminal medium, and Shimada (1987) confirmed the role of the Na^+/H^+ antiporter in the formation of the acid microclimate. Shimada (1987) also measured the microclimate pH and estimated the lowest value to be around 6.0 and the thickness of the microclimate layer to be around 600-700 μm .

Studies have shown that the acidic microclimate pH layer that is maintained in the normal small intestine can exert important effects on the absorption of weak acids or weak bases as suggested by Hogben et al. (1959). The microclimate pH will influence the ionisation of a drug at the membrane surface affecting the ratio of non-dissociated and dissociated species present and affecting the absorption of the drug via lipoidal or aqueous pathways, as shown by Ho et al. (1983) who reported that the surface pH significantly affected the intestinal absorption of weak acids.

Studies carried out by Shimada (1987) on changes in the microclimate pH in the presence and absence of dipeptides confirmed that dipeptides are co-transported with H^+ across the intestinal brush-border membrane. Changes in the content of the luminal fluid (particularly Na^+ and glucose concentrations) produced significant changes in the microclimate pH.

The changes in bulk luminal pH with time for different buffer systems perfused through the rat intestine were examined by Desai (1977). The pH of buffer systems, initially at pH 4.5, 6.5 and 9.5 respectively, all tended towards pH 6.5 with time as a result of the buffering actions of secretions, the rate of change depending on the degree of agitation and the buffer. Desai observed that, within the bulk lumen, a flux of relatively non-absorbable buffer species exists along with a constant flux of intestinal secretion of buffers which are mainly bicarbonates.

3.3.6 Buffer capacity of the intestine

Little data is available in the literature on the buffer capacity of the gastrointestinal contents under either fed or fasted conditions. It has been well established that the concentration of buffer in the dissolution media can have an influence on the dissolution rate of ionisable drugs (Higuchi et al., 1958; Mooney et al., 1981; McNamara and Amidon, 1988; Ozturk et al., 1988).

During *in situ* perfusion studies, a drug solution is perfused through a fixed length of intestine and its uptake is measured. In this situation, the buffer capacity of the perfusion solution may be of importance as it is apparent that the intestine has its own

intrinsic buffering ability (Desai, 1977). If the drug is a weak acid or base, any changes in the pH of the perfusion solution could have a significant effect on its degree of ionisation, and consequently on its absorption. From this perspective it is desirable to know the buffer capacity of the perfusion solution.

Buffer capacity (β), the ability of a solution to resist attempts to change its pH (Butler 1998), can be expressed numerically as the number of equivalents of strong base needed to change the pH value by one unit (Van Slyke, 1922). It can be calculated experimentally using Equation 3.6.

$$\beta = \frac{AB}{\Delta pH} \quad \text{Equation 3.6}$$

where AB is the amount of acid or base added and ΔpH is the pH change it produces. Once the composition of the buffer is known, its buffer capacity can be calculated using Equation 3.7 for a buffer based on a monoprotic acid (or a variation of it for di- or tri-protic systems).

$$\beta = 2.303 * \left(\left(\frac{K_w}{[H^+]} \right) + [H^+] + \left(C * K_a * \frac{[H^+]}{(K_a + [H^+])^2} \right) \right) \quad \text{Equation 3.7}$$

where C is the concentration of buffer salt in moles and K_a is the dissociation constant of the buffer salt. This shows that buffer capacity depends on the value of K_a , the concentration of the buffer and the pH (H^+ concentration). The buffer capacity of a particular system is at its maximum when the pH is equal to the pK_a of the buffer species.

K_a values are normally quoted at 25°C and consequently if a buffer is being used at room temperature or at 37°C, a correction is necessary. Also, K_a is affected by the ionic strength of the buffer, and this effect is described by the Deybe-Huckel relationship:

$$pK_a' = pK_a + (2z_a - 1) \cdot \left[\frac{A \cdot \sqrt{I}}{1 + \sqrt{I}} - 0.11 \right] \quad \text{Equation 3.8}$$

where Z_a is the charge on the conjugate acid species, I is the ionic strength and A is a temperature dependent constant with a value of approximately 0.5.

3.3.7 Gastric emptying and intestinal transit

The absorption of a compound from the small intestine is influenced by the time the drug is in contact with the intestinal surface. This will in turn be influenced by the gastric emptying and the intestinal transit, and consequently this is a controlling step in drug absorption. Gastric emptying is dependent on the gastric contents, but in general the gastric transit time for a meal is four hours (Granger et al., 1985). Gastric emptying is increased or decreased by a number of factors. Factors known to accelerate emptying include hunger, moderate physical exercise and drugs like metoclopramide. In the fasting state the stomach displays a complex temporal pattern of motor activity with alternating periods of quiescence and moderate contraction of varying frequency, culminating in an intense contraction, “the housekeeping wave”, that propels all gastric contents into the small intestine. In the presence of food, however, the irregular housekeeping waves are replaced by regular and more gentle contractions that continuously mix the gastric contents. Other factors also decrease gastric emptying such as emotion, pain, intensive physical exercise or certain drugs, for example anticholinergics.

Chyme traverses the human small intestine at a rate of $1\text{--}4\text{ cm min}^{-1}$ (Granger et al., 1985). This rate of transport is faster at the proximal portions of the small intestine (duodenum and proximal jejunum) and decreases as it approaches the ileum. On average, chyme traverses the entire small intestine in 3–4 hours. This relatively high rate of transit is of significance for the absorption of certain drugs where: (a) it penetrates the mucosa very slowly (*e.g.* acyclovir); (b) it has a slow dissolution rate or is in an extended release formulation; (c) it is absorbed selectively at a particular location in the intestine (*e.g.* lithium carbonate is absorbed by the small intestine but not by the colon). Transit time in the large intestine is considerably slower. Depending on the amount of fibre or other insoluble materials in the diet, transit time through the large intestine is 2–4 days (Granger et al., 1985). This relatively long transit time is an advantage when an extended release formulation is targeted to the large intestine. However, it is a disadvantage if the drug is incompletely absorbed on

its passage through the small intestine and is degraded by the colonic flora (e.g. indomethacin) (Macheras et al., 1995).

3.3.8 Mucus

Mucus is a complex visco-elastic layer that provides a protective covering over the epithelial surfaces of organs exposed to the external environment. The principal component of mucus is a high molecular weight glycoprotein, mucin, which is responsible for the viscous and gel-forming nature of mucus (Allen and Snary, 1972). Non-mucin components of mucus include lipids, water, sloughed epithelial cells, electrolytes and bacteria (Rathbone and Hadgraft, 1991) but the proportions of these may vary depending on the site of secretion. In the intestine, mucus is secreted by goblet cells that line the intestinal epithelium.

Mucin is found in two forms: soluble secretory mucin and membrane bound mucin (Filipe, 1979). Secretory mucins form viscous gels due to their high molecular weights and their ability to form intermolecular disulfide bridges. Membrane-bound mucins differ from secretory mucins as they contain a hydrophobic anchor holding the molecules in the plasma membrane and they lack intermolecular bonds. The carbohydrate chains of both types vary from 2 to 15 sugars in length and may be branched (Oates et al., 1974). These glycoproteins contain sulphate and sialic acid residues which have a pK_a of 2.6 (Johnson and Rainsford, 1972) which results in mucin having an overall negative charge at physiological pH. The repulsion between these anionic sialic acid and sulphate residues places the mucin glycoproteins in a stretched conformation. This expanded nature of the mucin network facilitates interpenetration of the glycoprotein molecules giving mucin a meshed structure. This mesh network of glycoproteins traps and immobilises water and produces the gel-like structure of mucus.

In addition to its roles in protection against mechanical damage and foreign bodies, preventing tissue water depletion, maintaining cellular integrity and acting as a lubricant to ease the transit of intestinal contents, various studies have indicated that it can also act as a potential barrier to drug absorption. The mucus layer promotes the formation of the UWL through which a drug must first diffuse before it can be absorbed across the epithelium (Thomson and Dietschy, 1977). The mesh of glycoproteins provide a physical barrier. Additionally, mucus can slow down or

reduce the absorption of some molecules by creating hydrogen or ionic bonds with the drug molecule (Bhat et al., 1995; Wikman-Larhed et al., 1997). This can occur particularly with low molecular weight, positively-charged drugs such as gentamicin, tobramycin and some β -lactam antibiotics (Niibuchi et al., 1986). However, the conformation of the mucin macromolecule is sensitive to its environment. Slight changes in pH, ionic strength, and the presence of drugs may significantly affect the extent of drug binding to mucus.

Despite the obstacle that mucus provides to drug delivery, it presents a unique opportunity for the sustained delivery of certain drugs via the development of mucoadhesive dosage forms (Longer et al., 1985). Over the past twenty years bioadhesives have been investigated as potential drug delivery systems for oral administration (Helliwell, 1993), and it is hoped that a delay in gastrointestinal transit brought about by an intimate and extended contact between bioadhesive and mucus lining will improve drug bioavailability.

3.3.9 Intestinal blood and lymph flow

The vessels of both the blood and lymphatic vascular systems supplying the intestine are contained in the lamina propria and are arranged in the villi as shown in Figure 3.4. The blood capillaries form a network that is located beside the basement membrane of the enterocytes. The density of this network varies with the region of the GI tract (Warwick and Williams, 1973) with the density decreasing from the proximal to the distal end of the small intestine. Not only are the vessels located close to the epithelia, but there are also openings in the endothelial cells of the blood vessels of approximately 500 Å in diameter that appear to facilitate the absorption of water and various water-soluble materials (Bloch and McCuskey, 1973).

The relationship between intestinal blood flow and absorption, secretion and metabolic activity of the intestinal mucosa is not very clear. It has been shown that reduced blood flow rates lead to reduced absorption rates. It has been suggested that this occurs by: (a) decreasing the effective concentration gradient across epithelial cells for passively absorbed solutes by not rapidly carrying the solute away, (b) lowering the oxygen supply to the absorptive cells which is needed to maintain the active transport mechanism and (c) causing metabolic changes which compromise the integrity of the membranes.

The lymphatic channels in the lamina propria consist of single sac-like vessels called lacteals that are located deeper in the lamina propria than the blood capillaries. They occupy the centres of the villi and their walls are composed of a single endothelial layer, but without fenestrations. They are permeable to even the largest chylomicrons (approximately 6000 Å in diameter) (Granger et al., 1985) due to the presence of permeable spaces between endothelial cells (Weiss, 1973).

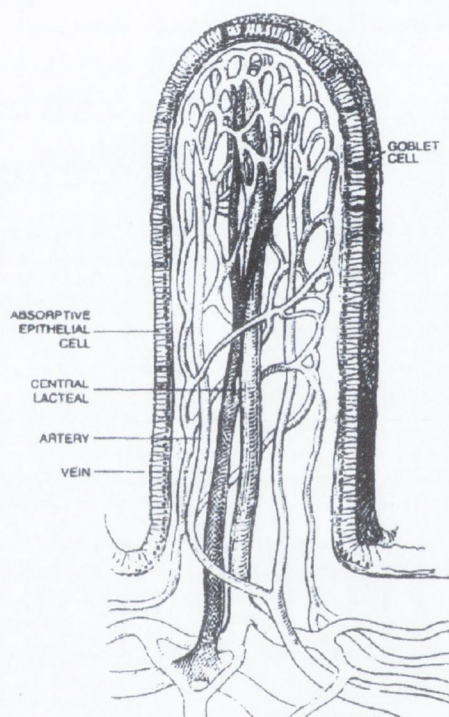


Figure 3.4 Arrangement of blood and lymphatic vessels within the villi.

Both vascular systems return their contents to the systemic circulation, but their routes of return vary. Blood from the small intestine flows directly to the liver where metabolism of drug molecules may occur in a process known as the first-pass effect. In contrast, lymphatic fluid from the small intestine eventually drains, via the thoracic duct, into the venous blood returning to the heart, and this allows drug molecules that are lymphatically absorbed to by-pass the liver and potential first-pass metabolism.

3.3.10 Intestinal secretions

The contents of the small intestine consist of, among other things, intestinal secretions, pancreatic enzymes and bile. These enzymes work to digest carbohydrates, proteins and fats into readily absorbed molecules. Others are involved in the metabolism of drug compounds which may be oxidised, hydrolysed and conjugated with large polar molecules in order to facilitate their excretion. From a digestion point of view, the most important secretions into the small intestine come from the pancreas and contain a range of enzymes responsible for digestion. These are listed in Table 3.2.

Table 3.2 *Enzymatic contents of pancreatic juice and their role in digestion.*

Secretion/Enzyme	Digestive Function
Bicarbonate	Keeps enteric contents at optimal pH for digestive enzymes
Amylase	Polysaccharides → di/oligosaccharides
Trypsin	Protein → small peptides
Chymotrypsin	Protein → small peptides
Elastase	Protein → small peptides
Carboxypeptidase	Cleaves amino acids from carboxy terminus of peptides
Lipase	Triglycerides → monoglycerides and free fatty acids
Phospholipase A2	Cleaves a free fatty acid from a phospholipid
Cholesterol esterase	Hydrolyses esters of cholesterol, Vitamin A and Vitamin D
Ribonuclease	Ribonucleic acids → oligoribonucleotides
Deoxyribonuclease	Deoxyribonucleic acids → oligodeoxyribonucleotides

The presence and activity of these enzymes may vary with age and sex, along the length of the digestive tract and the height of the villus, with time of day and diet, and among individuals (Chhabra and Eastin, 1984; Hoensch and Schwenk, 1984; Kaminsky and Fasco, 1992).

3.4 MODELS FOR STUDYING GASTROINTESTINAL DRUG ABSORPTION

Gastrointestinal permeability is one of the fundamental parameters controlling the rate and extent of drug bioavailability and this can be studied using *in vitro* and *in situ* models. Examples of *in vitro* absorption models include Ussing chambers (Ussing and Zerahn, 1951), cell culture models (*e.g.* the Caco-2 cell line established by Fogh et al., 1977), and everted gut sac technique (Wilson and Wiseman, 1954). The most common example of *in situ* absorption models is the *in situ* intestinal perfusion model (Komiya et al., 1980).

3.4.1 Cell culture models

The most commonly used cell culture models are obtained from immortalised cell lines. They can be obtained from a number of different tissues, and for intestinal permeability studies, cell lines derived from human colon cancers are the most commonly used. These cell lines have the capacity to differentiate and to become polarised in culture. There are three major intestinal cell models obtained from human colon cancer: Caco-2, HT-29 and T84, and of these the most widely used are Caco-2 monolayers. This cell line was established in 1974 (Fogh et al., 1977), and it is an immortal cell line derived from a human colon carcinoma that can be grown as a single layer on porous support. These cells morphologically resemble small intestine enterocytes with a well-defined brush border on the apical face expressing typical small intestine hydrolases, and well-formed tight junctions between the cells. The Caco-2 model enables both apical to baso-lateral and baso-lateral to apical transport to be studied, with the apical compartment mimicking the intestinal lumen and the basal compartment mimicking the blood stream. The two transport mechanisms (transcellular and paracellular) can be studied with this model (Gan et al., 1993; Inui et al., 1992; Boulenc et al., 1993). Several groups have demonstrated the presence of P-glycoprotein activity on the Caco-2 monolayers (Burton et al., 1993; Hunter et al., 1993). Thus the model can be useful to screen a large number of compounds likely to be P-glycoprotein substrates and in defining the limits produced by P-glycoprotein on the absorption of some drugs (Gan and Thakker, 1997). The Caco-2 model has also been used to evaluate the effects of a wide range of absorption enhancers (Anderberg

et al., 1992; Hovgaard and Bronstead, 1995). However, the Caco-2 junctions are tighter than those between normal small intestinal epithelial cells. Consequently, the effects of absorption promoters on the paracellular route (by opening tight junctions) will be exaggerated by this model and probably do not reflect the real effect of the potential enhancers at the small intestinal level *in vivo*. Another problem associated with the use of Caco-2 monolayers for the study of drug absorption is that they are a cancer-derived line and therefore have properties which differ from normal cells. Mature intestinal epithelial cells are highly developed biochemically, morphologically and functionally. There is a gradient of differentiation from the crypt regions of the small intestine to the main site of absorption at the villus tips. Also, the monolayers are devoid of mucin-producing goblet cells, so the impact of the mucus layer found on the intestinal epithelium cannot be evaluated. The main advantage of Caco-2 cells is that they are a human cell line and so do not suffer from the interspecies differences likely to be a factor when using animal models.

Cell lines that express specific transporters may be useful in transport or uptake studies of various drugs. An example of one of these is the Capan-2 cell line which has been derived from a pancreatic cancer (Oikawa et al., 1994) and which has been shown to express peptide transporters, and in particular the PepT1 transporter (Gonzalez et al., 1998). It can be used to study drug uptake by the peptide transporters and provides a convenient technique for the elucidation of new substrates, as well as for assessing compounds for the treatment of pancreatic cancer.

3.4.2 *In situ* perfusion studies

In situ experiments for studying intestinal drug uptake were first introduced in the late 1960s. Segments of the intestine of anaesthetised animals are cannulated and perfused by a solution of a drug and the amount of the drug which is taken up from the perfusate is calculated. Input of the drug compound can be closely controlled in terms of concentration, pH, osmolarity, intestinal region and flow rate. It is a good model of the *in vivo* system and is useful for producing kinetic data, particularly as the blood supply, innervation and clearance capabilities of the animal remain intact. *In situ* methods can also be used for regional absorption studies, and observing the gross effects of enhancers (Park and Mitra, 1992). The basic experimental procedure has been described by Komiya et al. (1980) and has been employed in several

intestinal drug absorption studies (Le Corre et al., 1998; Oguri et al., 1999; Pérez et al., 2002). Rats are fasted for at least 12 hours, anaesthetised and then an intestinal segment is isolated and cannulated. This segment is rinsed with an isotonic solution before being constantly perfused with a solution of the compound of interest. The perfusate is then collected at pre-determined intervals. If needed, marker molecules can be used to indicate that the viability of the intestinal tissue is maintained during the experiment. These are usually high molecular weight polyethylene glycol (PEG) molecules (*e.g.* PEG 4000) or phenol red and are used due to their lack of absorption from the intestine under physiological conditions (Fagerholm et al., 1996; Sutton and Rinaldi, 2001). Any drop in their intestinal concentration may indicate intestinal damage. They can also be used to monitor intestinal fluid flux (Raoof et al., 1998; Sutton and Rinaldi, 2001).

The measurement of the rate of decrease of the drug concentration in the perfusate does not always represent the rate of arrival of the drug in the vessels, in particular if pre-systemic or intracellular metabolism occurs. This problem can be overcome if measurements are made of the plasma drug concentration. Another disadvantage of these models is the large number of animals required compared with the rapid *in vitro* models. Also, studies are most often carried out on anaesthetised small animals and, recently it was shown that anaesthesia and surgical manipulation have pronounced effects on the absorption of some compounds. Uhing and Kimura (1995) showed that in a chronically catheterised rat model, the surgical manipulation of the intestine combined with anaesthesia caused an 86% decrease in the absorption rate of 3-O-methyl-glucose. Moreover, surgical manipulation and anaesthesia decreased the degree of intestinal flow and changed the distribution of intestinal blood flow, both parameters known to decrease intestinal absorption (Yuasa et al., 1993). Also, the *in situ* intestine is fully distended and the luminal hydrostatic pressure increases. This distension may affect the intestinal permeability or absorptive clearance (Chiou, 1994).

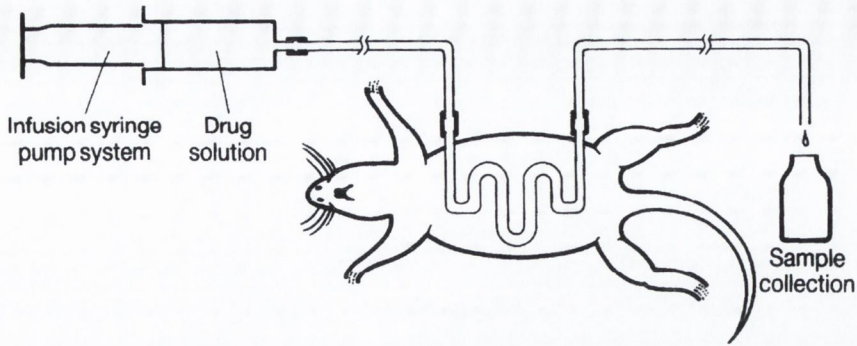


Figure 3.5 Schematic diagram of the *in situ* perfusion method of studying intestinal absorption.

Compared to *in vitro* systems (*e.g.* using chambers), the viability of the intestine is not such an issue as the *in situ* model maintains intact neural and endocrine environments as well as an intact blood and lymphatic supply. This ensures greater biological control of paracellular pathways (Lu et al., 1992). This model also allows access to the intestinal lumen which permits an estimate of intestinal drug metabolism that is not directly available from *in vivo* blood levels (Hui et al., 1994). This system also allows for sampling from both the intestinal lumen and the plasma compartment so that some evaluation of mass balance can be made.

3.5 MATHEMATICAL MODELS DESCRIBING GASTROINTESTINAL ABSORPTION

When drug absorption studies have been carried out using the *in situ* perfusion model described above, the results can be described mathematically by fitting them to one of two commonly used mathematical models. The first relates to non-steady-state fluid flow and absorption (Ni et al., 1980) and the second relates to steady-state drug absorption (Komiya et al., 1980). Both models consider the intestinal epithelium to be lipoidal in nature, perforated by a series of aqueous pores, to be in series with the UWL (or aqueous boundary layer) on the luminal side and with the blood/lymph vessels on the other, and any drop in perfusate drug concentration is due to absorption.

3.5.1 Non-steady-state model

As a drug in solution in the stomach flows into the intestine, a non-steady state concentration gradient occurs along the intestinal tract.

The following non-steady state expression describes the simultaneous effects of longitudinal spreading, fluid flow and drug absorption on the concentration of drug at any point 'x' along the intestine at time "t" (Ni et al. 1980).

$$\frac{dC_{(x,t)}}{dt} = \frac{\alpha dC_{(x,t)}}{dx^2} - \frac{\gamma dC_{(x,t)}}{dx} - \tau C_{(x,t)} \quad \text{Equation 3.9}$$

where $C_{(x,t)}$ is the concentraion of drug at any distance x along the intestine at time t (mg cm^{-3}), α is the longitudinal spreading coefficient ($\text{cm}^2 \text{sec}^{-1}$), γ is the linear flow velocity (cm sec^{-1}) and τ is the apparent first order absorption rate constant (sec^{-1})

The linear flow velocity (γ) which represents fluid flow can be further defined as follows by Equation 3.10:

$$\gamma = \frac{Q}{\pi r^2} \quad \text{Equation 3.10}$$

where Q is the bulk fluid flow rate ($\text{cm}^3 \text{sec}^{-1}$) and r is the effective radius of the intestinal lumen (cm).

The apparent first order constant (τ) can also be further defined:

$$\tau = 2 * P_e / r \quad \text{Equation 3.11}$$

where P_e is the apparent permeability coefficient for the absorption process.

The differential equation (first equation above) represents a general statement of the processes in the stomach and small intestine during simultaneous fluid flow and absorption. It thus provides a framework to quantify physiological, physical and physiochemical factors affecting bioavailability.

For an infinite drug reservoir with a constant infusion rate, Equation 3.9 can be simplified and solved using the Laplace transformation method (Ni et al., 1980), and the resulting equation can be expressed as follows:

$$F = 100 * \text{Exp}(B * X / (2 * A)) / 2 * (\text{Exp}(-W) * \text{erfc}(Y - Z) + \text{Exp}(W) * \text{erfc}(Y + Z))$$

$$\text{Equation 3.12}$$

where:

t = time (sec)

$A = D_e$

F = fraction of drug unabsorbed

$B = Q / (3.1416 * r^2)$

D_e = diffusion coefficient ($\text{cm}^2 \text{sec}^{-1}$)

$G = 2 * (P_e / r)$

Q = flow rate ($\text{cm}^3 \text{sec}^{-1}$)

$E = B^{2/(4 * A)} + G$

r = intestinal radius (cm)

$W = x * \sqrt{(E / A)}$

P_e = permeability coefficient (cm sec^{-1})

$Y = x / (2 * \sqrt{(A * T)})$

x = length of intestine (cm)

$Z = \sqrt{(E * t)}$

3.5.2 Steady state model

At the steady state, Equation 3.12 above reduces to Equation 3.13 (Ni et al., 1980).

$$P_{app} = \frac{-Q}{2\pi rl} \cdot \ln\left(\frac{C_l}{C_0}\right) \quad \text{Equation 3.13}$$

where P_{app} is the apparent permeability coefficient (cm sec^{-1}), C_0 is the input perfusate drug concentration, C_l is the outlet perfusate drug concentration, r is the effective lumen radius (cm), Q is the perfusate flow rate (ml sec^{-1}), and l is the length of intestinal segment (cm).

Komiya et al. (1980) used this model to establish the interplay of flow rate, aqueous boundary layer and membrane permeability, solute lipophilicity and intestinal length within an *in situ* intestinal perfusion model using a series of steroids. This study provided the experimental results to confirm the principles of gastrointestinal absorption described above.

The study utilised seven steroids ranging in three orders of magnitude in n-octanol / water partition coefficients. Flow rates varied from 0.1 to 4.2 ml min^{-1} . In the case of the membrane permeability (P_m) of the drug being much greater than the permeability of the aqueous boundary layer (P_{aq}) the experimental permeability coefficient P_{app} will approximate P_{aq} . In the case of absorption being membrane controlled the value of P_{app} will approximate P_m . Absorption of the most lipophilic steroids was found to be aqueous boundary layer controlled. This study provided experimental evidence of the sigmoidal relationship between the rate of absorption and lipophilicity of the diffusing species.

EXPERIMENTAL

Chapter 4

Materials and methods

4.1 MATERIALS

Material

Acetic acid (analytical grade) (CH_3COOH)
 Acetonitrile (CH_3CN)
 Buffer solution pH 4.0
 Buffer solution pH 7.0
 Calcium chloride ($\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$)
 Citric acid ($\text{C}_6\text{H}_8\text{O}_7 \cdot 6\text{H}_2\text{O}$)
 D-Glucose
 Deionised water
 Euthanal[®] (pentobarbitone sodium 200mg/ml)
 Filter holders, 13 mm, plastic
 Supor[®]-200 membrane filters (0.2 μm , 13 mm)
 Supor[®]-450 membrane filters (0.45 μm , 13 mm)
 Supor[®]-200 membrane filters (0.2 μm , 47 mm)
 Glycylsarcosine
³H-Glycylsarcosine
 Hanks Balanced Salt Solution
 Heparin injection BP (1000 Units ml^{-1})
 Ibuprofen acid
 Ibuprofen-alanine
 Ibuprofen-glycine
 Ibuprofen-L-arginine
 Ibuprofen-phenylalanine
 Ketoprofen acid
 Latex examination gloves
 Lipoid E PC phosphatidylcholine (lecithin)
 Liquid scintillation vials
 Magnesium sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)
 Methanol (HPLC grade)
 Naproxen acid
 Omnifix 5 ml syringes

Manufacturer

Scharlau
 Scharlau
 Merck
 Scharlau
 Riedel-de-Haen AG
 Merck
 Riedel-de-Haen AG
 Purite Prestige
 Rhone Merieux
 Gelman Sciences Inc.
 Gelman Sciences Inc.
 Gelman Sciences Inc.
 Gelman Sciences Inc.
 Sigma Chemical Co.
 Moravek Biochemicals
 Sigma Chemical Co.
 Leo Laboratories Ltd.
 Sigma Chemical Co.
 Dept. of Pharmaceutics
 Dept. of Pharmaceutics
 Dept. of Pharmaceutics
 Dept. of Pharmaceutics
 Sigma Chemical Co.
 Shermond Surgical Supplies
 Lipoid GMBH
 LIP Equipment Services
 BDH Chemicals Ltd.
 Labscan Ltd.
 Sigma Chemical Co.
 B.Braun

Omnifix-F 1 ml syringes	B.Braun
Orthophosphoric acid	BDH Chemicals Ltd.
Plastibrand [®] gilson pipette tips (50-1000 μ L)	Brand
Plastibrand [®] gilson pipette tips (2-200 μ L)	Brand
Plastibrand [®] microcentrifuge tubes (1.5 ml)	Brand
Polyethylene glycol 4000	Riedel-de-Haen AG
¹⁴ C-Polyethylene glycol 4000	Amersham Life Science
Potassium acid phosphate (KH ₂ PO ₄)	BDH Chemicals Ltd.
Potassium chloride (KCl)	BDH Chemicals Ltd.
Sagatal [®] (pentobarbitone sodium 60 mg ml ⁻¹)	Rhone Merieux
Sodium acid phosphate (NaH ₂ PO ₄ .2H ₂ O)	Merck
Sodium bicarbonate (NaHCO ₃)	BDH Chemicals Ltd.
Sodium chloride (NaCl)	Merck
Sodium hydroxide (NaOH)	Sigma Chemical Co.
Sodium phosphate (Na ₂ HPO ₄ .12H ₂ O)	Merck
Specimen glass vials (10 ml)	Samco
Sterican needles, 25G, 16 mm	B.Braun
Taurocholic acid (C ₂₆ H ₄₄ NO ₇ SNa)	Sigma Chemical Co.
Tolmetin sodium salt (C ₁₅ H ₁₄ NO ₃ Na.2H ₂ O)	Sigma Chemical Co.
Ultima Gold liquid scintillation cocktail	Packard
BD Vacutainers, 2 ml EDTA	Becton, Dickinson and Co.
Whatman filter paper no.1 (qualitative)	Whatman

4.2 INSTRUMENTATION

Instrument

Buchi vacuum system B-169
 Diode Array Spectrophotometer Model 8452A
 Electrothermal stirrer / hotplate
 Gallenkamp Economy Incubator (fan size 1)
 Gilson pipetman, P-200
 Gilson pipetman, P-1000
 Heidolph Stirrer RZR1
 Heto Temperature Controlled Water Bath
 Mettler Toledo MT5 Electronic Balance
 Omnifix-F 1ml syringes
 Orion 250A pH meter
 ROSS[®] semi-micro pH electrode
 Perfusor[®] V perfusion pump
 Plastibrand[®] microcentrifuge tubes
 PGT Spirit Energy Dispersive X-ray Analyser
 Reciprocal Shaking Water bath Model 25
 Laborota 4000 Rotary Evaporator
 Shimadzu LC-10AT liquid chromatograph
 Shimadzu SPD-10A UV-VIS detector
 Shimadzu SCL-10A system controller
 Shimadzu CLASS-VP Ver.6.1 software
 Shimadzu SIL-10AD autoinjector
 Sorvall RT 6000B refrigerated centrifuge
 Standard vial varisette sample holders
 Sterican needles 25G x 5/8"
 Titrino 702 SM autotitrator
 Tri-carb 2100TR Liquid Scintillation Analyzer
 Vortex
 Waters Spherisorb[®] 5 µm, C8, 4.6 x 250 mm

Manufacturer

Buchi
 Hewlett Packard
 Electrothermal
 Sanyo Gallenkamp PLC
 Gilson
 Gilson
 Heidolph
 Heto
 Mettler Toledo
 B. Braun
 Orion
 Orion
 B. Braun
 Lab. Equip. Manuf.
 Princeton Gamma-Tech Inc.
 Precision
 Heidolph
 Shimadzu Corporation
 Shimadzu Corporation
 Shimadzu Corporation
 Shimadzu Corporation
 Shimadzu Corporation
 Shimadzu Corporation
 Du Pont
 Packard
 B. Braun
 Metrohm
 Packard
 Scientific Equipment
 Labquip Ltd.

4.3 COMPUTER SOFTWARE

Software

cLog P for Windows[®] version 2.0.0b
Microsoft[®] Excel 2000
Microsoft[®] Word 2000
Minitab[®] Statistical Software version 13.1
Scientist[®] Experimental Data Fitting Programme
Sigma Plot[®] 8.0

Supplier

BioByte Corporation
Microsoft Corporation
Microsoft Corporation
Minitab Inc.
Micromath Scientific
SPSS Science, UK

4.4 METHODS

4.4.1 Buffer Systems

The compositions of the various buffer systems studied are given in Tables 4.1 to 4.3. The majority of the buffers were prepared by sequentially dissolving the individual components, in order of increasing concentration, in deionised water. The exceptions to this were: i) Hank's balanced salt solution (HBSS) which was supplied ready for use and ii) Fasted state simulated intestinal fluid (FaSSIF) and fed state simulated intestinal fluid (FeSSIF) which were prepared as described in Sections 4.4.1.1 and 4.4.1.2. respectively.

Table 4.1 *Compositions of Sorensen's phosphate buffering systems (PBS) (pHs 7.4, 6.8, 6.0 and 5.0) (Pharm. Handbook, 1980).*

Buffer Component	PBS pH 7.4	PBS pH 6.8	PBS pH 6.0	PBS pH 5.0
NaH ₂ PO ₄ ·2H ₂ O	13.46 mM	33.33 mM	53.20 mM	76.60mM
Na ₂ HPO ₄ ·12H ₂ O	53.31 mM	33.23 mM	13.40 mM	1.40 mM
NaCl	75.28 mM	82.14 mM	87.27 mM	71.87 mM

Table 4.2 *Compositions of fasted state simulated intestinal fluid (FaSSIF) and fed state simulated intestinal fluid (FeSSIF) (Galia et al., 1998).*

Buffer Component	FaSSIF pH6.5	FeSSIF pH5.0
KCl	103.29 mM	203.22 mM
KH ₂ PO ₄	28.66 mM	
Sodium taurocholate	3.0 mM	15.00 mM
Lecithin	0.75 mM	3.75 mM
NaOH	13.85 mM and q.s. pH 6.5	101.00 mM and q.s. pH 5.0
Acetic acid		144.11 mM

Table 4.3 *Compositions of Fagerholm's (Fagerholm et al., 1996), McIlvaine's (Pharm. Handbook, 1980) and Krebs' (Pharm. Codex, 1994) buffers and Hank's balanced salt solution (HBSS).*

<i>Buffer Component</i>	<i>Fagerholm's Buffer</i>	<i>McIlvaine's Buffer</i>	<i>Krebs' Buffer</i>	<i>HBSS</i>
NaH ₂ PO ₄ ·2H ₂ O	43.00 mM			
Na ₂ HPO ₄ ·12H ₂ O	28.00 mM	126.21 mM		0.34 mM
NaCl	48.00 mM		118.07 mM	136.89 mM
KCl	5.40 mM		4.69 mM	5.37 mM
D-Glucose	10.00 mM		10.09 mM	5.05 mM
Citric acid		36.64 mM		
MgSO ₄ ·7H ₂ O			1.18 mM	0.81 mM
KH ₂ PO ₄			1.18 mM	0.44 mM
NaHCO ₃			24.97 mM	4.17 mM
CaCl ₂ ·2H ₂ O			2.52 mM	1.26 mM

4.4.1.1 FaSSIF

This was prepared by dissolving the sodium hydroxide, potassium acid phosphate and potassium chloride in deionised water. The pH was adjusted to 6.5 using HCl or NaOH and the volume adjusted to 1 litre with deionised water. 0.825 g of sodium taurocholate was dissolved in approximately 125 ml of the pH 6.5 buffer and 2.95 mls of a 100 mg ml⁻¹ solution of lecithin in chloroform was added to this. The chloroform was then evaporated using a rotary-evaporator with the water bath at 40°C. Any remaining chloroform was blown off using a nitrogen stream. The final volume was adjusted to 500 ml using pH 6.5 buffer.

4.4.1.2 FeSSIF

This was prepared by dissolving the potassium chloride, acetic acid and sodium hydroxide in deionised water. The pH was adjusted to pH 5.0 using HCl or NaOH and the volume was adjusted to 1 litre with deionised water. 4.125 g of sodium taurocholate was dissolved in approximately 125 mls of the pH 5.0 buffer and 14.77 ml of a 100 mg ml⁻¹ solution of lecithin in chloroform was added to this. The chloroform was evaporated using a rotary-evaporator as before and any remaining chloroform was removed using a nitrogen stream. The final volume was adjusted to 500 ml using pH 5.0 buffer.

4.4.2 Solubility studies

4.4.2.1 Drug solubility in buffer

The solubility of a drug in a particular buffer was determined by the method of Chiou and Kyle, (1979). Excess drug was added to 50 ml of the buffer at 37°C in a jacketed vessel and the mixture was stirred at a rate of 300 rpm by an overhead stirrer. The temperature was maintained using a water bath connected to a Heto thermostat pumping motor. 3 ml samples were removed at regular time intervals over a two-hour time period. The samples were filtered through 0.2 µm membrane filters into glass vials. The filtrate was diluted immediately with buffer to prevent precipitation of the drug. After filtration and dilution, the samples were assayed spectrophotometrically using HPLC. All syringes, pipettes, filters, vials and needles used were preheated to 37°C in an oven. The solubilities quoted at each time point are an average of two determinations. The pH of both the mixture and filtrate was measured at each sampling point using an Orion 250A pH meter. In all cases, the pH of the mixture was reported as the pH values of a particular mixture and its corresponding filtrate were not significantly different.

4.4.2.2 pH-solubility profiles

Excess drug was added to 15 ml of deionised water at 37°C in a jacketed water vessel and the mixture was stirred at a rate of 300 rpm by an overhead stirrer. The temperature was maintained using a water bath connected to a Heto thermostat pumping motor. A 1 ml sample of the saturated solution was removed and filtered through 0.2 µm membrane filters into a glass vial. An appropriate quantity of the filtrate was diluted immediately with deionised water to prevent precipitation of the drug. The pH of the system was raised or lowered as appropriate by dropwise addition of 2M NaOH or 2M HCl respectively. The medium was allowed to equilibrate for at least 30 minutes and samples taken as before at different pH values. The pH of both the mixture and filtrate was measured at each sampling point using an Orion small volume electrode connected to an Orion 250A pH meter. The diluted samples were assayed by the appropriate HPLC method and the concentration of drug in solution was determined by reference to an appropriate calibration curve. pH solubility profiles were obtained in duplicate. All syringes, pipettes, filters, vials and needles used were preheated to 37°C in an oven.

4.4.3 High performance liquid chromatography (HPLC)

A modification of the HPLC method described by Healy and Corrigan (1992) for the quantitation of ibuprofen in solution was used. HPLC analysis of all compounds was performed using a Spherisorb[®] 5 μ m C8, 4.6 x 250 mm analytical column. The other components of the HPLC system were a Shimadzu LC-10AT liquid chromatograph, a Shimadzu SPD-10A UV-VIS detector and a Shimadzu SCL-10A system controller. For all compounds, the mobile phase used was methanol : 1% o-phosphoric acid (70:30) run at ambient temperature with a flow rate of 1.0 ml min⁻¹ and a sample injection volume of 20 μ L.

The wavelength of uv detection depended on the compound being assayed. Ibuprofen and its amino acid derivatives were detected at 222 nm. Ketoprofen was detected at 258 nm and naproxen was detected at 262 nm.

Peak area was used for quantitation. A calibration curve was produced each day the HPLC was run, using at least four standard solutions of the compound under assay.

4.4.4 Ibuprofen-calcium salt

4.4.4.1 Preparation of ibuprofen-calcium salt in Krebs buffer

Ibuprofen sodium was added to 25 ml of Krebs buffer in 10 mg increments with continuous stirring until a visible precipitate was formed and the pH of the system was measured. The precipitate was isolated by filtration through a 0.2 μ m membrane filter, rinsed with methanol, dried at 37°C and analysed by energy dispersive X-ray analysis (Section 4.4.4.2) and assayed for ibuprofen (Section 4.4.4.3).

4.4.4.2 Energy dispersive X-ray analysis of ibuprofen-calcium precipitate

Samples were mounted on aluminium slabs and subsequently analysed by energy dispersive x-ray analysis to provide information on the elemental composition of the samples.

4.4.4.3 Assay of ibuprofen-calcium precipitate for ibuprofen

A known amount of ibuprofen-calcium precipitate was dissolved in HPLC mobile phase and the quantity of ibuprofen in the sample was determined by HPLC (Section 4.4.3) against a standard ibuprofen acid solution.

4.4.5 Buffer capacity

Buffer capacity was measured by titrating 25 ml samples of buffer with 0.2M HCl or 0.2M NaOH in aliquots of 100 μ L using an auto-titrator, while continuously stirring. The titration with both acid and base was performed in duplicate. The pH of the solution was recorded after the addition of each aliquot and the buffering capacity (β) (expressed as equivalents/litre/pH unit) at each pH value was calculated using Equation 3.6:

$$\beta = \frac{AB}{\Delta pH} \quad \text{Equation 3.6}$$

where AB is the amount of acid or base added that produced the change in pH, ΔpH .

The buffer capacity for a monoprotic acid system is given by Equation 3.7:

$$\beta = 2.303 * \left(\left(\frac{K_w}{[H^+]} \right) + [H^+] + \left(C * K_a * \frac{[H^+]}{(K_a + [H^+])^2} \right) \right) \quad \text{Equation 3.7}$$

where C is the concentration of buffer salt in moles and K_a is the dissociation constant of the buffer salt. Buffer capacity was plotted versus pH and best-fit profiles were generated by fitting the experimental data to variations of Equation 3.7 (depending on whether the buffer concerned contained salts that were mono-, di-, or triprotic, and on the number of buffer species present; Appendices 1-4) using the Scientist curve fitting programme.

A buffer should show greatest buffer capacity (*i.e.* $\beta = \beta_{\max}$) when its pH is equal to the pK_a of the buffer salt (*i.e.* when $[H^+] = K_a$). Between pH 3-11, Equation 3.7 can be reduced to Equation 4.1 to predict reasonably the maximum buffer capacity.

$$\beta_{\max} = 2.303 * \frac{C}{4} \quad \text{Equation 4.1}$$

where C is the concentration of buffer salt in moles.

4.4.6 Purification of ibuprofen-amino acid derivatives

4.4.6.1 Ibuprofen-alanine, ibuprofen-glycine and ibuprofen-phenylalanine

To purify further a batch of ibuprofen-amino acid derivative after its synthesis, it was dissolved in deionised water and the mixture was made alkaline by the drop-wise addition of 1M NaOH to precipitate the basic impurities. The mixture was heated to 37°C and stirred for one hour to dissolve the drug. The mixture was then filtered through a 0.45 µm membrane filter to remove any undissolved impurities. The filtrate was cooled and acidified by the drop-wise addition of concentrated HCl to precipitate the drug. The drug was collected by filtration through filter paper and dried on a hot-plate at 37°C. A sample of the drug was assayed by HPLC using the method described in Section 4.4.3 to assess its purity.

4.4.6.2 Ibuprofen-L-arginine

To purify further the ibuprofen-L-arginine after its synthesis the compound was added to deionised water and the mixture was made alkaline by the drop-wise addition of 1 M NaOH to precipitate the basic impurities. The mixture was then filtered through a 0.45 µm membrane filter to remove any undissolved impurities. The filtrate was neutralised using 1 M HCl. The water in the filtrate was evaporated off using a rotary evaporator leaving behind the ibuprofen-L-arginine and sodium chloride. These two compounds were separated by dissolving the ibuprofen-L-arginine in methanol and removing the NaCl by filtration through a 0.45 µm membrane filter. The methanol was evaporated from the filtrate using a hot-plate and the ibuprofen-L-arginine remained in the beaker. A sample of the drug was assayed by HPLC using the method described in Section 4.4.3 to assess its purity.

4.4.7 *In situ* rat intestinal perfusion study

In situ absorption studies were carried out according to the single pass perfusion method described by Komiya et al. (1980) and is shown in Figure 3.5.

Male Wistar rats (280-320g) were used throughout the experiment and were fasted for 18-24 hours prior to the experiment but were allowed free access to water.

The rats were anaesthetised by an intraperitoneal injection of pentobarbitone sodium (50 mg kg⁻¹). A midline abdominal incision was made, and the small intestines were

removed from the body cavity, taking care to avoid disturbing the circulatory system. The proximal jejunum was cannulated with a glass L-shaped cannula which had an internal diameter of 2 mm and an outer diameter of 4 mm. The cannula was connected to an infusion pump and the intestine was perfused with buffer which had been preheated to 37°C. The small intestine became distended and the 33.3 cm required for the infusion was measured along the distended intestine using thread. The distal end of the intestine was then cannulated by a glass cannula of the same dimensions. The intestine was rearranged in a uniform multi-S arrangement in order to ensure a degree of uniform hydrodynamics. Perfusion of the segment with buffer was continued until the perfusate was clear.

The body temperature of the rat was kept as close as possible to normal using a heating mat placed under the animal and by means of an overhead lamp. The intestines were kept moist throughout the experiment using tissue which had been soaked in saline at 37°C.

The drug solution to be perfused (also heated to 37°C) was transferred to the infusion pump. The solution was then advanced through the cannula to the proximal cannulation point. At the start of the experiment (t_0) the pump was switched to a flow rate of 0.2 ml min⁻¹.

Perfusate samples were collected at ten minute intervals for a period of 120 minutes. Sample vials were weighed prior to use and after perfusate collection in order to monitor flow rate and to determine any variation in the volume of liquid collected. Blood samples were taken at 30 minute intervals and centrifuged to separate the plasma. Both perfusate and plasma samples were frozen until analysis could be carried out.

The animals were sacrificed at the end of the experiment with an injection of an overdose of pentobarbitone sodium.

4.4.8 Radiolabelled marker molecules

4.4.8.1 Preparation of ³H-Glycylsarcosine (Gly-Sar) solutions

The ³H-Gly-Sar was supplied as a solution in 40% ethanol. The solution had a radioactive concentration of 1 mCi per ml. 0.35 µL of this solution was included in each ml of perfusion solution. A 1.0 mM solution of 'cold' Gly-Sar was prepared in

deionised water and 10 μL of this solution was also included in each ml of perfusion solution.

4.4.8.2 Preparation of ^{14}C -PEG 4000 solutions

The ^{14}C -PEG 4000 was supplied as a solution in 3% ethanol. The solution had a radioactive concentration of 50 μCi per ml. 0.1 ml of this solution was diluted to 10 ml with deionised water. 40 μL of this solution was included in each ml of perfusion solution. 8.4 mg of 'cold' PEG 4000 was also included in each ml of perfusion solution.

4.4.9 Assay of perfusate pH

The pH values of the perfusate samples were measured prior to sample freezing using an Orion small volume electrode connected to an Orion 250A pH meter.

4.4.10 Validation of perfusion pump

Teflon tubing of internal diameter 2.0 mm and length 33.3 cm was connected between the two L-shaped cannulae and the junctions were sealed with Parafilm. The pump's syringe was filled with PBS 6.8 and the flow rate was set to 0.2 ml min^{-1} . The pump was started and the buffer was perfused through the tubing and collected in pre-weighed sample vials at 10-minute intervals. The average sample weight and standard deviation were recorded.

4.4.11 Assay of perfusate samples

4.4.11.1 Assay for drug content

Perfusate samples to be assayed for drug content were filtered through a $0.45 \mu\text{m}$ membrane filter and appropriately diluted prior to assay by the appropriate HPLC method as described in Section 4.4.3.

4.4.11.2 Assay for ^{14}C -PEG 4000

Samples were assayed for ^{14}C -PEG 4000 using a Tri-Carb liquid scintillation counter. A 0.1 ml volume of the sample was pipetted into a liquid scintillation vial and 10 ml of liquid scintillation cocktail (Ultima Gold) were added. This was mixed thoroughly and the disintegrations per minute (dpm) of the mixture were measured by the counter over a 10-minute period.

4.4.11.3 Assay for ^3H -Gly-Sar

Samples were assayed for ^3H -Gly-Sar using a Tri-Carb liquid scintillation counter. A 0.1 ml volume of the sample was pipetted into a liquid scintillation vial and 10 ml of liquid scintillation cocktail (Ultima Gold) were added. This was mixed thoroughly and the disintegrations per minute (dpm) of the mixture were measured by the counter over a 10-minute period.

4.4.12 Calculation of the apparent permeability coefficient (P_{app})

The fraction of compound unabsorbed was calculated for each perfusate sample by dividing the concentration in the perfusate sample (C_l) by the input perfusate concentration (C_0). At each time point these values were averaged over the number of rats studied and fraction unabsorbed against time profiles were plotted. The fraction unabsorbed values were converted to permeability coefficients using steady-state data and Equation 3.13.

$$P_{\text{app}} = \frac{-Q}{2\pi r l} * \ln\left(\frac{C_l}{C_0}\right) \quad \text{Equation 3.13}$$

where C_0 is the input perfusate drug concentration, C_l is the outlet perfusate drug concentration, r is the effective lumen radius (0.18 cm), Q , is the perfusate flow rate (ml sec^{-1}), and l is the length of intestinal segment (33.3 cm). For a particular system, the P_{app} values for each rat were averaged and a standard deviation calculated.

4.4.13 Corrections in P_{app} calculation

4.4.13.1 Alteration of 'Q' using perfusate sample weights

Perfusate sample weights were recorded and averaged over the perfusion experiment in each rat. These were then averaged over the number of rats studied in a particular system. This average sample weight could be converted to 'Q' by assuming that the sample weight is equal to its volume and dividing this by time:

$$Q = \frac{\text{Average Sample Weight}}{\text{Time}} \quad \text{Equation 4.2}$$

4.4.13.2 Correcting fraction unabsorbed (C_i/C_0) for fluid flux

This is based on the method described by Sutton and Rinaldi (2001) and Pérez et al. (2002) and involves the use of Equation 4.3 and assumes that perfusate sample weight is equal to its volume.

$$C_i' = C_i * \frac{W_{out}}{W_{in}} \quad \text{Equation 4.3}$$

where W_{out} is the average sample weight, W_{in} is 2.0 g (as the input flow rate is 0.2 ml min⁻¹ and samples are collected over a 10-minute period), C_i is the concentration of compound in the sample and C_i' is the concentration of compound in the sample after it has been corrected for fluid flux.

4.4.14 Calculation of amount absorbed

The amount of drug absorbed from the intestine was calculated as follows:

- a The amount of drug in each perfusate sample was calculated by multiplying concentration by volume.
- b These values were averaged at each time point over the number of rats studied in a particular system.
- c These average values were summed. This sum was equal to the amount of drug that left the intestine unabsorbed in an average rat for that system.

- d** Some drug remained behind in the intestine unabsorbed. This was estimated by multiplying the volume of the intestine by the steady-state perfusate concentration. The volume of the intestine was estimated as the volume of a cylinder of length 33.3 cm and radius 0.18 cm.
- e** The values from **c** and **d** were added to give the amount of drug that was unabsorbed.
- f** The amount of drug that entered the intestine was calculated by multiplying the inlet perfusate drug concentration (C_0) by the volume perfused (*i.e.* flow rate multiplied by time).
- g** The amount absorbed was then calculated by subtracting **e** from **f**.

4.4.15 Assay of plasma samples

4.4.15.1 Assay for ibuprofen, ibuprofen-alanine, ibuprofen-phenylalanine or ibuprofen-L-arginine

The assay is based on two previously reported methods by Lockwood and Wagner (1982) and Lalande et al. (1986). 100 μL of the plasma sample was added to a centrifuge tube and acidified with one drop of o-phosphoric acid (85% w/v). 1 ml of internal standard solution (tolmetin 30 $\mu\text{g ml}^{-1}$ acetonitrile) was added and the tube was sealed with parafilm and vortexed for 15 seconds to ensure thorough mixing and denaturation of the plasma proteins. The samples were centrifuged at 5000 G at 4°C for 15 minutes using a Sorvall refrigerated centrifuge. The supernatant was pipetted into an HPLC autosampler vial without dilution and analysed for drug and internal standard by HPLC as described in Section 4.4.3.

4.4.15.2 Assay for ketoprofen and naproxen

This is the same as the plasma assay described in Section 4.4.15.1, with the only change being the internal standard solution. In this assay ibuprofen (25 $\mu\text{g ml}^{-1}$ in acetonitrile) is used due to the similarity in retention times between tolmetin and ketoprofen or naproxen.

4.4.15.3 Assay for ibuprofen-glycine

This is the same as the plasma assay described in Section 4.4.15.1, with the only change being the internal standard solution. In this assay ibuprofen-phenylalanine ($35 \mu\text{g ml}^{-1}$ in acetonitrile) is used due to the similarity in retention times between tolmetin and ibuprofen-glycine. Ketoprofen and naproxen were also unsuitable as internal standards for the same reason.

4.4.16 Validation of plasma assays

The ability of the plasma assays to reliably extract and detect the relevant drug compound in plasma samples was validated by assessing their extraction efficiency, their precision and the linearity of response.

Extraction efficiency was calculated by comparing the peak areas of freshly prepared sample extracts (which had been obtained from plasma samples containing low, medium and high concentrations of the drug substance) with the peak areas obtained from analysis of corresponding directly injected standards (Campanero et al., 1999).

Precision was determined both within-day and between-day using the relative standard deviation (*RSD*) of peak areas obtained from replicate analyses of samples at appropriate concentrations (Ha et al., 1997). *RSD* was calculated using Equation 4.4.

$$RSD = \frac{s.d. \text{ of peak areas}}{\text{Average peak area}} \cdot 100\% \quad \text{Equation 4.4}$$

where *s.d.* refers to conventional standard deviation.

The linearity of the assays were assessed by the r-squared (r^2) values of the calibration curves.

The limit of quantitation (LOQ) of each assay was also assessed using a modification of the technique described by Paino and Moore (1999). A series of spiked plasma samples were prepared with each sample being half the concentration of the previous one. Each sample was extracted three times and a *RSD* value was calculated for each sample. The LOQ was taken as the lowest concentration for which the *RSD* was less than 10%.

For ibuprofen, ketoprofen and naproxen, all of these validation techniques were employed. Due to the limited availability of the amino acid derivatives of ibuprofen,

the linearity of the assays and their LOQ values were the only techniques used. Validation results are given in Appendix 14.

4.4.17 Histology

4.4.17.1 Preparation of tissue samples

Tissue samples for histological evaluation were prepared from the perfused intestinal segment after the animal had been sacrificed, based on the method described by Swenson et al. (1994). The intestinal tissues were fixed *in situ* by immediately perfusing a 20 ml volume of 10% phosphate buffered formalin (pH 7) at a flow rate of 0.2 ml min⁻¹. The perfused intestinal segment was then removed and immersed in the same fixative. Cross-sections were serially sectioned at 5 µm, stained with haematoxylin-eosin and mounted on glass slides.

4.4.17.2 Histological evaluation of tissue samples

Tissue samples were examined using light microscopy by an experienced pathologist in a blinded fashion, based on the method described by Swenson et al. (1994). Various measures of histological abnormality were quantitated on an arbitrary scale of 0–3 with 0 indicating no effect and 3 indicating an extensive effect. The measures of histological abnormality included the presence of mucus/debris, villous shortening, erosion, swollen epithelial cells, flat epithelial cells and goblet cells.

Mucus/debris refers to the presence of basophilic material and lysed cells in the lumen. Villous shortening refers to apparent retraction of villi. Erosion refers to loss of epithelium, exposing the lamina propria without ulceration. Swollen epithelial cells are an indication of cytoplasmic fluid gain. Flat epithelial cells refers to cells which are short and have spread laterally in an apparent attempt to cover voids in the epithelium. Goblet cells refers to the concentration of goblet cells and is an indication of relative loss of columnar epithelial cells.

4.4.18 Cell culture drug uptake studies

These were carried out in tissue culture models established by the University of Michigan using a modification of the method previously described by Gonzalez et al.

(1998). Capan-2 cells were grown in McCoy's 5A medium containing 10% heat-inactivated foetal bovine serum, 2 mM glutamine and 100 units ml⁻¹ penicillin-100 mg ml⁻¹ streptomycin mixture and non-essential amino acids.

Cells were washed twice with transporter buffer (pH 6.0, 1 mM CaCl₂, 1 M MgCl₂, 150 mM NaCl, 3 mM KCl, 1 mM NaH₂PO₄, 5 mM D-Glucose, 5 mM MES) and incubated with 10 µM Gly-Sar (9.94 µM Gly-Sar and 0.06 µM ³H-Gly-Sar) in 1 ml transporter buffer for 30 minutes at room temperature. In the drug inhibition study, the compound under study was also included in the transporter buffer. After 30 minutes, the uptake was stopped by the addition of 0.5 ml ice-cold transporter buffer. Cells were washed 3 times with ice-cold transporter buffer, collected in 0.5 ml 1.5% Triton X-100, and sonicated 3 times for 10 seconds. Two hundred µL of sonicated cell suspension was used for scintillation counting.

Gly-Sar uptake in the presence of the drug under study was expressed as a percentage of the Gly-Sar uptake in the absence of drug.

4.4.19 Statistical techniques used for data analysis

All statistical analyses were performed with the aid of Microsoft Excel 97 for Windows on a PC.

4.4.19.1 Student's t-test

Student's t-tests were applied when comparing two independent samples. The null hypothesis was tested using a t-ratio complying with a student's t-distribution having $n-1$ degrees of freedom, where n is the sample number for each sample group. All tests were conducted at the 0.05 level of significance. The t-ratio of two sample means is given by Equation 4.5:

$$t = \frac{\bar{Y}_2 - \bar{Y}_1}{\sqrt{\frac{2s^2}{n}}} \quad \text{Equation 4.5}$$

where t is the t-ratio, $\bar{Y}_1$ is the mean of sample 1, $\bar{Y}_2$ is the mean of sample 2 and s^2 is given by Equation 4.6:

$$s^2 = \frac{s.d._1^2 + s.d._2^2}{2} \quad \text{Equation 4.6}$$

where $s.d._1$ is the standard deviation of sample 1 and $s.d._2$ is the standard deviation of sample 2.

4.4.19.2 ANOVA

The generalisation of the t-test to permit comparisons of more than two sample means is called analysis of variance (ANOVA). This involves the statistical separation of the total sum of squares and the total degrees of freedom into i) random variation within experiments and ii) variation between experiments. These were performed using Minitab Statistical Software version 13.1.

4.4.19.3 Curve fitting

Goodness-of-fit statistics for various models were calculated, where indicated, by Scientist (version 2.0) or by Sigma Plot 8.0. These are experimental data fitting programs which are capable of fitting data to non-linear curves.

When using Scientist, two statistical parameters were used to determine this goodness-of-fit: the model selection criterion (MSC) and the r-squared (r^2) (Scientist Handbook, 1995). When using Sigma Plot, only the r^2 was used. The MSC is calculated using Equation 4.7:

$$MSC = \ln \left[\frac{\sum_{i=1}^n w_i (Y_{obs_i} - \bar{Y}_{obs})^2}{\sum_{i=1}^n w_i (Y_{obs_i} - \bar{Y}_{cal_i})^2} \right] - \frac{2 p'}{n} \quad \text{Equation 4.7}$$

where n is the number of data points, w_i are the weights applied to each point, Y_{obs_i} , are the observed data points, Y_{obs} the predicted data points, Y_{cal_i} the weighted mean of the observed data and p' is the number of parameters estimated. The MSC value is affected by the number of parameters being estimated and will place a higher burden on models with a greater number of parameters, such that, models fitted with different numbers of parameters can be compared (Scientist Handbook, 1995). Equal weighting was used for all data sets during data fitting.

The r^2 for curve fitting is calculated using Equation 4.8:

$$r^2 = \frac{\left| \sum_{i=1}^n w_i^2 \cdot Y_{obs_i}^2 - \sum_{i=1}^n w_i^2 (Y_{obs_i} - Y_{cal_i})^2 \right|}{\sum_{i=1}^n w_i^2 \cdot Y_{obs_i}^2} \quad \text{Equation 4.8}$$

where n is the number of points and w_i are the weights applied to each point.

4.4.19.4 Regression analysis

Regression analysis was performed to produce a linear function to predict the value of one of the variables, given a new value for the other. Regression equations are accompanied by an r^2 value, which gives an estimate of the level of variation of the experimental data attributed to the regression equation. The remainder of the variation may be attributable to random effects. Given n pairs of variables (X_1, Y_1) , $(X_2, Y_2), \dots, (X_n, Y_n)$ of variables X and Y , the correlation coefficient, r , is defined in Equation 4.9:

$$r = \frac{\frac{1}{n} \sum (X_n - \bar{X})(Y_n - \bar{Y})}{S_x S_y} \quad \text{Equation 4.9}$$

where S_x and S_y are the standard deviations of the samples of X and of Y , $\bar{X}$ is the mean of samples X , $\bar{Y}$ is the mean of samples Y , r^2 is the coefficient of determination. A high value of r^2 , approaching 1, would give an indication of a strong relationship between the variables involved in the regression model.

4.4.20 Calculation of cLog P

The cLog P value of a compound is a calculated Log P value. This was determined using the SMILES based program cLog P for Windows version 2.0.0b.

4.4.21 Calculation of topological polar surface area

The topological polar surface area (TPSA) of a compound was calculated using a SMILES based program supplied by Ertl et al., (2003).

RESULTS AND DISCUSSION

Chapter 5

Effect of buffer composition on the solubility and absorption of ibuprofen

5.1 INTRODUCTION

The key biopharmaceutical characteristics impacting on bioavailability and on the likelihood of obtaining a good *in vitro in vivo* correlation (IVIVC) are the drug's solubility (C_s) and permeability coefficient (P_{app}) (Amidon et al., 1995). However these properties may be sensitive to experimental medium composition, particularly if the drug is ionisable in the medium in which the parameters are determined.

Particularly for ionisable drugs, the dissolution medium pH is important because of the influence of pH on solubility, dissolution and the level of sink conditions (Mooney et al., 1981; Aunins et al., 1985). Thus media containing HCl, acetate, citrate, phosphate or Tris in the pH range 1-7.6 are often used. However, the buffer capacity of such media of equivalent pH often varies despite evidence that buffer capacity at a given pH can substantially influence the dissolution rate of ionisable drugs and excipients (Mooney et al., 1981; Aunins et al., 1985; Ramtoola and Corrigan, 1989). In this regard both Prasad et al. (1983) and Skelly et al. (1986) studied dissolution rates of quinidine gluconate products. The dissolution rates of these products were widely different in water, acetate buffer of pH 5.4 and phosphate buffer of pH 5.4. The results highlighted the importance of buffer composition as well as pH on the dissolution rates.

A variety of *in vitro* and *in vivo* models for studying drug absorption have been developed. These include Ussing chambers, everted gut sac techniques, cell culture models, *in situ* perfusions and intestinal perfusions in man (Stewart et al., 1997). Using these methods, researchers have employed a variety of different buffer solutions to assess the absorption properties of drug substances. These range from simple phosphate based systems (PBS) at pH 7.4 (Iwanaga et al., 1999) to more complex systems containing lipids and surfactants to simulate intestinal contents i.e. the fasted (FaSSIF) and fed (FeSSIF) state simulated intestinal fluids (Galia et al., 1998). Xiang et al. (2002) used McIlvaine's citrate-phosphate buffer to study transbuccal drug delivery, while Hank's balanced salt solution (Crowe and Lemaire, 1998) and Krebs' buffer (Kim et al., 1994; Leone-Bay et al., 1996) have been employed for intestinal absorption studies.

Fagerholm et al. (1996) used a phosphate based buffer system (pH 6.5) to compare the P_{app} values of a range of drugs between rat and human jejunum. They obtained a high correlation between perfused rat and human jejunum P_{app} estimates, and

therefore concluded that the perfused rat model can be used with precision to predict *in vivo* oral absorption in man.

In this section the influence of a range of commonly used 'physiological' media on ibuprofen C_s and P_{app} was examined, the latter obtained using the *in situ* perfused rat model.

5.2 BUFFER SYSTEMS

The buffer systems studied are listed below and their compositions are given in Section 4.4.1.

- i. Sørensen's Phosphate buffer pH 7.4 (Pharm. Handbook, 1980; Iwanaga et al., 1999)
- ii. Sodium phosphate perfusion solution (Fagerholm et al., 1996)
- iii. McIlvaine's buffer (Pharm. Handbook, 1980; Xiang et al., 2002) pH 6.0
- iv. Hank's balanced salt solution (HBSS) (Crowe and Lemaire, 1998)
- v. Krebs' buffer (Pharm. Codex, 1994; Leone-Bay et al., 1996)
- vi. Sørensen's Phosphate buffer pH 6.8 (Pharm. Handbook, 1980)
- vii. Fasted state simulated intestinal fluid (FaSSIF) (Galia et al., 1998)
- viii. Fed state simulated intestinal fluid (FeSSIF) (Galia et al., 1998)

5.3 SOLUBILITY STUDIES

The solubility of ibuprofen in a particular buffer was determined by the method of Chiou and Kyle (1979) as described in Section 4.4.2.1. The solubilities quoted at each time point are an average of two determinations.

Figure 5.1 shows the best-fit solubility profiles (Scientist[®]) of ibuprofen in each of the eight buffer systems.

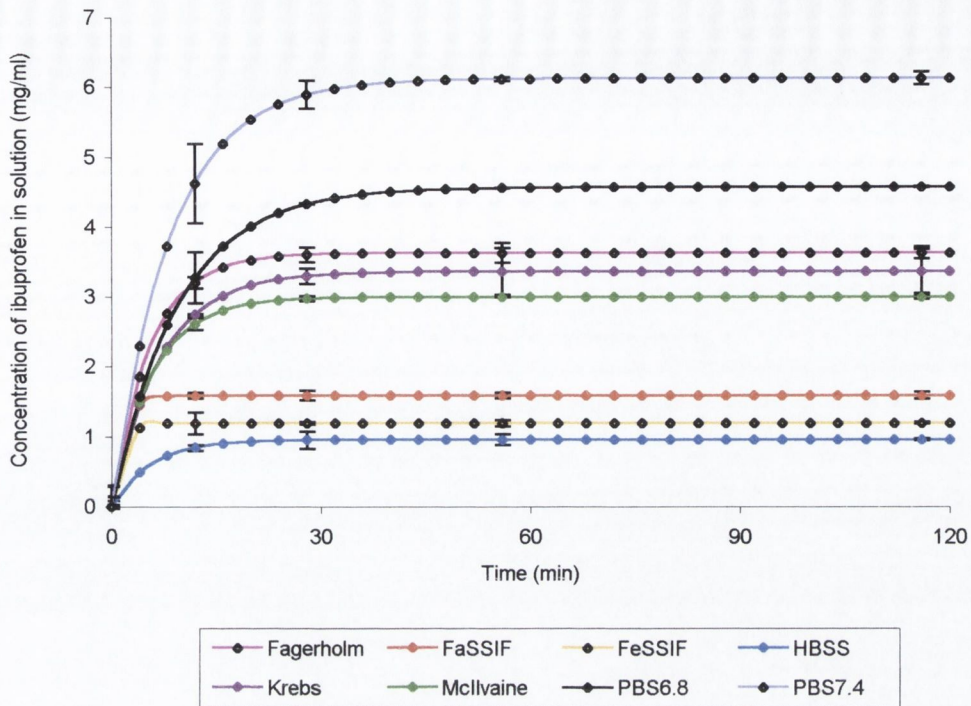


Figure 5.1 Ibuprofen concentration versus time profiles in each of the buffer systems.

The solubility of ibuprofen varied significantly with the buffer, with a six-fold difference between HBSS and PBS 7.4. The solubility profiles in Figure 5.1 were fitted to Equation 5.1.

$$C = C_s (1 - e^{-k \cdot t}) \quad \text{Equation 5.1}$$

where C is the concentration at a particular time point t , C_s is the saturated solubility and k is the rate constant. Saturation was reached within 1 hour. There were slight differences in the rate of attainment of steady state. The k values in Table 5.1 indicate that ibuprofen in FaSSIF and FeSSIF attained a steady state 3-4 times faster than the simple buffer systems. FaSSIF and FeSSIF differ from the other systems as they contain sodium taurocholate, which has surfactant properties thus promoting better wetting of the ibuprofen particles which would explain the higher solubility rate.

Table 5.1 *Solubility of ibuprofen in each of the buffers at 37 °C, ionic strengths (IS), pH values of the saturated solutions and buffer osmolarities.*

Buffer System	Solubility C_s (mg ml ⁻¹)	s.d.	k (min ⁻¹)	IS	Initial pH of buffer	Final pH	Osmolarity (milliosmoles/l)
PBS 7.4	6.02	0.10	0.12	0.21	7.21	6.35	337.5
PBS 6.8	4.55	0.04	0.11	0.20	6.72	6.25	330.6
Fagerholm (pH6.5)	3.65	0.09	0.18	0.17	6.56	6.15	286.8
Krebs	3.45	0.09	0.16	0.16	8.22	6.93	317.8
McIlvaine (pH6.0)	2.99	0.03	0.17	0.37	6.19	6.05	415.3
FaSSIF (pH6.5)	1.56	0.04	0.61	0.15	6.38	5.85	270.0
FeSSIF (pH5.0)	1.16	0.05	0.74	0.32	5.01	4.98	635.0
HBSS	0.97	0.05	0.18	0.15	7.56	5.52	310.7

The eight buffers studied varied in pH over a range of 3.21 pH units from 5.01 to 8.22. The final pH values of the saturated solutions of ibuprofen in each of the systems and the corresponding solubility values were fitted to Equation 5.2 (Scientist[®]) to generate the pH-solubility profile (Figure 5.2):

$$C_s = C_0 \left(1 + 10^{pH - pK_a} \right) \quad \text{Equation 5.2}$$

where C_0 is the intrinsic solubility. The best-fit values for C_0 and the pK_a of ibuprofen are 0.068 mg ml⁻¹ (0.33 mM) and 4.43 at 37°C respectively, which when corrected for ionic strength gives a pK_a of 4.57. These best-fit values were determined in the absence of the FeSSIF and Krebs' data points as they were shown to be outliers. These agree closely with previously reported values of 0.078 mg ml⁻¹ (0.38 mM) and 4.55 at room temperature by Fini et al. (1995). The slightly higher intrinsic solubility determined by these authors may be due to ionic strength. Fini et al. (1995) have also shown that in an aqueous medium containing 0.5 M NaCl (ionic strength 0.5), the solubility of the sodium salt of ibuprofen is 42.23 mg ml⁻¹ (185 mM) at 25°C. The ionic strength, sodium ion concentration and solubility are all significantly higher than in any of the systems used in this study (Table 5.1), indicating that the solubility of ibuprofen in the systems studied is not limited by the solubility of the ibuprofen sodium salt.

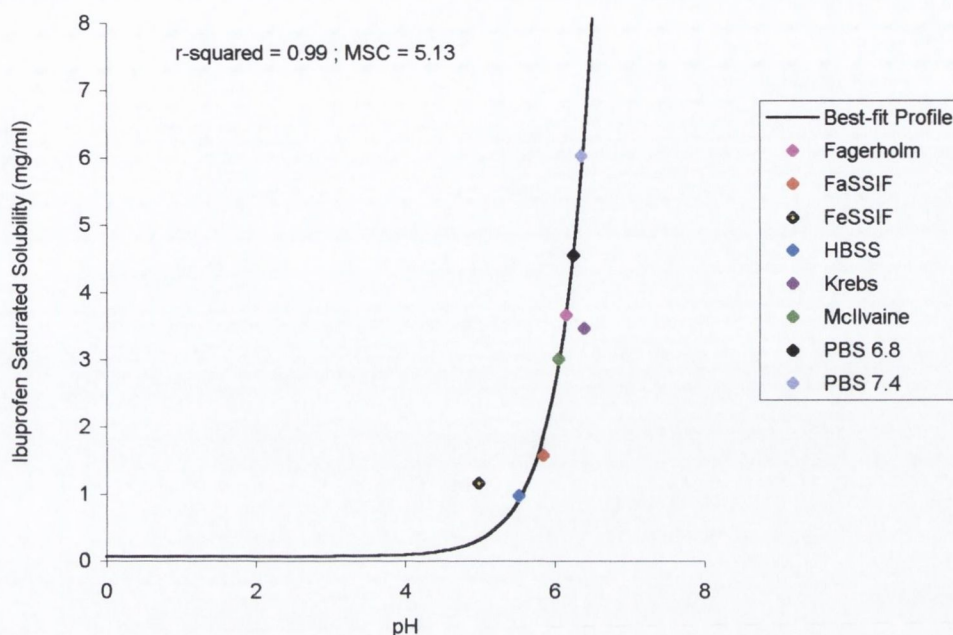


Figure 5.2 pH-solubility profile of ibuprofen acid.

5.3.1 Effect of sodium taurocholate

The solubility of ibuprofen in FeSSIF is significantly higher than predicted from Equation 5.2 even when the pK_a is corrected (to 4.43) to account for ionic strength. The higher solubility is likely due to the presence of sodium taurocholate (15 mM) which is above its critical micellar concentration (Poelma et al., 1990; Staggers et al., 1990) leading to aggregation with lecithin in the buffer, and mixed micelle formation. The hydrophobic nature of the micelle centre allows a higher than expected amount of ibuprofen to dissolve in the buffer at pH 4.98 as the ibuprofen partitions into the micelle.

5.3.2 Effect of divalent ions

The solubility of ibuprofen in Krebs' is significantly lower than predicted at pH 6.39 from Equation 5.2, again using a pK_a of 4.43 to account for ionic strength. The presence of divalent ions (Ca^{2+} 2.52 mM; Mg^{2+} 1.18 mM) in Krebs' buffer was investigated as a possible cause, using the method described in Section 4.4.4.1 which involved the gradual addition of excess ibuprofen sodium to Krebs' buffer.

After the addition of 90 mg of ibuprofen sodium to 25 mls of Krebs' buffer (13.6 mM solution), a precipitate was formed which was collected and examined by energy dispersive X-ray analysis. The scan obtained (Figure 5.3) shows two major peaks corresponding to carbon (0.3 keV) and calcium (3.6 keV). HPLC analysis of the precipitate quantitated the amount of ibuprofen in the precipitate (92.69 % w/w).

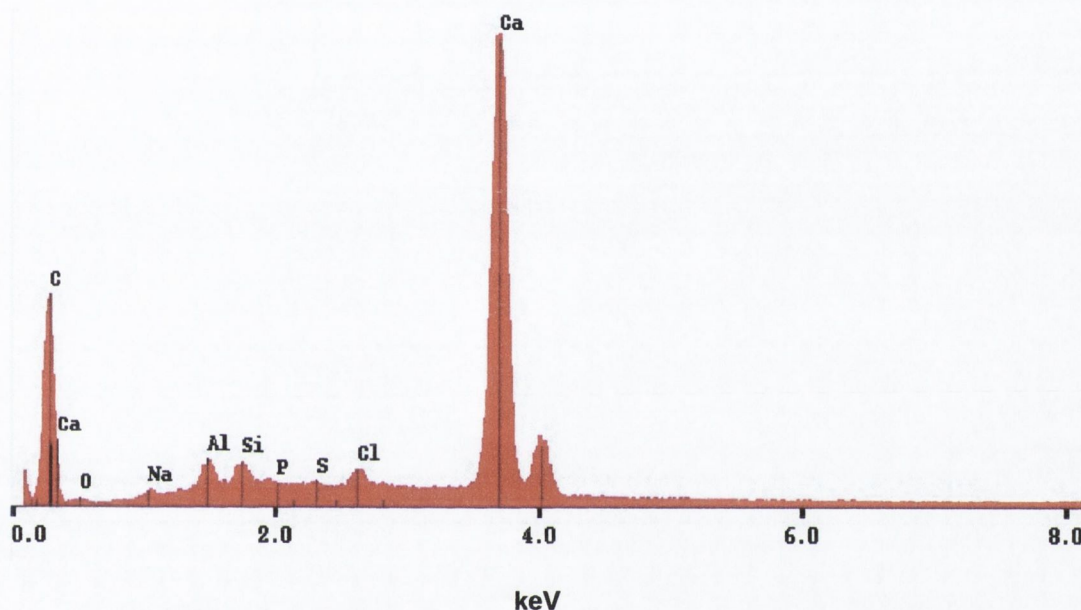


Figure 5.3 Energy dispersive X-ray analysis of the ibuprofen- Ca^{2+} precipitate.

From the results of the energy dispersive X-ray analysis and HPLC determination of the ibuprofen present in the precipitate, it can be concluded that the precipitate is a salt of ibuprofen- Ca^{2+} , having a stoichiometric ratio of 2:1.

Fini et al. (2001) have shown that indomethacin forms a sparingly soluble salt with Ca^{2+} when the molar ratio of indomethacin/ Ca^{2+} reaches the stoichiometric value (2:1). This supports the view that the solubility of ibuprofen acid in Krebs' buffer is limited by the relatively insoluble salt that it forms with Ca^{2+} .

5.3.3 Buffer osmolarity

Water flux across the intestinal mucosa is influenced by the osmotic gradient across the intestinal mucosa (Lennernas, 1995), which is in turn dependent on the osmolarity of the intestinal contents. In this respect, the osmolarity of a buffer being used in an intestinal perfusion study may influence drug absorption.

The osmolarities of the eight buffers are given in Table 5.1. The osmolarities of six of the eight buffers lie within, or close to, the physiological range (280-320 milliosmoles litre⁻¹), the exceptions being FeSSIF and McIlvaine's buffers.

5.4 BUFFER CAPACITY

Buffer capacity, the ability of a solution to resist attempts to change its pH (Butler 1998), can be expressed numerically as the number of equivalents of strong base needed to change the pH value by 1 unit (Van Slyke, 1922). The buffer capacities of the systems were measured as described in Section 4.4.5.

5.4.1 Effect of varying pH on buffer capacity

The experimentally determined buffer capacity versus pH profiles, together with the best-fit curves, for FaSSIF, FeSSIF, HBSS and PBS 6.8, are shown in Figure 5.4, and for McIlvaine's, Krebs', Fagerholm's and PBS 7.4 are shown in Figure 5.5. The best-fit profiles for all eight buffers are compared in Figure 5.6 and the initial pH and buffer capacity values are annotated on each profile with the standard deviations at the maximum buffer capacities.

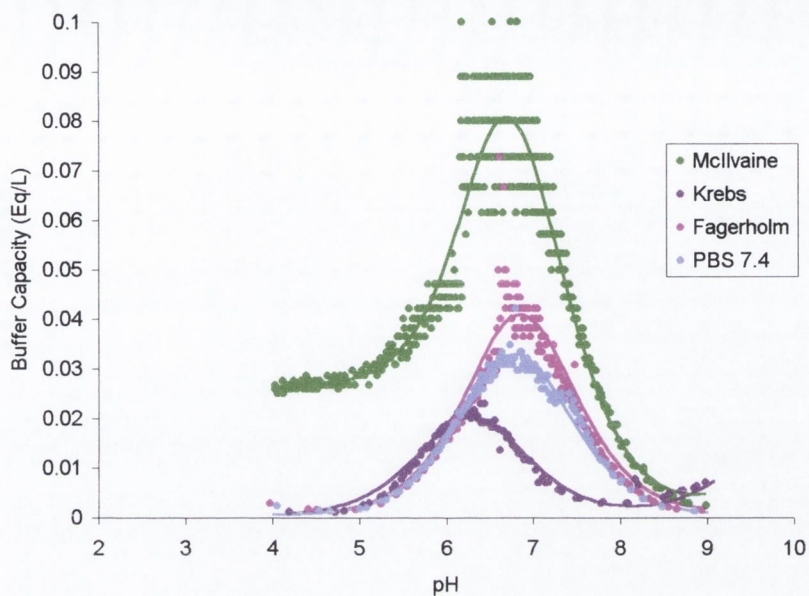


Figure 5.4 Best-fit buffer capacity versus pH profiles with experimental data for McIlvaine, Krebs, Fagerholm and PBS 7.4.

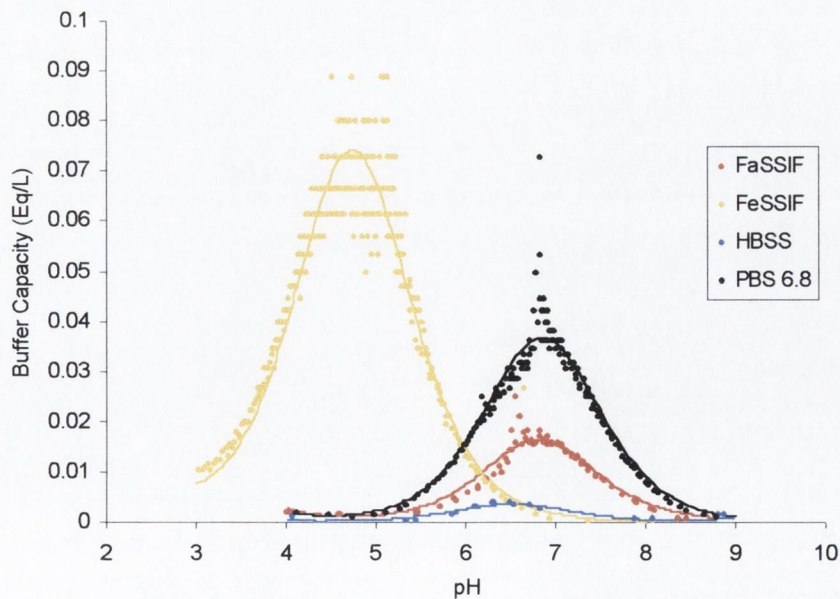


Figure 5.5 Best-fit buffer capacity versus pH profiles with experimental data for FaSSIF, FeSSIF, HBSS and PBS 6.8.

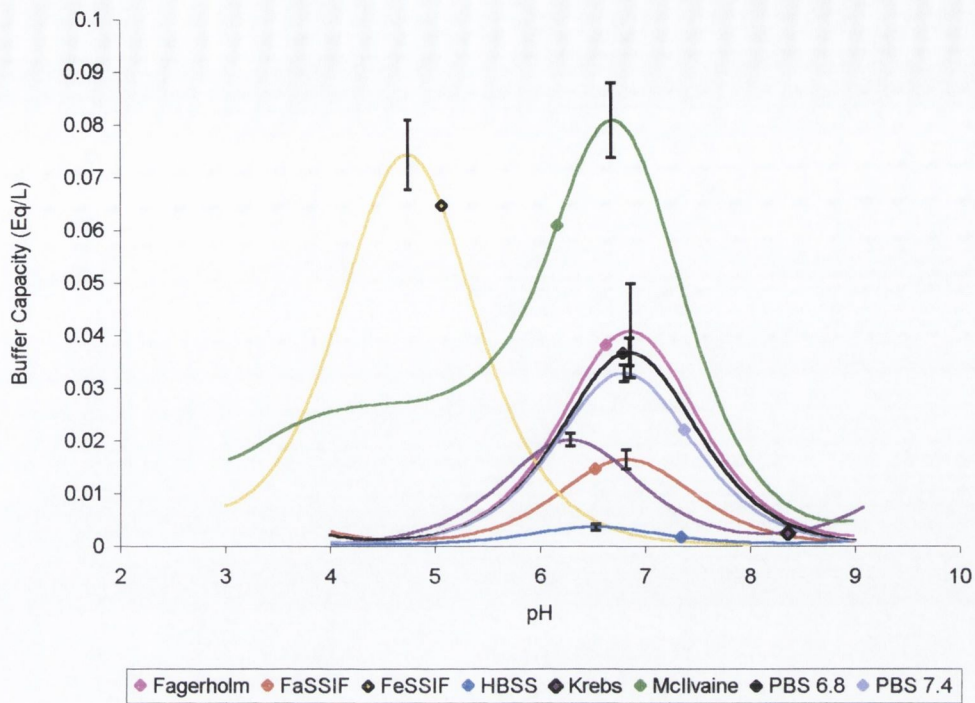


Figure 5.6 Best-fit buffer capacity versus pH profiles of each of the eight buffers, their initial pH and buffer capacities, and standard deviations at maximum buffer capacity.

Table 5.2 shows the buffer capacities of each of the buffers at their initial pHs (β). There is a 40-fold difference between the lowest (HBSS) and the highest (FeSSIF). Also indicated in the table are the maximum buffer capacities (β_{max}) and it is apparent that all of the buffers are used at their suboptimal pH, with the exception of PBS 6.8 (Figure 5.6). This is of significance as similar buffer capacities were observed at the initial pH values for FeSSIF and McIlvaine’s. However, for FeSSIF the initial pH is on the downward slope of its profile, while for McIlvaine’s the initial pH is on its upward slope (Figure 5.6). Consequently, it takes a greater amount of base to shift the pH of one litre of McIlvaine’s by one pH unit (1.84 millimoles of NaOH) than for FeSSIF (0.94 millimoles of NaOH).

Table 5.2 Buffer capacity (β) results.

Buffer System	Buffer Type	^a Conc. (mM)	pH	^b β	^c $\beta_{\max}$	^d $\beta_{\max}$ predicted	^e pK _a	^f pK _a '	^g Best-fit pK _a
Fagerholm	H ₃ PO ₄	71.00	6.63	0.0381	0.0408	0.0409	7.21	6.84	6.86
FaSSIF	H ₃ PO ₄	29.00	6.53	0.0146	0.0164	0.0167	7.21	6.85	6.82
FeSSIF	CH ₃ COOH	144.00	5.06	0.0647	0.0742	0.0829	4.76	4.61	4.74
HBSS	H ₃ PO ₄	0.80	7.35	0.0016	0.0035	0.0005	7.21	6.84	6.56
	H ₂ CO ₃	4.20			0.0035	0.0024	6.40	6.25	6.52
Krebs	H ₃ PO ₄	1.20	8.36	0.0025	0.0201	0.0007	7.21	6.85	6.29
	H ₂ CO ₃	4.20			0.0201	0.0024	6.40	6.25	6.29
McIlvaine	H ₃ PO ₄	126.00	6.17	0.0608	0.0807	0.0725	7.21	6.77	6.72
	C ₆ H ₈ O ₇	37.00			0.0326	0.0213	6.40*	5.65	5.40
PBS 6.8	H ₃ PO ₄	66.56	6.79	0.0366	0.0368	0.0383	7.21	6.82	6.85
PBS 7.4	H ₃ PO ₄	66.79	7.37	0.0219	0.0328	0.0385	7.21	6.80	6.80

^aConcentration of each buffer salt (mM); ^bBuffer capacity at the initial pH of the buffer (Eq/L/pH unit); ^cMaximum buffer capacity (Eq/L/pH unit); ^dMaximum buffer capacity predicted from the formulation using Equation 4.3; ^eThermodynamic pK_a at 25°C (Beynon and Easterby, 1996; Martindale, 1977); ^fpK_a corrected for ionic strength and temperature; ^gBest-fit pK_a using Scientist® curve fitting programme.

5.4.2 Effect of temperature and ionic strength

Seven of the eight buffer systems contain phosphate salts either as the sole buffer component or in combination with a second buffer. The buffer capacity versus pH profiles show that the pK_a (of the second ionisation) of phosphoric acid for each of the buffers differ slightly from each other and differ significantly from the thermodynamic value of 7.21. This is due to the effect of temperature and ionic strength. The effect of ionic strength is described by the Deybe-Huckel relationship:

$$pK_a' = pK_a + (2z_a - 1) \cdot \left[\frac{A \cdot \sqrt{I}}{1 + \sqrt{I}} - 0.11 \right]$$

Equation 3.8

where Z_a is the charge on the conjugate acid species, I is the ionic strength and A is a temperature dependent constant with a value of approximately 0.5.

When the thermodynamic value for the pK_a of the buffer component is corrected for temperature and ionic strength there is good agreement with the experimental data (Table 5.2).

5.4.3 Effect of concentration

The concentration of phosphate in each of these buffers varies from 0.0008 M (HBSS) to 0.126 M (McIlvaine's) and as predicted from Equation 4.1 there is a corresponding increase in buffer capacity with an increase in phosphate concentration.

Equation 4.1 describes how the maximum buffering capacity is dependent only on the concentration of the buffering species present. When it is applied to each of the buffers to predict $\beta_{\max}$ there is a good agreement with the best-fit $\beta_{\max}$ values for all of the buffers, with the exception of Krebs'. This may be due to carbon dioxide dissolving into the buffer and contributing to the buffer effect of the carbonate component. This occurrence was not seen to the same extent in HBSS despite its similar composition of phosphate and carbonate buffer species. A possible reason for this is that Krebs' has a more alkaline pH (8.36) than HBSS (7.35) and this may make Krebs' buffer more likely to dissolve carbon dioxide from the atmosphere than HBSS.

5.4.4 Conclusions

The solubility of ibuprofen in a particular buffer depends principally on the pH of the buffer and to a lesser extent on ionic strength. However it may be reduced by the presence of divalent metal ions or increased by the presence of micelles in the buffer.

FeSSIF and McIlvaine's buffers are hyperosmotic with the remaining six having osmolarities close to or within the physiological range.

The majority of the buffers are phosphate based and buffer over the same pH range, with the exception of FeSSIF, which buffers over a lower pH range due to its composition of acetic acid. The buffer capacity varies significantly between the buffers and is due to differences in buffer salt concentrations.

5.5 IN SITU ABSORPTION STUDIES

In situ absorption studies were carried out on male Wistar rats (280-320 g) according to the rat gut perfusion method described by Komiya et al. (1980) and outlined in Section 4.4.7. Perfusate samples were collected every 10 minutes for a period of 120 minutes and blood samples were taken at 30-minute intervals.

5.5.1 Ibuprofen permeability coefficients

The fraction of ibuprofen unabsorbed was calculated for each perfusate sample as described in Section 4.4.12. At each time point, for a particular buffer, these values were averaged over the number of rats studied ($n \geq 4$). Fraction unabsorbed against time profiles were plotted for each buffer (Figure 5.7).

The absorption data was fitted to two models:

- i) the non-steady-state model described by Ni et al. (1980) (Appendix 6).
- ii) the steady-state model described by Komiya et al. (1980).

Attempts were made to fit the fraction unabsorbed versus time profiles to the non-steady-state model using Scientist, but the fits were generally poor. The steady-state model, as described by Equation 3.13, converts fraction unabsorbed values to permeability coefficients (P_{app}) using steady-state data.

$$P_{app} = \frac{-Q}{2\pi r l} \cdot \ln\left(\frac{C_l}{C_0}\right) \quad \text{Equation 3.13}$$

where C_0 is the input perfusate drug concentration, C_l is the outlet perfusate drug concentration, r is the effective lumen radius (0.18 cm), Q is the perfusate flow rate (ml sec^{-1}), and l is the length of intestinal segment (33.3 cm). The flow rate of perfusate into the intestine was set at 0.2 ml min^{-1} .

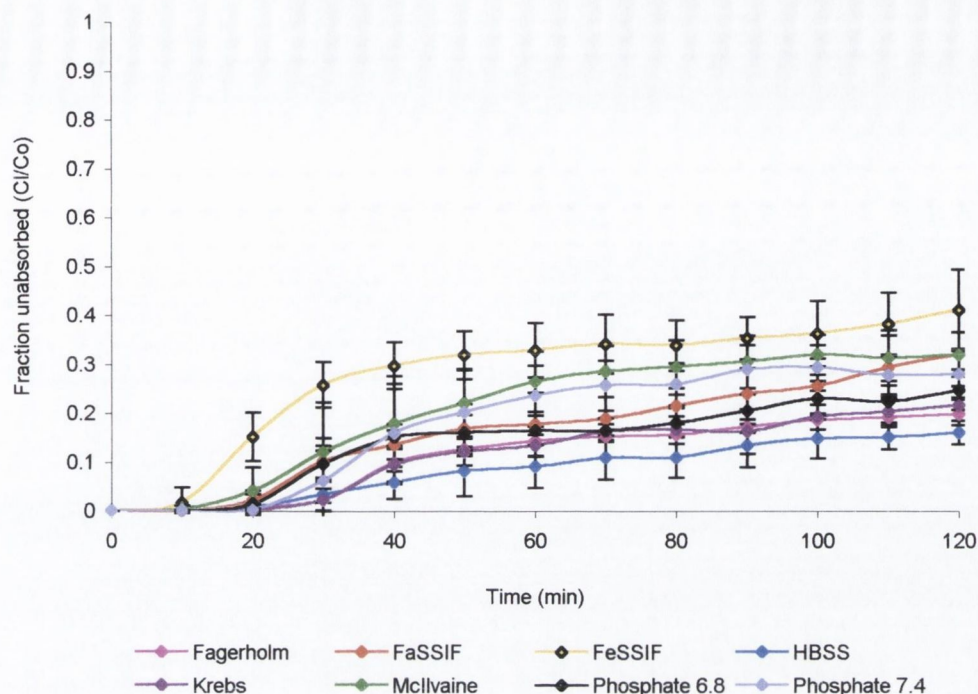


Figure 5.7 Fraction unabsorbed versus time profiles for each of the buffer systems studied.

The steady-state was taken to be between 100-120 minutes. The ibuprofen P_{app} value was obtained by averaging the P_{app} values over 100-120 minutes for each perfusion experiment. For a particular buffer, the P_{app} values for each rat were averaged and a standard deviation calculated. Tukey and Fisher pair-wise comparisons were performed using a one-way analysis of variance (ANOVA) using Minitab™ Statistical Software (version 13.1).

5.5.1.1 Impact of fluid flux on P_{app} calculation

Perfusate samples were monitored by gravimetric analysis and the average sample weight was determined for each system. Variation in average sample weights between the eight systems was observed. Validation of the perfusion pumps as described in Section 4.4.10 showed that they were operating accurately (Table 5.3) and variation in average sample weight was not due to pump error.

Table 5.3 *Average flow rates and standard deviations of each of the perfusion pumps.*

	Average Flow Rate (ml min ⁻¹)	s.d.
Pump 1	0.20	0.01
Pump 2	0.20	0.00

This suggested that the variation in average sample weight between the eight systems was due to intestinal fluid flux. Several studies have shown that fluid flux occurs across the intestinal wall and is probably driven by an osmotic gradient across the intestinal mucosa (Kitazawa et al., 1975; 1978). Two approaches were taken to correct for fluid flux when calculating P_{app} :

5.5.1.1.1 Alteration of ‘Q’ using sample weights

The first approach replaces the pump flow rate ‘Q’ (0.2 ml min⁻¹) in Equation 5.5 with a value based on sample weights from gravimetric analysis as described in Section 4.4.13.1. These values are shown in Figure 5.8 together with the pump flow rate of 0.2 ml min⁻¹.

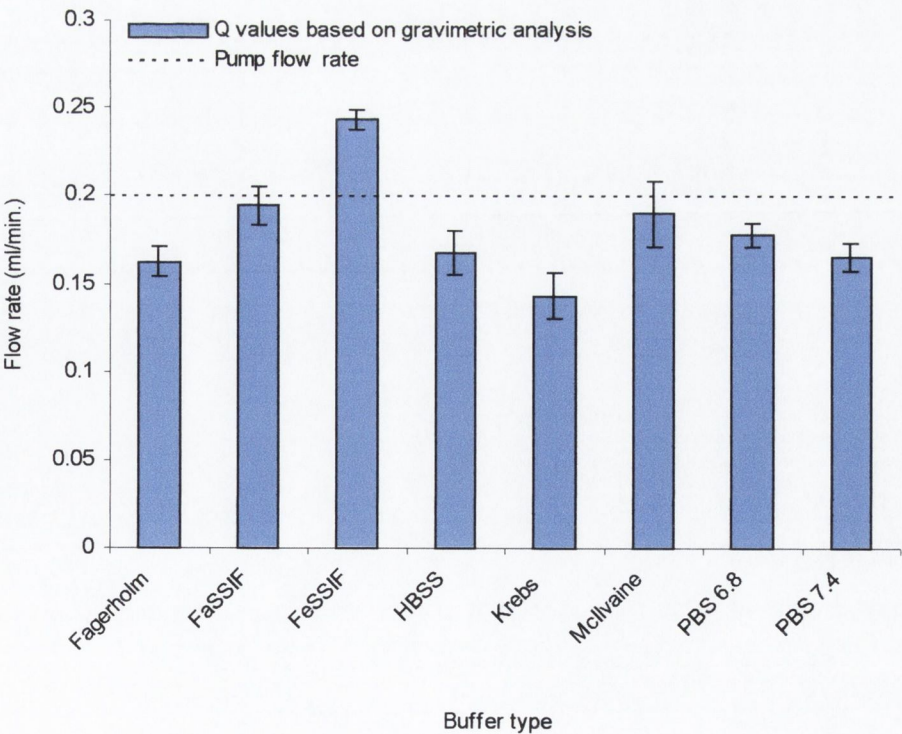


Figure 5.8 Q values (ml min⁻¹) of the eight buffers based on gravimetric analysis.

The P_{app} values for ibuprofen in each of the buffers using the Q values based on average sample weight for each buffer are given in Table 5.4 and ranged from 0.93×10^{-4} to 1.40×10^{-4} cm sec⁻¹.

The P_{app} for HBSS was significantly higher than six other buffers (FaSSIF, FeSSIF, Krebs', McIlvaine's, PBS 6.8, PBS 7.4). Fagerholm's buffer was significantly higher than McIlvaine's buffer and PBS 7.4. PBS 6.8 was significantly higher than PBS 7.4 ($p < 0.05$).

Table 5.4 Permeability coefficients (P_{app}) of ibuprofen in each buffer calculated using Q values based on average sample weight for each system to correct for intestinal fluid flux.

Buffer System	Flow Rate (ml min ⁻¹)	s.d.	P_{app} cm sec ⁻¹ x10 ⁻⁴	s.d.	n
Fagerholm	0.16	0.01	*1.20	0.14	5
FaSSIF	0.19	0.01	1.10	0.19	5
FeSSIF	0.24	0.01	1.05	0.17	6
HBSS	0.17	0.01	*1.40	0.10	4
Krebs	0.14	0.01	1.01	0.09	5
McIlvaine	0.19	0.02	0.97	0.13	4
PBS 6.8	0.18	0.01	*1.15	0.12	6
PBS 7.4	0.17	0.01	0.93	0.11	4

* P_{app} values that are significantly different based on ANOVA ($p < 0.05$) using pair-wise comparisons as indicated in the text.

Although the Q values based on average sample weight vary significantly with the buffer used, only that with FeSSIF is significantly higher than all the others as well as from 0.2 ml min⁻¹ (Figure 5.8). This suggests that water is being secreted into the perfusate, contributing to an apparent increased flow rate.

5.5.1.1.2 Correcting C_i/C_0 values for fluid flux

The second approach to correct for intestinal fluid flux in the calculation of P_{app} involves calculating the effect that the fluid flux has on the concentration of ibuprofen in the perfusate samples, and consequently on the steady-state C_i/C_0 values. This is based on the method described by Sutton and Rinaldi (2001) and Pérez et al. (2002) in Section 4.4.13.2. Any fluid flux that occurs, either by secretion or absorption, will alter the concentration of ibuprofen in the perfusate samples. If uncorrected this change in ibuprofen concentration will be assumed to be the result of absorption and lead to an error in P_{app} . Fluid flux can be measured using a number of approaches, the two most common of which involve non-absorbable markers (*e.g.* PEG 4000, phenol red) or gravimetric analysis. Both methods have been compared in rat single pass intestinal perfusion studies by Sutton and Rinaldi (2001) and the gravimetric method was found to be the most accurate, least variable, easiest and was also not subject to the error associated with possible absorption of the non-absorbable marker (Lane et al., 1996).

Experimental fraction unabsorbed values (C_i/C_0) were corrected for fluid flux by the gravimetric method and used in Equation 5.5 to calculate ibuprofen P_{app} values. The flow rate was taken as 0.2 ml min^{-1} as confirmed from validation studies of the perfusion pumps (Section 5.5.1.1). These corrected P_{app} values are given in Table 5.5.

Table 5.5 *Permeability coefficients (P_{app}) of ibuprofen in each buffer calculated using corrected C_i/C_0 values to correct for fluid flux.*

Buffer System	P_{app} $\text{cm sec}^{-1} \times 10^{-4}$	s.d.	n
Fagerholm	*1.66	0.17	5
FaSSIF	1.17	0.19	5
FeSSIF	*0.68	0.14	6
HBSS	*1.83	0.12	4
Krebs	*1.72	0.12	5
McIlvaine	1.07	0.14	4
PBS 6.8	*1.40	0.14	6
PBS 7.4	1.30	0.13	4

* P_{app} values that are significantly different based on ANOVA ($p < 0.05$) using pair-wise comparisons as indicated in the text.

Fagerholm, HBSS and Krebs' are all significantly higher than FaSSIF, FeSSIF, McIlvaine, PBS 6.8 and PBS 7.4. PBS 6.8 is significantly higher than McIlvaine and FeSSIF. FeSSIF is significantly lower than the other seven buffers ($p < 0.05$).

5.5.1.1.3 Conclusion

The P_{app} values calculated using the two different correction methods are shown in Figure 5.9. P_{app} values calculated using the second method show more significant differences between the buffers than when corrected by flow rate.

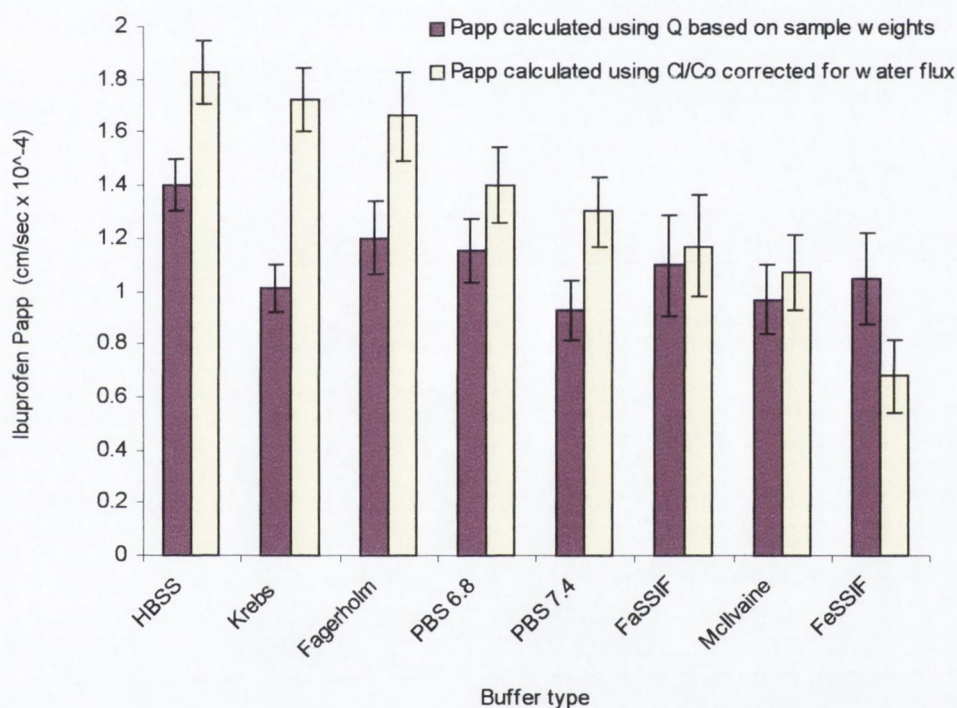


Figure 5.9 P_{app} values for each buffer calculated using a 'Q' value based on average sample weight and a C_1/C_0 values that have been corrected for fluid flux.

The second method of correcting for water flux directly reverses the diluting or concentrating effect of the water movement into or out of the intestine and uses the actual flow rate (0.2 ml min^{-1}) in the calculation of P_{app} . This is a more accurate method than by using Q values based on average sample weight to correct for fluid flux when calculating P_{app} and yields more significant differences in P_{app} values between the eight systems.

These are the values that will be discussed further.

5.5.2 Effects of buffer osmolarity and fluid flux on P_{app}

The osmolarities of the eight buffers are given in Table 5.1. The osmolarities of six of the eight buffers lie within, or close to, the physiological range (280-320 milliosmoles litre⁻¹), the exceptions being FeSSIF and McIlvaine buffers, which are hyperosmotic (Figure 5.10).

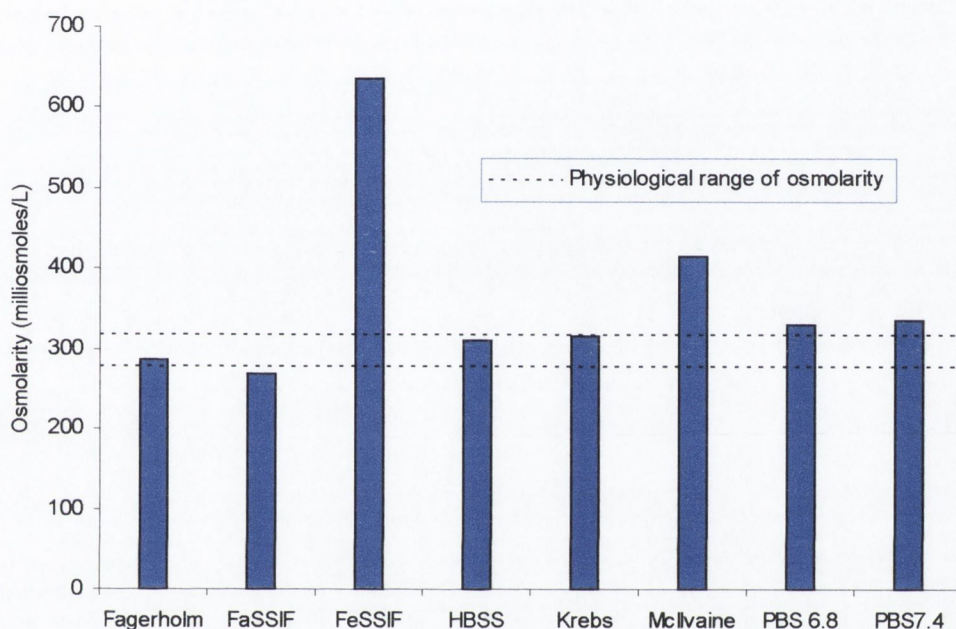


Figure 5.10 Osmolarities (milliosmoles litre⁻¹) of the buffer systems.

As well as having the highest osmolarities, these two buffers also show the lowest ibuprofen P_{app} values (Table 5.5). The ibuprofen P_{app} in FeSSIF (0.68×10^{-4} cm sec⁻¹) is significantly lower than for all the other buffers. The P_{app} in McIlvaine (1.07×10^{-4} cm sec⁻¹) is the second lowest and is significantly lower than four other buffers. The relationship between buffer osmolarity and ibuprofen P_{app} was investigated and is shown in Figure 5.11.

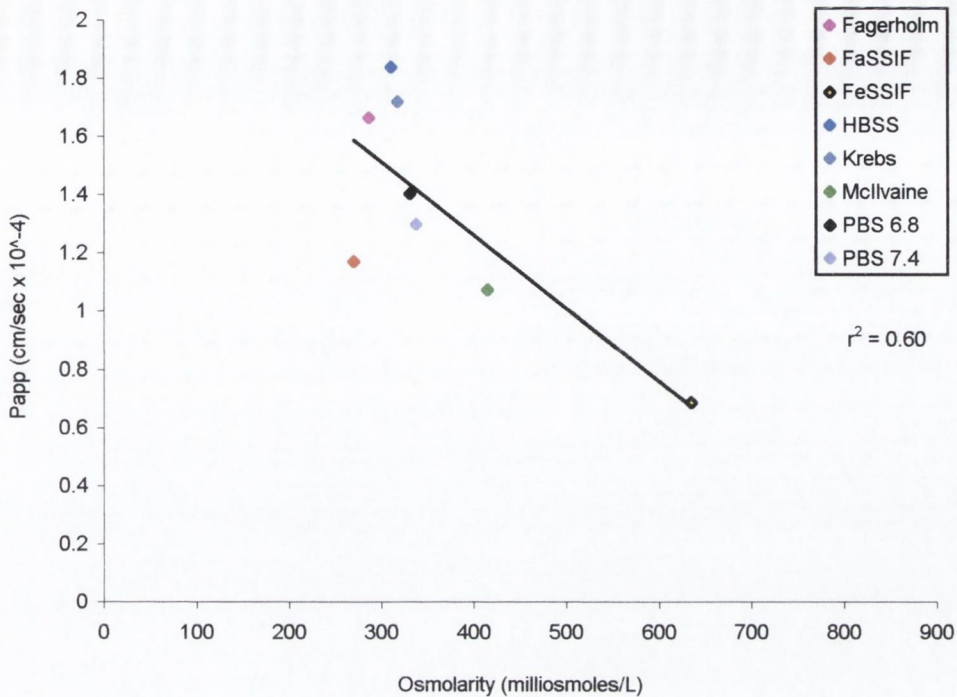


Figure 5.11 Relationship between P_{app} and buffer osmolarity.

Kitazawa et al. (1975) have shown that transmucosal fluid movement influences drug absorption. This may be mediated by 'solvent drag' by the fluid absorbed through the paracellular pathway, a phenomenon whereby as water is absorbed it brings with it any dissolved drug and effectively drags it across the intestinal wall (Andersson and Ussing, 1957; Papenheimer et al., 1987; Krugliak et al., 1989). The driving force for this water flux is probably an osmotic gradient across the intestinal mucosa (Lennernas, 1995) with hypotonic solutions driving water absorption and hypertonic solutions hindering it. Significantly, Thomas et al. (1984) have shown that a hypertonic system induced water flux across rat rectal mucosa into the lumen. This suggests that osmolarity, through its effect on water flux, will have an enhancing or retarding effect on drug absorption depending on the direction of water movement. Figure 5.12 shows the relationship between fluid flux and ibuprofen P_{app} in the eight systems studied.

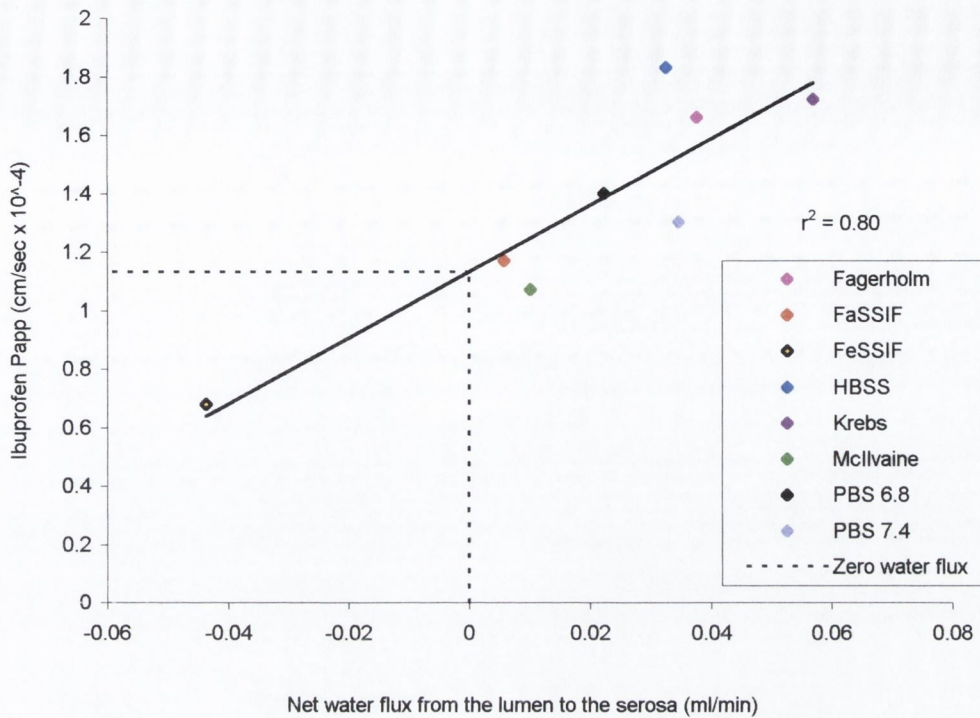


Figure 5.12 Relationship between ibuprofen P_{app} and net water flux across the intestinal wall from the lumen to the serosa (a negative value for net water flux represents secretion into the lumen and a positive value represents absorption out of the lumen).

Figure 5.12 shows that as water flux increases from the mucosal to the serosal side it increases P_{app} and, conversely, as it increases from the serosal to the mucosal side it reduces P_{app} . The hyperosmotic buffers, FeSSIF and McIlvaine's, may be generating reduced estimates of ibuprofen P_{app} by producing changes in water flux across the intestine relative to the other six buffers. Extrapolating a line from zero water flux up to the line of best-fit and across to P_{app} on the y-axis generates a value for ibuprofen P_{app} when there is no water flux. This value is $1.13 \times 10^{-4} \text{ cm sec}^{-1}$.

The concentration of ibuprofen in the perfusion solution (prior to entry into the gut) before and at the end of each experiment was, on analysis, the same in all systems indicating that precipitation of ibuprofen salts of low solubility (e.g. calcium salt) over the course of the experiment did not occur.

5.5.3 pH Monitoring

The pH values of the perfusate samples were monitored over the course of each experiment as described in Section 4.4.9 and the pH versus time profiles are shown in Figure 5.13. Over the time course of each perfusion experiment, the pH values of the samples gradually change from their initial values towards a median value of approximately 6.5, with some of the systems being shifted upwards and others shifted downwards from their initial values.

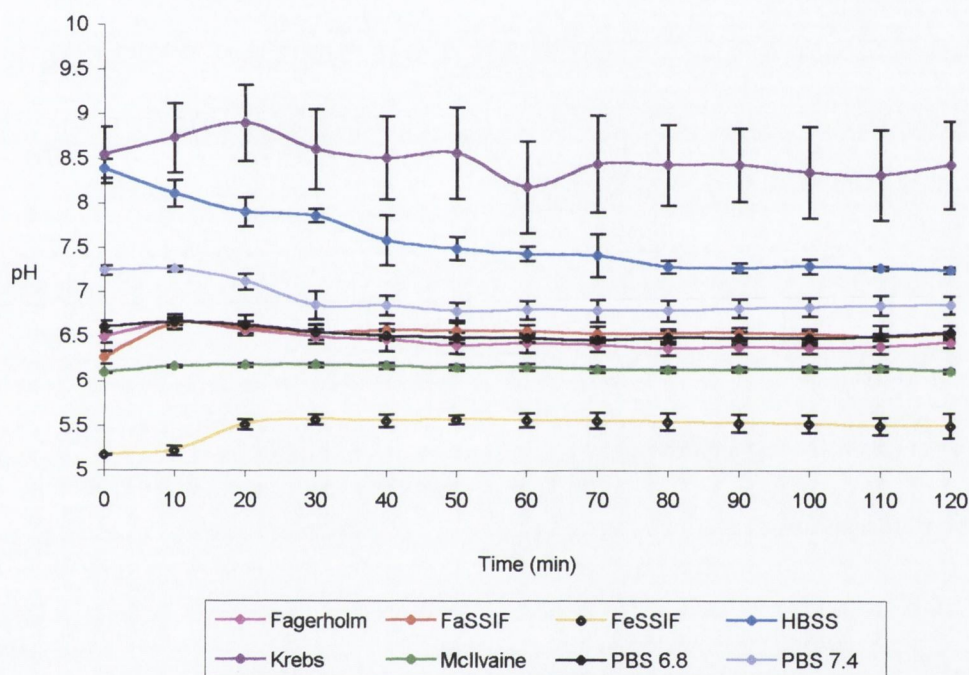


Figure 5.13 pH versus time profiles of perfusate samples.

The magnitude of these pH changes is significant for four of the buffers: FaSSIF, FeSSIF, HBSS and PBS 7.4 ($p < 0.05$). This would be expected from the buffer capacity profiles as FaSSIF, HBSS, PBS 7.4 and Krebs' correspond to the four lowest buffer capacity values of the perfusion solutions. However, despite its low buffer capacity, the pH change for Krebs' is not significant as it shows no definite trend in its pH values. This may be due to carbon dioxide dissolving in the perfusate samples. Despite its high buffer capacity, FeSSIF shows a significant change in pH over the course of the perfusion. This may be due to its relatively low pH (compared to the microclimate pH) that may stimulate the intrinsic intestinal buffering system to produce the pH change observed. In the case of most of the buffers, the change in pH

occurs over ~50 minutes after which there is little change. This suggests that the intestinal buffering system reaches a steady-state after ~50 minutes. Desai (1977) reported that the pH of buffered solutions, initially pH 9.5 and 4.5 respectively, of low buffer capacity tended rapidly towards a pH of 6.5 when placed in the rat intestine and Ikuma et al. (1996) have shown that the jejunal microclimate pH in young adult rats is 6.12 ± 0.04 . It is well established that the cell surface of the rat jejunum has an acidic microclimate *in vitro* (Lucas et al., 1975; Iwatsubo et al., 1989) and this microclimate appears to be maintained by a dynamic equilibrium of H^+ secretion and absorption across the luminal membrane and the diffusion from the microclimate towards the luminal fluid (Shimada, 1987). The shift in pH of the perfusate samples over the first 50 minutes is maintained over the course of the perfusion indicating that there is no exhaustion of the intestinal buffering system. It also provides evidence of the viability of the intestine as it indicates that the intestinal segment is functioning normally.

Several studies have shown that the absorption processes of some drugs and nutrients are influenced by the microclimate pH (Lister, 1997).

The changes in the pHs of the perfusate samples produce changes in the percentage of drug ionised. Despite some of the pH changes being significant, the corresponding change in fraction of ibuprofen ionised is negligible for all buffers except FeSSIF. The starting pH of the FeSSIF perfusion solution (5.17) corresponds to 84.6% ionised and its pH at the steady-state (5.51) corresponds to 92.3% ionised. At the steady-state, the remaining seven buffers give 98-99.9% of drug ionised.

5.5.4 Conclusions from ibuprofen P_{app} studies

Based on research in oral drug delivery studies, a compound is considered to be completely absorbed in humans (90-100% absorbed) if its P_{app} is greater than approximately $0.2 \times 10^{-4} \text{ cm sec}^{-1}$ in the rat (Fagerholm et al., 1996). The values reported in the present work are all greater than this and when they are converted to human fraction absorbed values (Fagerholm et al., 1996), they show 99% absorption with no statistically significant differences observed.

Eight ibuprofen P_{app} values were calculated from *in situ* absorption studies in eight buffers of differing composition. Each system caused a certain amount of water flux across the intestinal mucosa, which influenced the P_{app} values. From a plot of P_{app} against net water flux, the P_{app} of ibuprofen in the absence of water flux was calculated as $1.13 \times 10^{-4} \text{ cm sec}^{-1}$. This also corresponds to 99% absorption in humans.

Komiya et al. (1980) investigated the intestinal permeability in the rat of a range of steroids. When using similar experimental conditions, the permeability of hydrocortisone in phosphate buffer (pH 6.0) was found to be membrane controlled with a P_{app} of $0.30 \times 10^{-4} \text{ cm sec}^{-1}$. In contrast, the corresponding P_{app} for progesterone was $1.08 \times 10^{-4} \text{ cm sec}^{-1}$ and its transport was aqueous boundary layer controlled. From this work, the P_{app} of ibuprofen in a similar buffer (citrate-phosphate pH 6.0) was $0.97 \times 10^{-4} \text{ cm sec}^{-1}$. This suggests that the absorption of ibuprofen in the rat is aqueous boundary layer controlled.

Compounds with molecular weights less than 200 are able to pass through the aqueous pores (Lennernas, 1995), but transmembrane transport of the unionised fraction can occur simultaneously (Chan and Stewart, 1996). Hydrophilic and charged solutes have a low membrane permeability due to a low partition into the lipid membrane and/or a slower transcellular diffusion coefficient. They are therefore expected to be absorbed through the paracellular route, which is a result of diffusion and a convective volume flow through the water filled intercellular space (Pappenheimer and Reiss, 1987). The high permeability of ibuprofen, along with its molecular weight (206) and degree of ionisation, implies that the drug may be absorbed principally by the paracellular route or through the aqueous pores. This is further supported by the significant effect of water flux on the permeability

coefficient of ibuprofen, as these two routes are widely thought to be the principal routes of water movement across the intestine (Pappenheimer and Reiss, 1987; Tripathi and Boulpaep, 1989; Van Os et al., 1994). These comments must be viewed in the context of our increasing knowledge of transporters with an affinity for carboxylic acid compounds (Tamai et al., 1995).

5.5.5 Plasma profiles

Plasma samples were collected at 30-minute intervals over the course of each two-hour perfusion for each buffer. In addition, for PBS 6.8, PBS 7.4, HBSS and Krebs' buffers, plasma samples were collected at 30-minute intervals for an additional two hours after the perfusions had stopped to generate both absorption and elimination data. Samples were assayed for drug content using the method described in Section 4.4.15.1.

5.5.5.1 Pharmacokinetic modelling of plasma profiles

The plasma ibuprofen concentration versus time profiles were fitted (using the Scientist[®] curve fitting program) to two pharmacokinetic models:

- i. One compartment model with constant input and first-order output.
- ii. Two compartment model with constant input and first-order output.

The ibuprofen plasma concentration versus time profiles for PBS 6.8, PBS 7.4, HBSS and Krebs' buffers were fitted to these two models. The second showed the best-fit of the absorption and elimination data from the four buffers. This model is described in Appendix 5.

5.5.5.2 Two compartment model with constant input and first order output

The Scientist[®] data fitting results suggests that ibuprofen in the rat displays two compartment pharmacokinetics with first order output as reported in previous studies by Itoh et al. (1997), Parrott and Christensen (1984), and Shah and Jung (1987). The parameters in this model are k_{elim} (elimination rate constant), k_{12} (rate constant for transfer from the central to the peripheral compartment), k_{21} (rate constant for transfer from the peripheral to the central compartment) and volume of distribution (V). The best-fit values are given in Table 5.6 and are compared with values previously reported by Itoh et al. (1997). Dose and t_{iv} (120 mins) were fixed at the experimental values. Dose was calculated from the cumulative amount of drug absorbed over the 2 hour perfusion as described in Section 4.4.14.

Table 5.6 *Best-fit ibuprofen pharmacokinetic parameter values for HBSS, Krebs, PBS 6.8, PBS 7.4 and literature values (*Itoh et al., 1997).*

Parameter type	HBSS	Krebs	PBS 6.8	PBS 7.4	Average	Literature values*
$k_{elim} \text{ (min}^{-1}\text{)}$	0.449	0.486	0.325	0.183	0.361	0.051-0.106
$k_{12} \text{ (min}^{-1}\text{)}$	0.090	0.074	0.044	0.040	0.062	0.072-0.081
$k_{21} \text{ (min}^{-1}\text{)}$	0.039	0.060	0.081	0.041	0.055	0.080-0.091
$V \text{ (ml kg}^{-1}\text{)}$	27.463	30.157	52.878	54.257	41.189	67.4-69.9
n	4	5	6	4		
MSC	3.15	3.85	4.02	4.21		
r^2	0.99	0.99	0.99	0.99		

The average best-fit parameters were used in Scientist to fit the data from the eight buffer systems to the same model. All parameters were ‘fixed’ except the dose, which was allowed to float and settle at a best-fit value for each buffer system. The best-fit plasma ibuprofen concentration versus time profiles together with the experimental data points for HBSS, Krebs’, PBS 6.8 and PBS 7.4 are shown in Figure 5.14 and for Fagerholm’s, FaSSIF, FeSSIF and McIlvaine’s in Figure 5.15. The best-fit profiles for all eight systems are shown in Figure 5.16.

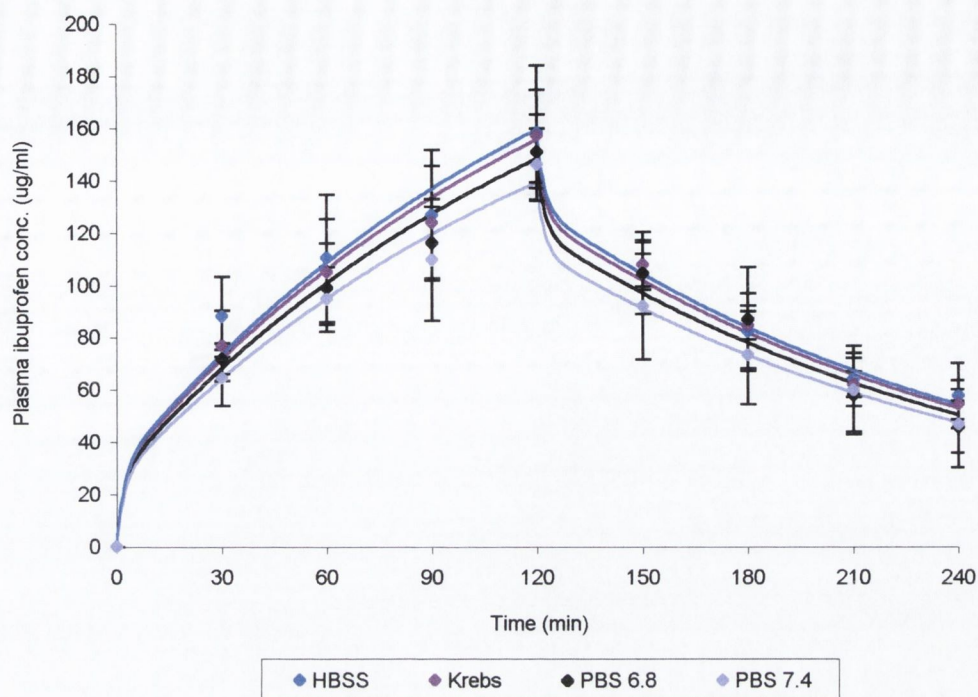


Figure 5.14 The best-fit plasma ibuprofen concentration versus time profiles together with the experimental data points for HBSS, Krebs', PBS 6.8 and PBS 7.4.

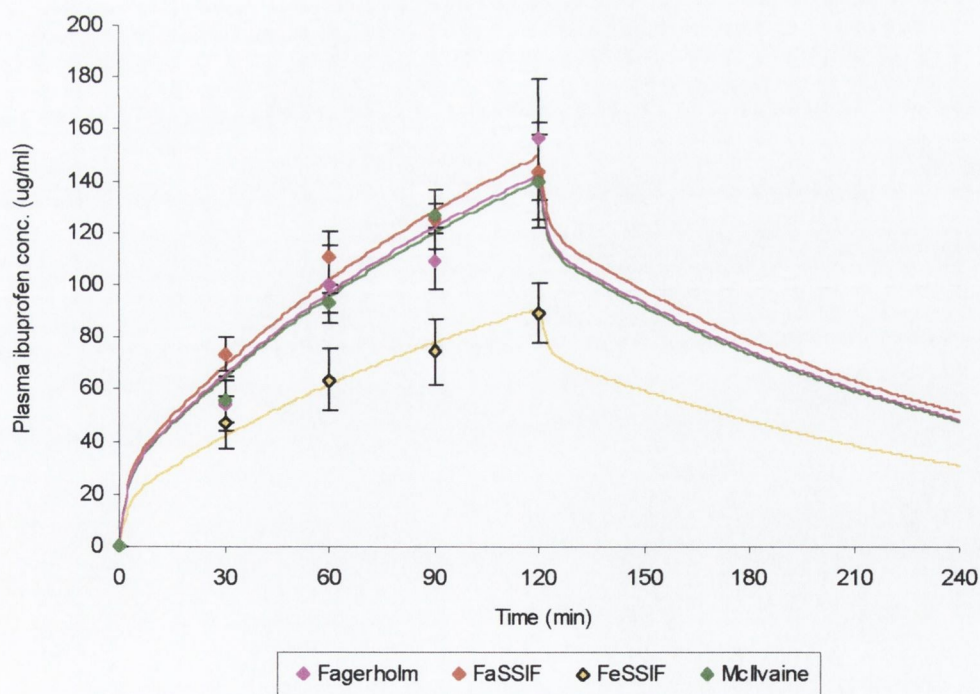


Figure 5.15 The best-fit plasma ibuprofen concentration versus time profiles together with the experimental data points for Fagerholm's, FaSSIF, FeSSIF and McIlvaine's.

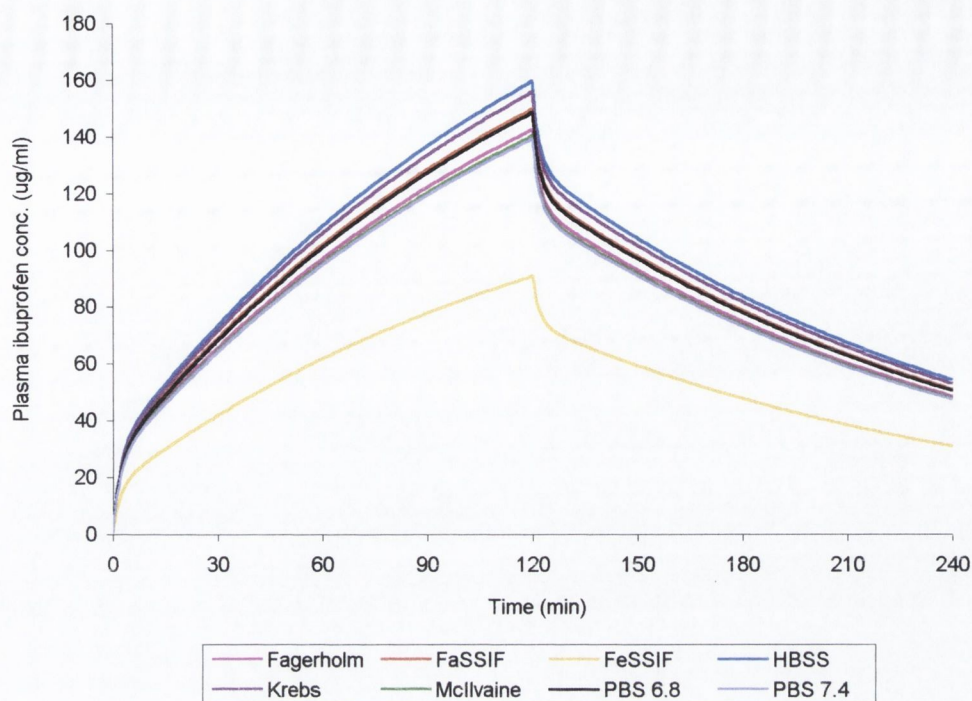


Figure 5.16 The best-fit plasma ibuprofen concentration versus time profiles for all eight systems.

Good correlations were obtained between the lines of best-fit and the experimental data points. These are given in Table 5.7 together with the best-fit doses.

Table 5.7 Results from fitting the plasma data for each buffer to the model described above including the best-fit dose and the goodness of fit (using $k_{10} = 0.361 \text{ min}^{-1}$; $k_{12} = 0.062 \text{ min}^{-1}$; $k_{21} = 0.055 \text{ min}^{-1}$; $V = 41.189 \text{ ml kg}^{-1}$; $t_{iv} = 120 \text{ mins}$).

Buffer	Best-fit Dose ($\mu\text{g rat}^{-1}$)	MSC	r^2
Fagerholm	20392	2.95	0.99
FaSSIF	21421	4.11	0.99
FeSSIF	12992	4.26	0.99
HBSS	22826	3.62	0.99
Krebs	22272	4.26	0.99
McIlvaine	20060	4.22	0.99
PBS 6.8	21256	3.63	0.99
PBS 7.4	19927	4.34	0.99

5.5.5.3 Relationship between intestinal permeability data and plasma data

The best-fit dose values for each system given in Table 5.7 can be used to calculate a zero order input rate constant using Equation 5.3:

$$k_0(\text{plasma}) = \frac{\text{Dose}}{\text{Time}} \quad \text{Equation 5.3}$$

where $k_0(\text{plasma})$ is the input rate constant calculated from plasma data; *dose* is the best-fit dose for each system; *time* is the duration of the perfusion (120 mins.).

A second input rate constant can be calculated from the rate of ibuprofen absorption from the gut at the steady-state, $k_0(\text{gut})$, and is calculated using Equation 5.4:

$$k_0(\text{gut}) = \frac{\pi * r^2 * l * (C_0 - C_l)}{t} \quad \text{Equation 5.4}$$

where r is the effective lumen radius (0.18 cm); l is the length of intestinal segment (33.3 cm); C_0 is the input perfusate drug concentration; C_l is the outlet perfusate drug concentration at the steady-state; t is the time taken for a drug molecule to pass through the length of intestinal segment.

The $k_0(\text{plasma})$ and $k_0(\text{gut})$ values for each system are given in Table 5.8 and are plotted against each other in Figure 5.17.

Table 5.8 $k_0(\text{plasma})$, $k_0(\text{gut})$ and peak plasma ibuprofen concentration values for each system.

Buffer System	$k_0(\text{plasma})$ (mg min ⁻¹)	$k_0(\text{gut})$ (mg min ⁻¹)	Peak Plasma Conc. (µg ml ⁻¹)
Fagerholm	0.170	0.169	156.13
FaSSIF	0.179	0.149	143.74
FeSSIF	0.108	0.106	89.38
HBSS	0.190	0.174	158.28
Krebs	0.186	0.171	157.01
McIlvaine	0.167	0.140	139.97
PBS 6.8	0.177	0.158	151.00
PBS 7.4	0.166	0.153	145.92

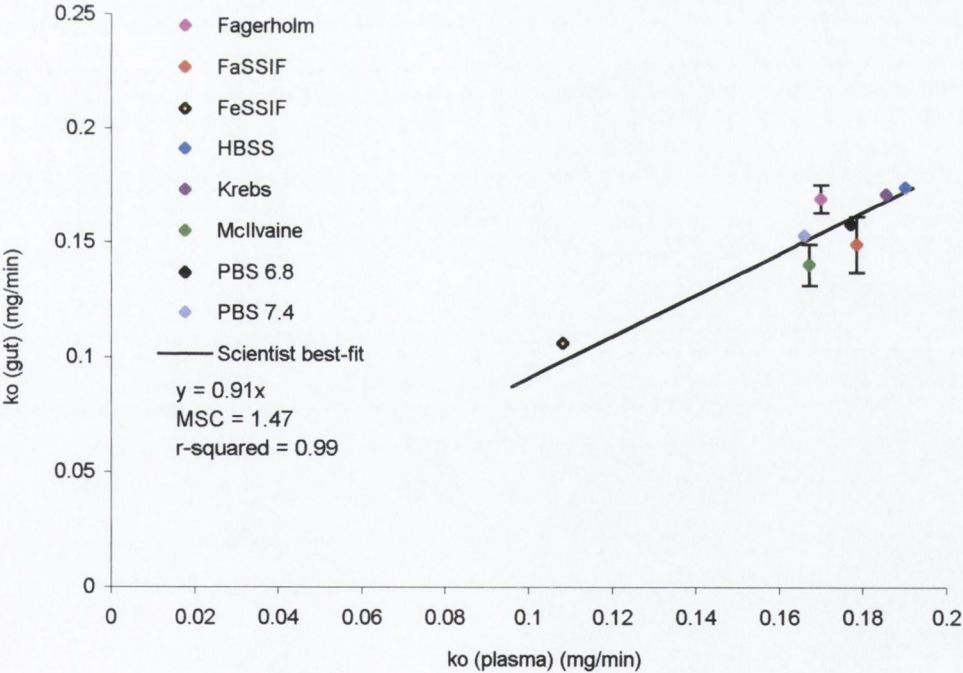


Figure 5.17 Relationship between $k_0(\text{gut})$ and $k_0(\text{plasma})$.

Figure 5.17 shows the relationship between the ibuprofen input rate constants for each system calculated using plasma data and perfusate data. The data points were fitted to Equation 5.5 using Scientist.

$$k_o(gut) = m * k_o(plasma)$$

Equation 5.5

where *m* is the slope of the line of best-fit in Figure 5.17. The best-fit value for *m* was 0.91 with a standard deviation of 0.02 and a 95% confidence interval of 0.86-0.95. This linear relationship provides validation of the pharmacokinetic parameters listed in Table 5.6. It shows the direct relationship between rate of disappearance of ibuprofen from the intestine and the rate of appearance in the plasma.

5.5.5.4 **Impact of intestinal water flux on rate of ibuprofen appearance in the plasma**

The principal factor that was responsible for the differences in ibuprofen *P_{app}* values between the different buffers was intestinal water flux. The relationship between *k_o*(plasma) and water flux was investigated and is shown in Figure 5.18.

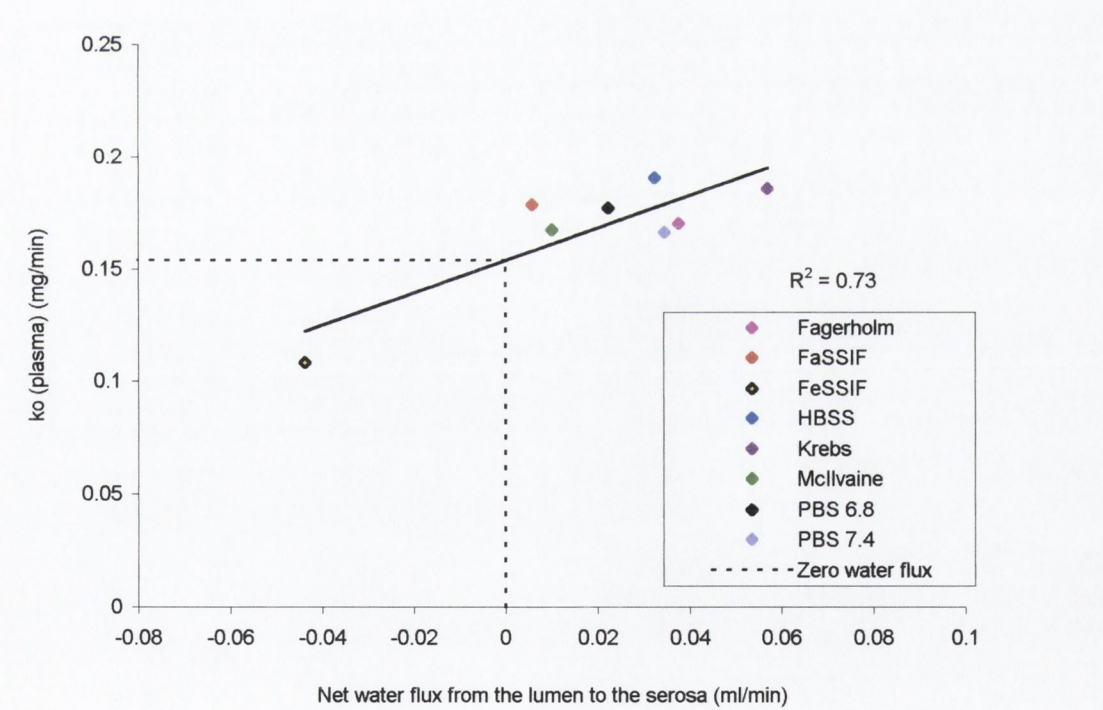


Figure 5.18 Relationship between *k_o*(plasma) and net water flux across the intestinal wall from the lumen to the serosa (a negative value for net water flux represents secretion into the lumen and a positive value represents absorption out of the lumen).

Figure 5.18 shows that as water flux increases from the mucosal to the serosal side it increases the rate of appearance of ibuprofen in the plasma and, conversely, as it increases from the serosal to the mucosal side it reduces the rate of appearance. This mirrors what is happening in the intestine: as water flux increases from the mucosal to the serosal side it increases P_{app} and, conversely, as it increases from the serosal to the mucosal side it reduces P_{app} , as described in Section 5.5.2. Extrapolating a line from zero water flux up to the line of best-fit and across to $k_0(\text{plasma})$ on the y-axis generates a value for ibuprofen $k_0(\text{plasma})$ when there is no net water flux. This value is $0.154 \text{ mg min}^{-1}$.

5.6 RACEMIC IBUPROFEN AND S-IBUPROFEN

Ibuprofen has a chiral centre and is used clinically as a mixture of the two enantiomers, R and S. For this reason the racemate was used in the work carried out in this chapter. However, its pharmacological activity mainly resides in the S-enantiomer (Adams et al., 1976) and it has been reported that conversion of the R-enantiomer to the S-enantiomer occurs *in vivo* in humans and rats (Kaiser et al., 1976; Sattari and Jamali, 1994). It has also been shown by Sattari and Jamali (1994) that when ibuprofen is administered in a sustained release formulation this R to S chiral inversion may occur in the intestine. However, when ibuprofen was administered in a solution from which it was readily absorbed, Jeffrey et al. (1991) proposed that the site of chiral inversion was the liver. It is of relevance to the results reported in this chapter on the absorption characteristics and pharmacokinetics of racemic ibuprofen to investigate if pure S-ibuprofen yields similar results from absorption studies.

5.6.1 Absorption profiles

In situ absorption studies were carried out using a solution of S-ibuprofen in PBS 6.8 (1 mg ml⁻¹) as described in Section 4.4.7. The fraction unabsorbed versus time profile of S-ibuprofen is shown in Figure 5.19 together with the profile of racemic (RS) ibuprofen.

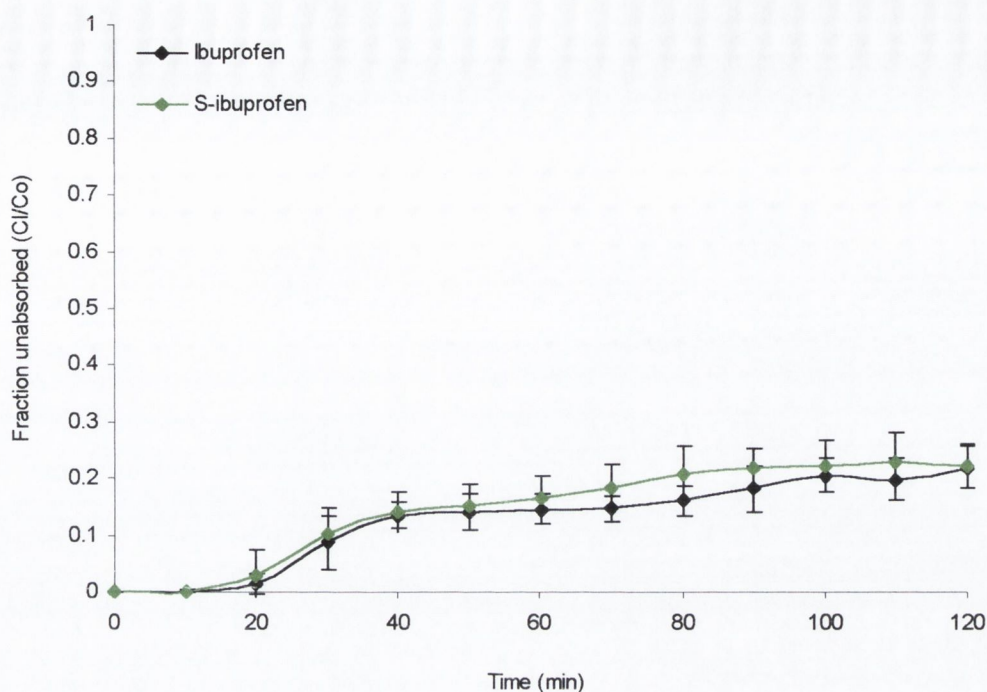


Figure 5.19 Fraction unabsorbed versus time profiles for RS-ibuprofen and S-ibuprofen in PBS 6.8.

The fraction unabsorbed values at the steady-state were converted to P_{app} values as described in Section 4.4.12. The steady-state was taken to be between 100-120 minutes. The P_{app} values for each of the systems were corrected for intestinal water flux as described in Section 4.4.13.2 and are given in Table 5.9.

Table 5.9 Permeability coefficients (P_{app}) of RS- and S-ibuprofen (1 mg mL^{-1}) in PBS 6.8

NSAID	P_{app} ($\text{cm sec}^{-1} \times 10^{-4}$)	s.d.	n
RS-ibuprofen	1.40	0.14	6
S-ibuprofen	1.34	0.17	9

Based on ANOVA, no significant difference was observed between the P_{app} values of RS- and S-ibuprofen ($p > 0.05$).

5.6.2 pH profiles

The pHs of the perfusate samples were monitored over the course of each S-ibuprofen perfusion study as described in Section 4.4.9. A pH versus time profile was plotted for S-ibuprofen along with the profile for RS-ibuprofen in PBS 6.8 described in Section 5.5.2 and this is shown in Figure 5.20.

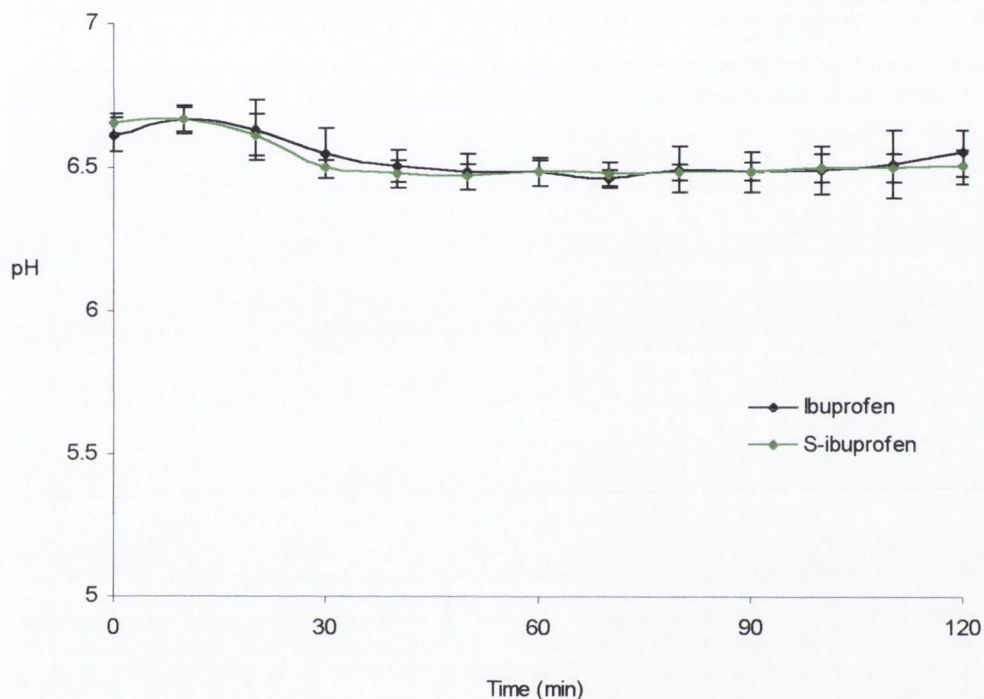


Figure 5.20 pH profiles of the perfusate samples from the RS- and S-ibuprofen absorption studies.

Over the time course of the perfusion experiments the pH values of the S-ibuprofen samples gradually change from their initial values towards a median value in a similar pattern to RS-ibuprofen. The pHs of the samples are shifted down from their initial values towards a steady-state pH of approximately 6.5. As reported in Section 5.5.2, the change in pH is most dramatic over the first forty minutes, after which there is very little change.

5.6.3 Plasma profiles

Plasma samples were collected at 30-minute intervals over the course of each two-hour perfusion of S-ibuprofen and for two hours after the perfusions had stopped to generate both absorption and elimination data for S-ibuprofen. Samples were assayed for drug content using the method described in Section 4.4.15.1. The plasma S-

ibuprofen concentration versus time profiles were fitted (using the Scientist curve fitting program) to the two compartment model applied to RS-ibuprofen in Section 5.5.3.1. The best-fit profile obtained for S-ibuprofen is shown in Figure 5.21 together with the best-fit profile for RS-ibuprofen obtained in Section 5.5.3.1.

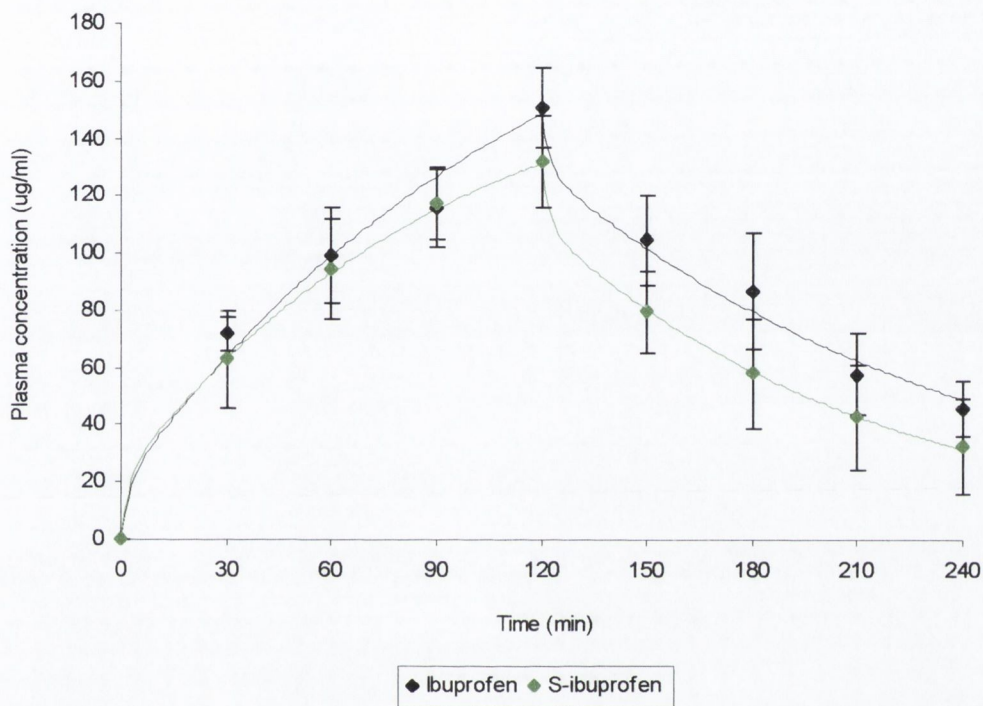


Figure 5.21 The best-fit plasma concentration versus time profiles together with the experimental data points for RS- and S-ibuprofen.

The pharmacokinetic parameters describing these two plasma profiles are given in Table 5.10.

Table 5.10 Results ($\pm$ s.d.) from fitting the plasma data for RS- and S-ibuprofen to the model described in Section 5.5.3.1 showing the best-fit ibuprofen pharmacokinetic parameter values.

Parameter type	S-ibuprofen	RS-ibuprofen
k_{elim} (min^{-1})	0.333 ± 0.158	0.325 ± 0.005
k_{12} (min^{-1})	0.066 ± 0.001	0.044 ± 0.016
k_{21} (min^{-1})	0.073 ± 0.010	0.081 ± 0.003
V (ml kg^{-1})	47.27 ± 8.91	52.88 ± 0.42
Dose ($\mu\text{g rat}^{-1}$)	19584	19405
n	9	6
MSC	4.58	4.02
r^2	0.99	0.99

No significant difference was observed between RS- and S-ibuprofen with regard to their elimination rate constants (k_{elim}) or their volumes of distribution (V) (ANOVA; $p > 0.05$). Given that Itoh et al. (1997) observed a significant difference in elimination between R- and S-ibuprofen in the rat, this may indicate that the R enantiomers in RS-ibuprofen are being converted *in vivo* to S-ibuprofen in this study, as described by Sattari and Jamali (1994).

5.7 CONCLUSIONS

Within the range of the eight buffers studied, the solubility of ibuprofen differed six fold from the lowest (HBSS) to the highest (PBS 7.4). These differences reflected the pHs of the buffers when saturated with drug. The results were consistent with Equation 5.2 with two exceptions, FeSSIF and Krebs' buffers, due to the presence of micelles and divalent ions respectively.

Significant differences were also observed in the buffer capacities of the buffers. When reporting buffer capacity values it is important to specify the pH of the buffer and whether an acid or a base was used to measure it, as each may produce different results unless the pH is at the pK_a .

Significant differences in the P_{app} values were observed between some of the buffer systems. FeSSIF was significantly lower than the other seven buffers (Fagerholm's, FaSSIF, HBSS, Krebs', McIlvaine's, PBS 6.8 and PBS 7.4). Fagerholm's, HBSS and Krebs' buffers were all significantly lower than the remaining five buffers. PBS 6.8 was observed to be significantly higher than McIlvaine's and FeSSIF.

Physicochemical factors such as pH and buffer capacity should be considered for determining the P_{app} values of ionisable compounds. Care needs to be exercised when comparing P_{app} values from different laboratories as buffer composition can have a significant effect on the permeability of an acidic drug that is substantially ionised over the pH range of the buffers.

Within the range of buffers studied, the osmolarities of the buffer systems appeared to influence the P_{app} values. As the osmolarities of the buffer systems increased, the corresponding P_{app} values decreased.

The intestinal microclimate buffering system appears to maintain the pH of the rat small intestinal contents somewhere in the range of 6.13-7.27.

Results from the *in situ* perfusion studies suggest that ibuprofen is principally absorbed via the aqueous pores or paracellular route. Alternatively, active transporters present in the intestinal mucosa may be involved (Tamai et al., 1995).

In the rat, ibuprofen obeys a two compartment pharmacokinetic model with first order output. The rate of appearance of ibuprofen in the plasma ($k_0(\text{plasma})$) was proportional to the rate of intestinal absorption ($k_0(\text{gut})$) at the steady-state.

There is no significant difference between the intestinal permeability properties of RS- and S-ibuprofen. The agreement between the pharmacokinetic parameters of RS- and S-ibuprofen may be due to chiral inversion of the R- to the S-enantiomer.

Chapter 6

Absorption and pharmacokinetic studies of NSAIDs

6.1 INTRODUCTION

Ibuprofen, ketoprofen and naproxen belong to a group of NSAIDs called the propionic acids. They have been the focus of several intestinal permeability studies including site-specific absorption (Raoof, 2001) and stereoselective absorption studies (Sattari and Jamali, 1994). However, their exact mechanism of absorption is still unknown. Results from Chapter 5 suggest that ibuprofen is absorbed by the paracellular route or via the aqueous pores. This assumes that active transport mechanisms are not involved. Carrier-mediated drug transport has been relatively unexplored as a potential absorption mechanism for these compounds. Recent results from other researchers suggest that the possible active transport of NSAIDs may have been overlooked (Tsuji and Tamai, 1996). Aspects of the absorption of ibuprofen, ketoprofen and naproxen will be investigated *in situ* and *in vitro* in this section with a view to exploring these issues.

6.2 IBUPROFEN AND ACTIVE TRANSPORTERS

A wide range of carrier-mediated intestinal transporters have been identified in the intestine and are described in Section 3.3.2.3. One of these in particular, the intestinal dipeptide transporter PepT1, has captured the most attention due to its wide substrate specificity (Dantzig and Bergin, 1990; Ganapathy et al., 1995; Ganapathy and Lieblich, 1982, 1986; Hidalgo et al., 1993, 1995; Hu et al., 1995; Liang et al., 1995; Zhu et al., 2000) which has been shown to include di- and tripeptides, β -lactam antibiotics, angiotensin-converting enzyme inhibitors, and even compounds without an obvious peptide bond or equivalent, such as δ -amino-levulinic acid (Temple et al., 1998) and ω -amino fatty acids (Döring et al., 1998). This high tolerance for structural diversity implies that the chemical criteria for substrate recognition are not very discriminating. Gonzalez et al. (1998) reported that the pancreatic cancer cell line, Capan-2, as well as expressing a population of peptide transporters, expresses higher levels of the PepT1 transporter than the Caco-2 intestinal epithelial cell line. Consequently this cell line is a useful tool for looking at drug uptake by peptide transporters.

Glycylsarcosine (Gly-Sar) is a dipeptide of molecular weight 146.1 containing glycine and sarcosine, the structures of which are shown in Figure 6.1.

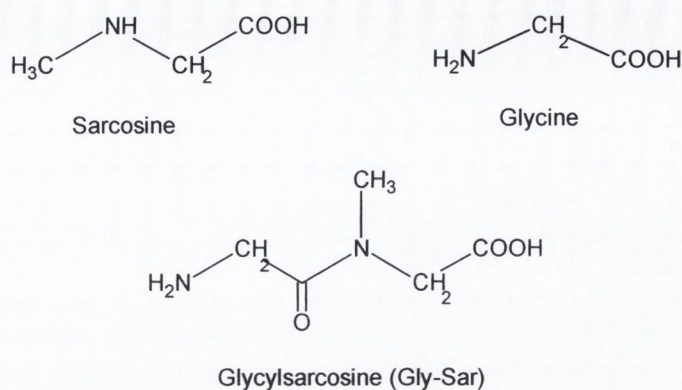


Figure 6.1 Chemical structures of sarcosine, glycine and glycylsarcosine (Gly-Sar).

The pK_a values of the amino and carboxylic acid groups of glycine and sarcosine are given in Table 6.1 (Dawson et al., 1986) and the cLog P of Gly-Sar is -3.07 (calculated as described in Section 4.4.20).

Table 6.1 pK_a values of the amino and carboxylic acid groups of glycine and sarcosine.

	Amino group	Carboxylic acid group
Glycine	9.78	2.35
Sarcosine	10.20	2.12

Gly-Sar is a substrate for peptide transporters and in its radiolabelled form has been used as a marker for PepT1 binding (Brandsch et al., 1997). Also, its peptide bond is resistant to hydrolysis by peptidases and is not subjected to the extensive cleavage seen with normal peptide substrates (Payne et al., 2001). Consequently, it is potentially a useful tool for assessing binding and/or possible uptake of compounds by peptide transporters using competitive inhibition studies. This will be investigated at different concentrations of ibuprofen using both the *in situ* rat gut perfusion method and Capan-2 cell culture uptake studies.

6.2.1 Influence of pH on Gly-Sar absorption

Some transport proteins are proton (H^+)-coupled (Section 3.3.2.3) and Brandsch et al. (1997) have shown that in cell culture the kinetics of the PepT1 transporter are influenced by proton concentration. Increasing the proton concentration outside the cells resulted in an increase in the maximal velocity of Gly-Sar uptake by PepT1. In this section, the impact of different pHs (and consequently H^+ concentrations) on Gly-Sar uptake with and without ibuprofen was investigated using phosphate buffers of different pHs in the *in situ* rat gut as described in Section 4.4.7. Perfusate samples were assayed for Gly-Sar by liquid scintillation and for ibuprofen by HPLC as described in Sections 4.4.11.3 and 4.4.11.1 respectively.

6.2.1.1 Ibuprofen and Gly-Sar in PBS 6.8

Solutions of radiolabelled Gly-Sar (3H -Gly-Sar) in PBS 6.8 with and without ibuprofen (1 mg ml^{-1}) were prepared as described in Section 4.4.8.1. The composition of PBS 6.8 is given in Section 4.4.1. The absorption profiles of Gly-Sar in the two systems are given in Figure 6.2.

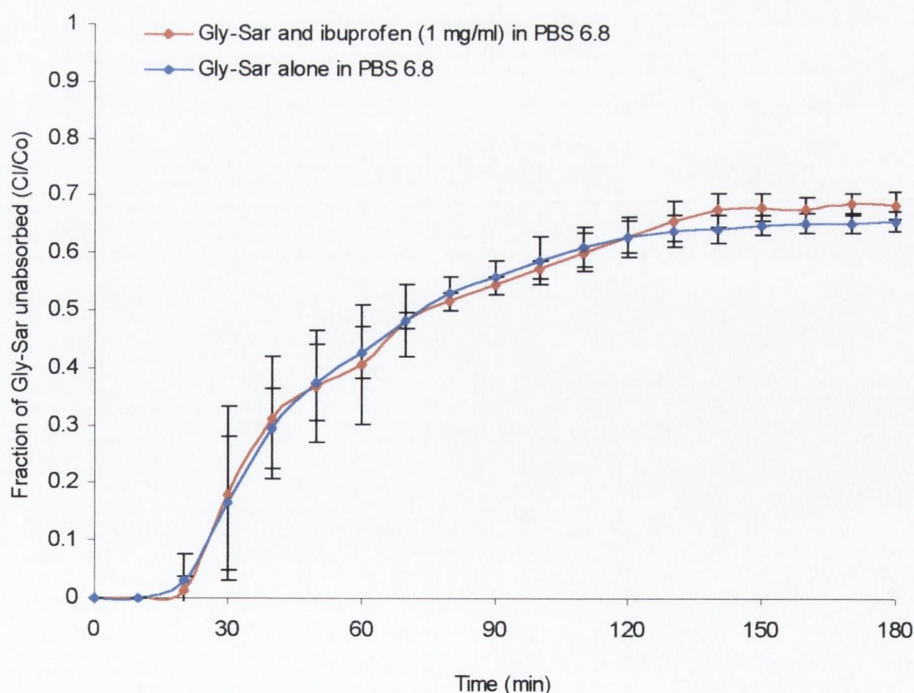


Figure 6.2 Absorption profiles of Gly-Sar in PBS 6.8 with and without ibuprofen (1 mg ml^{-1}).

The steady-state was taken to be between 150-180 minutes. The absorption profiles were converted to permeability coefficients (P_{app}) as described in Section 4.4.12 after correcting for water flux as described in Section 4.4.13.2. The Gly-Sar P_{app} value in a particular system was obtained by averaging the P_{app} values over 150-180 minutes for each perfusion experiment. For a particular system, the P_{app} values for each rat were averaged and a standard deviation calculated and these are given in Table 6.2.

Table 6.2 *Gly-Sar permeability coefficients (P_{app}) in the presence and absence of ibuprofen (1 mg ml⁻¹) in PBS 6.8.*

	n	Gly-Sar P_{app} (cm sec ⁻¹ x 10 ⁻⁴)	s.d.
Gly-Sar alone	4	0.38	0.02
Gly-Sar and ibuprofen	4	0.35	0.03

No significant differences were observed between the P_{app} values for Gly-Sar in the presence and absence of ibuprofen (1 mg ml⁻¹) (ANOVA; $p > 0.05$).

6.2.1.2 Ibuprofen and Gly-Sar in PBS 6.0

Solutions of radiolabelled Gly-Sar (³H-Gly-Sar) in PBS 6.0 with and without ibuprofen (1 mg ml⁻¹) were prepared as described in Section 4.4.8.1. The composition of PBS 6.0 is given in Section 4.4.1. *In situ* perfusion studies were carried out as described in Section 4.4.7 and the steady-state (150-180 minutes) fraction unabsorbed values were converted to P_{app} values as described in Section 4.4.12. These are given in Table 6.3.

Table 6.3 *Gly-Sar permeability coefficients (P_{app}) in the presence and absence of ibuprofen (1 mg ml⁻¹) in PBS 6.0.*

	n	Gly-Sar P_{app} (cm sec ⁻¹ x 10 ⁻⁴)	s.d.
Gly-Sar alone	4	0.48	0.01
Gly-Sar and ibuprofen	4	0.44	0.04

No significant difference between the P_{app} values was observed (ANOVA: $p > 0.05$).

Komiya et al. (1980) studied the intestinal absorption characteristics of a range of steroids of varying lipophilicity, including progesterone and hydrocortisone, in a pH 6.0 phosphate buffer using the same experimental procedure as this work. They reported that the absorption of the highly lipophilic steroid progesterone (Log P 3.99) was aqueous boundary layer controlled with a P_{app} of $1.08 \times 10^{-4} \text{ cm sec}^{-1}$. In contrast, the absorption of the less lipophilic steroid hydrocortisone (Log P 1.53) was membrane controlled with a P_{app} of $0.30 \times 10^{-4} \text{ cm sec}^{-1}$. The P_{app} values for Gly-Sar in a similar system (PBS 6.0) given in Table 6.2 are much closer to the P_{app} value for hydrocortisone than to progesterone. This suggests that the intestinal absorption of Gly-Sar is membrane controlled. The physicochemical properties of Gly-Sar support this as it is 99% ionised at pH 6.0, has a relatively low molecular weight (146.1) together and a low cLog P value of -3.07 . This indicates that it is hydrophilic and would be expected to pass through the aqueous boundary layer faster than through the membrane.

6.2.1.3 Ibuprofen and Gly-Sar in PBS 5.0

Solutions of radiolabelled Gly-Sar (^3H -Gly-Sar) in PBS 5.0 with and without ibuprofen (0.25 mg ml^{-1}) were prepared as described in Section 4.4.8.1. The composition of PBS 5.0 is given in Section 4.4.1. *In situ* perfusion studies were carried out as described in Section 4.4.7 and the steady-state (150-180 minutes) fraction unabsorbed values were converted to P_{app} values as described in Section 4.4.12. These are given in Table 6.4.

Table 6.4 Gly-Sar permeability coefficients (P_{app}) in the presence and absence of ibuprofen (0.25 mg ml^{-1}) in PBS 5.0.

	n	Gly-Sar P_{app} ($\text{cm sec}^{-1} \times 10^{-4}$)	s.d.
Gly-Sar alone	4	0.57	0.02
Gly-Sar and ibuprofen	4	0.60	0.09

As with Gly-Sar in PBS 6.0, there is no significant difference between the P_{app} values of Gly-Sar with and without ibuprofen (0.25 mg ml^{-1}) in PBS 5.0 (ANOVA: $p > 0.05$).

This suggests that altering the pH of the perfusion solution, and consequently altering the proton concentration, has no effect on the impact of ibuprofen on Gly-Sar uptake. This shows that any variation in Gly-Sar absorption at different pHs is not due to any effect that ibuprofen may have on its absorption.

6.2.1.4 Gly-Sar absorption at varying pH

Although varying the pH of the perfusion solutions had no effect on the impact of ibuprofen on Gly-Sar uptake, the impact of varying pH on Gly-Sar uptake and transporter activity *in situ* was investigated.

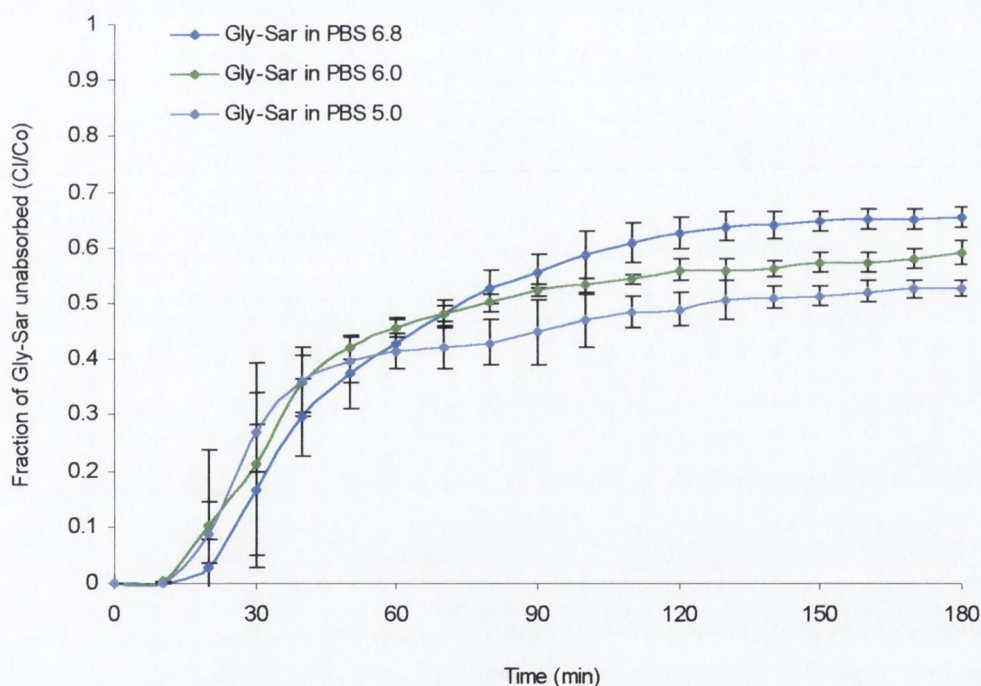


Figure 6.3 Absorption profiles of Gly-Sar in phosphate buffers of three different pHs (PBS 6.8, PBS 6.0 and PBS 5.0).

It is apparent from Figure 6.3 that as the pH of the perfusion solutions increase, the fractions unabsorbed also increase, suggesting that the uptake of Gly-Sar from the intestine decreases with increasing pH. This was confirmed when the fraction unabsorbed values were converted into P_{app} values, given in Table 6.5.

Table 6.5 Gly-Sar permeability coefficients (P_{app}) in phosphate buffers of three different pHs (PBS 6.8, PBS 6.0 and PBS 5.0).

	n	pH at the steady-state	s.d.	Gly-Sar P_{app} ($\text{cm sec}^{-1} \times 10^{-4}$)	s.d.
Gly-Sar in PBS 6.8	4	6.63	0.02	0.38	0.02
Gly-Sar in PBS 6.0	4	6.07	0.01	0.48	0.01
Gly-Sar in PBS 5.0	4	5.66	0.01	0.57	0.02

The differences between the Gly-Sar P_{app} values in Table 6.5 were tested for statistical significance using ANOVA. The values in the three different systems are all significantly different from each other ($p < 0.05$). The P_{app} values of Gly-Sar were plotted against steady-state pH and are shown in Figure 6.4.

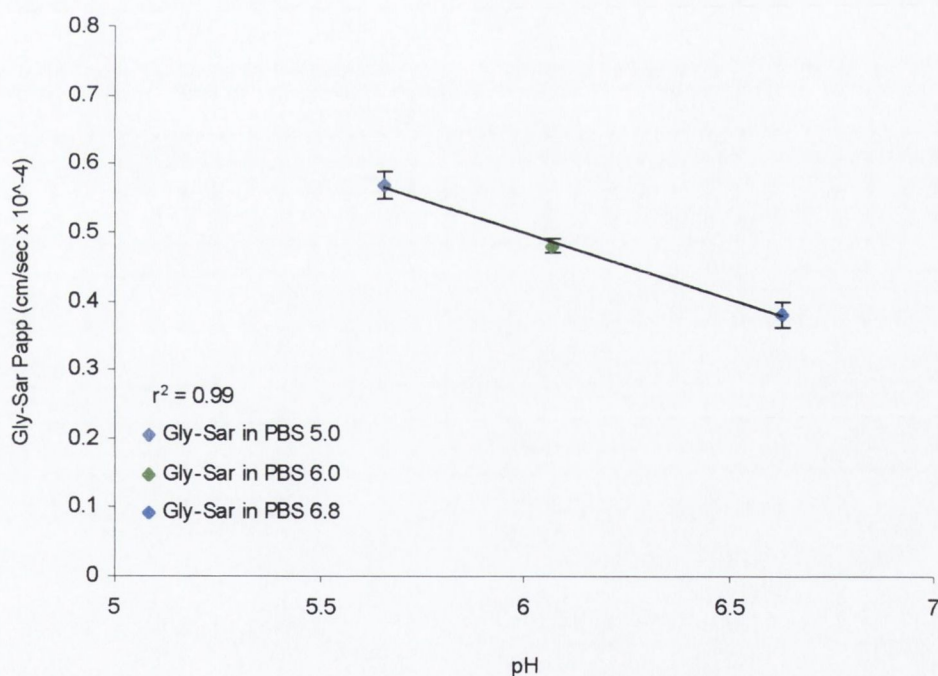
**Figure 6.4** Correlation between Gly-Sar P_{app} ($\text{cm sec}^{-1} \times 10^{-4}$) and the steady-state pH of the perfusate samples.

Figure 6.4 shows that there is a good correlation between Gly-Sar P_{app} and the pH of the perfusate samples at the steady-state ($r^2 = 0.99$). As pH decreases, the concentration of protons in the perfusate increases and the P_{app} for Gly-Sar also

increases. This is in agreement with the cell culture results published by Brandsch et al. (1997), which showed that increasing the proton concentration outside the cells resulted in an increase in the maximal velocity of Gly-Sar uptake by PepT1. The correlation between Gly-Sar P_{app} and perfusate pH seen here suggests that proton-dependent transporters are involved and it can be used as a model for active transport of Gly-Sar.

6.2.2 *In situ* absorption studies of ibuprofen and Gly-Sar

The absorption of radiolabelled Gly-Sar (^3H -Gly-Sar) in PBS 6.8 at various concentrations of ibuprofen was investigated using *in situ* intestinal perfusion studies as described in Section 4.4.7. The composition of PBS 6.8 is given in Section 4.4.1. Perfusate samples were assayed for Gly-Sar by liquid scintillation counting and for ibuprofen by HPLC as described in Sections 4.4.11.3 and 4.4.11.1 respectively. PBS 6.8 was chosen from the results in Chapter 5, as its osmolality and pH values are close to physiological levels.

6.2.2.1 Influence of ibuprofen (1 mg ml⁻¹) on Gly-Sar absorption

Solutions of radiolabelled Gly-Sar (^3H -Gly-Sar) in PBS 6.8 with and without ibuprofen (1 mg ml⁻¹) were prepared as described in Section 4.4.8.1. The absorption profiles of Gly-Sar in the two systems have been shown previously in Figure 6.2 confirming no significant differences in Gly-Sar absorption in the presence and absence of ibuprofen.

6.2.2.2 Influence of ibuprofen (5 mg ml⁻¹) on Gly-Sar absorption

In situ intestinal perfusions were also performed using solutions of radiolabelled Gly-Sar (^3H -Gly-Sar) in PBS 6.8 with and without ibuprofen (5 mg ml⁻¹), which were prepared as described in Section 4.4.8.1. The Gly-Sar absorption profiles obtained are given in Figure 6.5 together with the absorption profile of Gly-Sar in the 1 mg ml⁻¹ ibuprofen system.

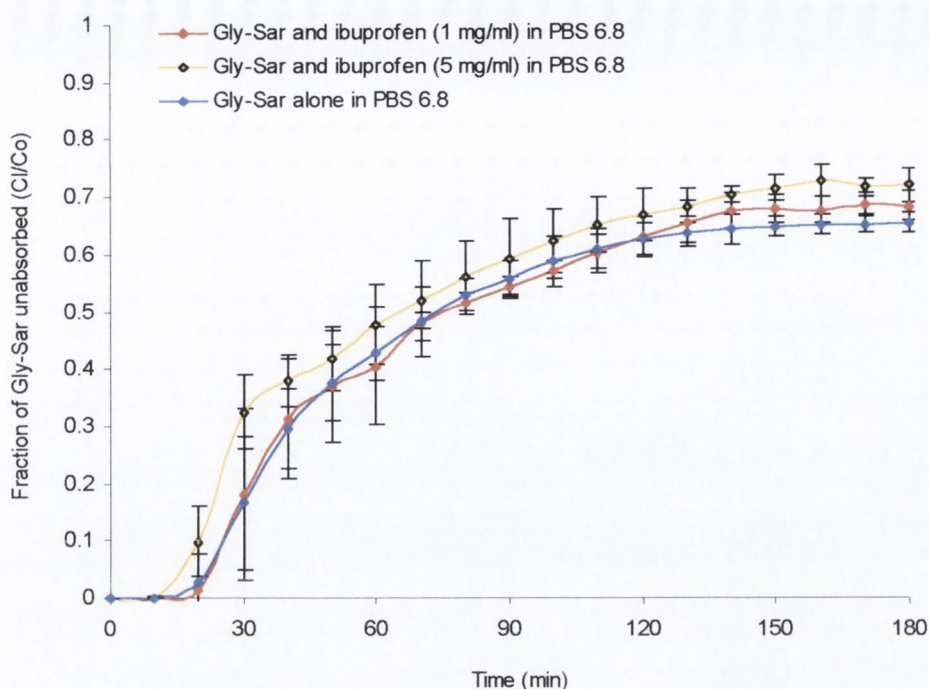


Figure 6.5 Absorption profiles of Gly-Sar in PBS 6.8 with ibuprofen 1 mg ml⁻¹, ibuprofen 5 mg ml⁻¹ and without ibuprofen.

The fraction unabsorbed values for Gly-Sar at the steady-state were corrected for water flux and converted into P_{app} values as described in Section 4.4.12. The results are given in Table 6.6 together with the steady-state ibuprofen concentrations.

Table 6.6 Gly-Sar permeability coefficients (P_{app}) ($\pm$ s.d.) in PBS 6.8 with ibuprofen 1 mg ml⁻¹, ibuprofen 5 mg ml⁻¹ and without ibuprofen together with their steady-state ibuprofen concentrations and pH values ($\pm$ s.d.).

	n	Gly-Sar P_{app} (cm sec ⁻¹ x 10 ⁻⁴)	Ibuprofen conc. (mg ml ⁻¹)	Steady- state pH
Gly-Sar alone	4	0.38 $\pm$ 0.02	0.00 $\pm$ 0.00	6.63 $\pm$ 0.02
Gly-Sar with ibup. (1 mg ml ⁻¹)	4	0.35 $\pm$ 0.03	0.20 $\pm$ 0.02	6.62 $\pm$ 0.01
Gly-Sar with ibup. (5 mg ml ⁻¹)	4	0.29 $\pm$ 0.03	1.07 $\pm$ 0.13	6.64 $\pm$ 0.04

ANOVA was used to test for any significant differences between the three P_{app} values. The P_{app} for Gly-Sar in the presence of ibuprofen (1 mg ml^{-1}) is not significantly different from the P_{app} for Gly-Sar alone or the P_{app} for Gly-Sar in the presence of ibuprofen (5 mg ml^{-1}). However, the P_{app} for Gly-Sar in the presence of ibuprofen (5 mg ml^{-1}) is significantly different from the P_{app} for Gly-Sar alone ($p < 0.05$). The relationship between Gly-Sar uptake and ibuprofen concentration is shown in Figure 6.6.

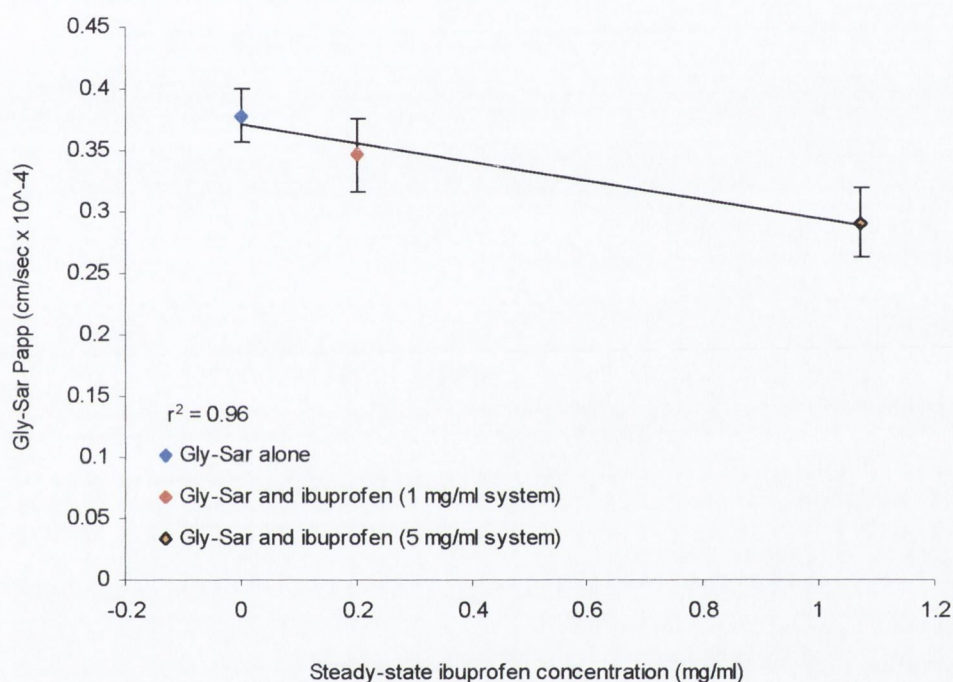


Figure 6.6 Relationship between Gly-Sar P_{app} and ibuprofen concentration in PBS 6.8.

Ibuprofen appears to reduce the uptake of Gly-Sar by the intestinal mucosa *in situ*, assuming that there is no interaction between Gly-Sar and ibuprofen and that ibuprofen does not adversely affect the transporter. In the 5 mg ml^{-1} ibuprofen system (1.07 mg ml^{-1} at steady-state), there is a significant 23.0% decrease in Gly-Sar uptake. This implies that, at relatively high concentrations, ibuprofen may interact with the transport proteins through non-specific binding. This significant reduction in Gly-Sar uptake at high concentration (5 mg ml^{-1}) *in situ* may reflect the importance of other routes of transport or binding. These results have been obtained from an *in situ* model which involves the whole animal. The binding of ibuprofen to transport proteins may be more apparent in cell culture where the cells being used express high concentrations of membrane bound transporters such as PepT1.

6.2.3 pH monitoring of perfusate samples from Gly-Sar perfusion studies

The pHs of the perfusate samples were monitored over the course of each experiment as described in Section 4.4.9 and the pH versus time profiles are shown in Figure 6.7. Over the time course of the perfusion experiments, the pH of the samples gradually changed from their initial values towards a median value of six.

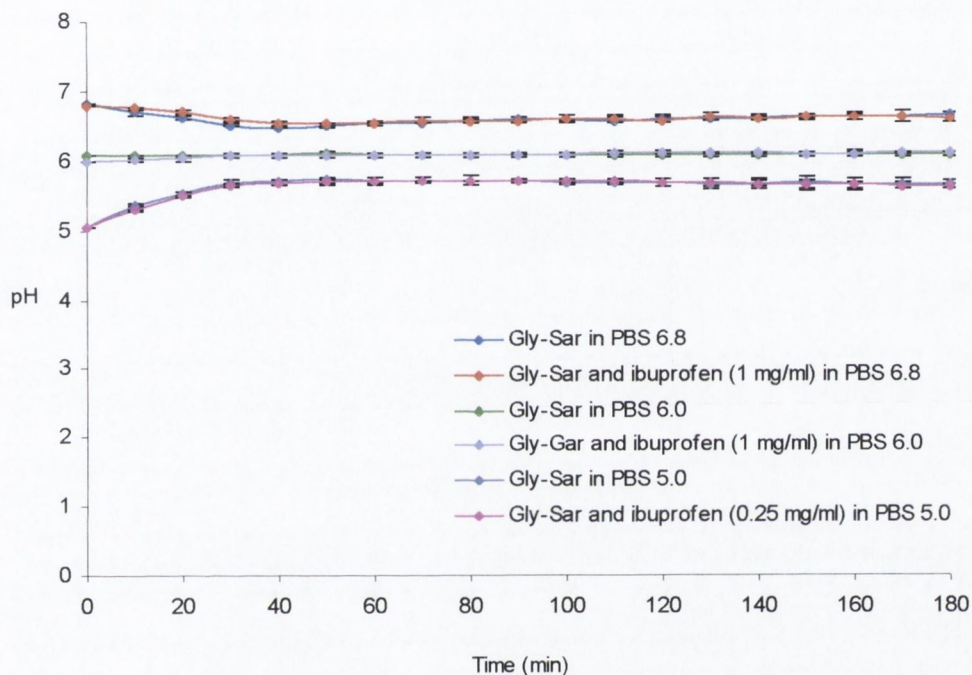


Figure 6.7 pH versus time profiles of the perfusate samples from the Gly-Sar absorption studies.

The shifts in pH seen in the profiles in Figure 6.7 are expressed numerically in Table 6.7.

Table 6.7 Changes in pH of the perfusate samples from time zero to steady-state.

System	pH of perfusate		pH of perfusate		n	Change in pH
	before perfusion	s.d.	samples at the steady-state	s.d.		
Gly-Sar in PBS 6.8	6.83	0.01	6.63	0.02	4	0.20
Gly-Sar in PBS 6.0	6.08	0.01	6.07	0.01	3	0.01
Gly-Sar in PBS 5.0	5.06	0.01	5.66	0.01	4	0.60

Over the time course of the perfusions, the pHs of the PBS 6.8 samples are shifted downwards by 0.20 pH units, from an initial pH of 6.83 to a steady-state pH of 6.63. The pHs of the PBS 5.0 samples are shifted upwards by 0.60 pH units from an initial pH of 5.06 to a steady-state pH of 5.66. For these two systems the shifts in pH are significant (t-test: $p < 0.05$). The shift in pH is much greater for PBS 5.0 than for PBS 6.8 due to differences in buffering capacity between the two systems, as discussed in Section 5.4.1.

The shift in perfusate pH is most dramatic over the first 40 minutes, after which there is very little change. This suggests that the intestinal buffering system reaches a steady-state after 40 minutes. The shift in perfusate pH that is maintained over the course of the perfusion, indicating that there is no exhaustion of the intestinal buffering system. It also provides evidence of the viability of the model as it indicates that the intestinal segment is functioning normally.

The pHs of the PBS 6.0 samples do not undergo any significant change (t-test: $p > 0.05$) and stay at approximately pH 6 over the course of the perfusion, despite having a lower buffering capacity than PBS 6.8. This suggests that the intestinal microclimate pH in the rat is close to pH 6, as proposed by Ikuma et al. (1996).

6.2.3 Cell culture studies

In addition to *in situ* absorption studies, the effect of ibuprofen on Gly-Sar uptake was also studied in cell culture using Capan-2 cells as described in Section 4.4.18. The level of Gly-Sar uptake was assessed in the presence of different concentrations of ibuprofen ranging from 0–1.01 mg ml⁻¹ in transporter buffer pH 6.0. The percentage of Gly-Sar uptake at each concentration was determined as described in Section 4.4.18 and the results are given in Table 6.8. Ibuprofen does not affect the viability of the cells (Lane, 2002).

Table 6.8 *Results of the effect of varying ibuprofen concentration on Gly-Sar uptake in cell culture.*

Ibuprofen concentration (mg ml ⁻¹)	% Gly-Sar Uptake	s.d.
0.00	100.00	0.00
0.03	67.42	0.64
0.06	61.31	1.99
0.13	47.97	3.57
0.25	29.97	6.23
0.51	18.40	5.45
1.01	11.13	7.86

The data points in Table 6.8 were fitted to Equation 6.1 describing a 4-parameter logistic curve, using Sigma Plot 8.0.

$$y = min + \frac{max - min}{1 + 10^{(LogIC_{50} - x) Hillslope}}$$

Equation 6.1

where *y* is the % Gly-Sar uptake, *x* is the ibuprofen concentration, *min* and *max* are the minimum and maximum percent Gly-Sar uptake values respectively, *IC*₅₀ is the inhibitory or binding constant and *hillslope* is an additional parameter which characterises the slope of the curve at its midpoint (Sigma Plot, 2003). *IC*₅₀ is the concentration of the compound under test that will produce a 50% reduction in Gly-Sar uptake by the transporters.

The best-fit profile of percentage GlySar uptake versus ibuprofen concentration (mg ml^{-1}) together with the experimental data points is shown in Figure 6.8. The best-fit value for the IC_{50} was 0.10 mg ml^{-1} or 0.48 mM ($r^2 = 0.99$).

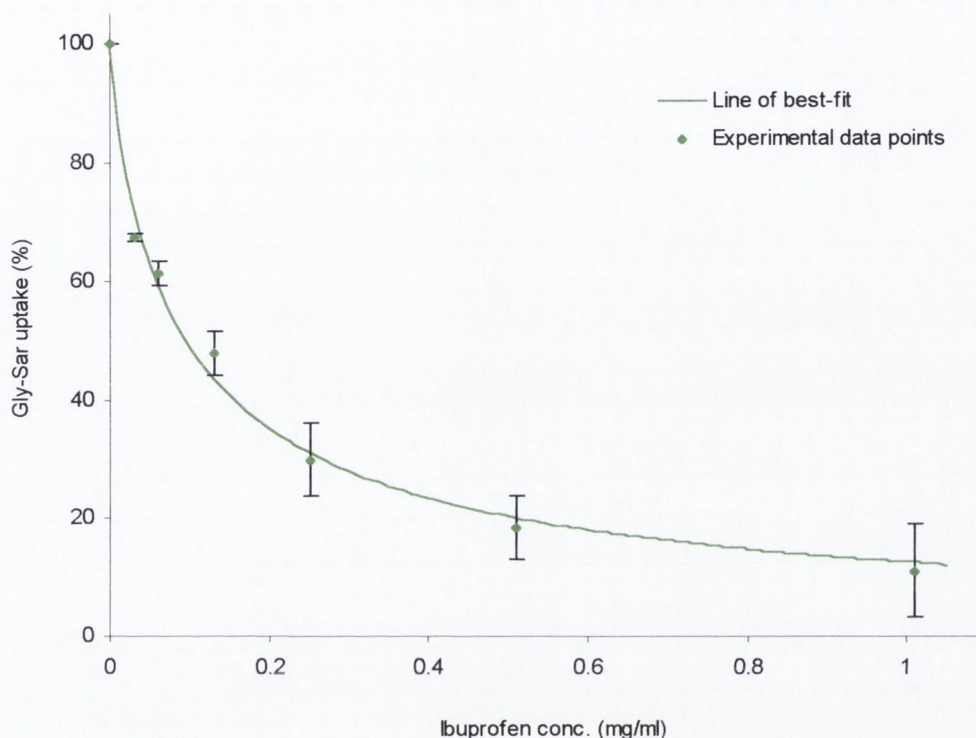


Figure 6.8 Best-fit profile of percentage GlySar uptake expressed in Capan-2 cells in cell culture versus ibuprofen concentration (mg ml^{-1}) together with the experimental data points.

The profile in Figure 6.8 shows the effect of varying ibuprofen concentration on Gly-Sar uptake in cell culture. It clearly shows that as the concentration of ibuprofen increases, the percentage of Gly-Sar uptake decreases, suggesting that ibuprofen binds to the same transport proteins *in vitro*.

Section 6.2.2. shows that there may be some binding of ibuprofen to the same transporters *in situ*, but the results were not as conclusive as they are from these cell culture studies. This may be due to the nature of the *in situ* model, which encompasses a more diverse array and variable expression of transporters and absorption pathways.

6.2.5 Conclusions

The uptake of Gly-Sar by the intestinal mucosa *in situ* is reduced by ibuprofen. This reduction is much more apparent *in vitro* using cell culture studies. This may be due to the higher concentration of peptide transporters, such as PepT1, expressed in Capan-2 cells (Gonzalez et al., 1998) compared to rat small intestinal enterocytes.

Gly-Sar uptake *in situ* was significantly influenced by the pH of the perfusion solution, with a low pH (high proton concentration) producing a high rate of uptake by the proton-dependent transporters.

The intestinal absorption of Gly-Sar appears to be membrane controlled.

The pHs of the perfusate samples in each of the systems were shifted from their initial values towards a median value of approximately six. In all cases the shift in pH mainly occurred over the first forty-minutes of perfusion.

6.3 IBUPROFEN, KETOPROFEN AND NAPROXEN

Ibuprofen, ketoprofen and naproxen all belong to a group of NSAIDs called the propionic acids. However, they differ in terms of their physicochemical properties (*e.g.* pK_a , Log P, solubility and molecular weight) and it is of interest to see if these differences impact on their intestinal absorption properties.

6.3.1 Solubility profiles

The solubilities of ketoprofen and naproxen in PBS 6.8, PBS 7.4, Fagerholm's and HBSS buffers were determined by the method of Chiou and Kyle (1979) as described in Section 4.4.2.1. The compositions of the buffers are given in Section 4.4.1. The concentrations quoted at each time point are an average of two determinations.

6.3.1.1 Ketoprofen

Figure 6.9 shows the best-fit solubility profiles (Scientist) of ketoprofen in each of the buffers.

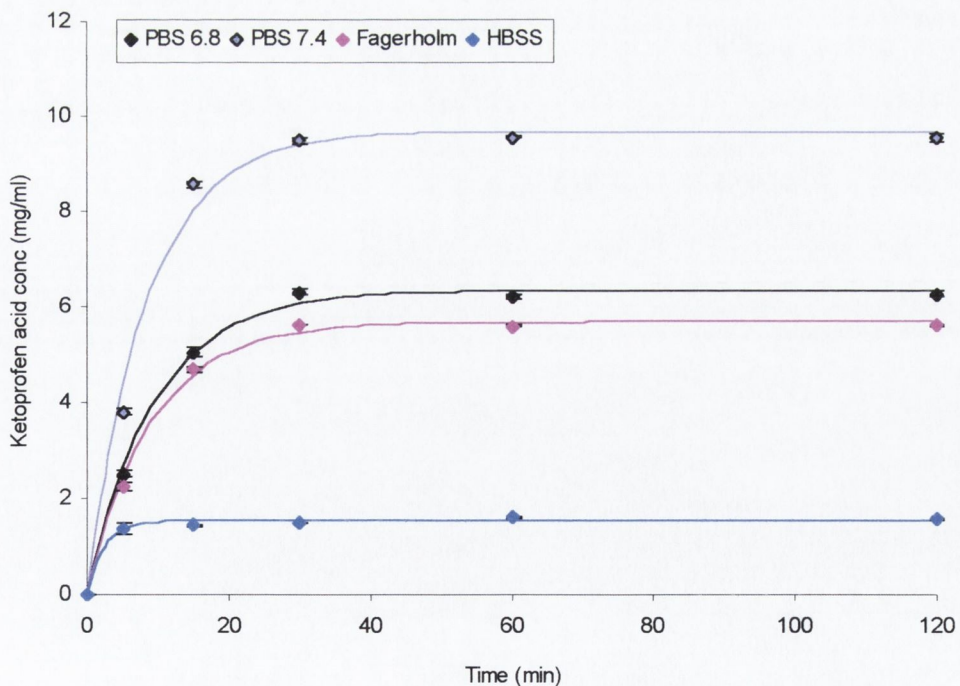


Figure 6.9 Ketoprofen concentration versus time profiles in PBS 6.8, PBS 7.4, Fagerholm and HBSS.

The solubility of ketoprofen varied significantly with the buffer, with a six-fold difference between HBSS and PBS 7.4. Saturation appeared to be reached within one hour and the solubility profiles were fitted to Equation 5.1. The 'k' values in Table 6.9 indicate that the rate of attainment of steady-state in HBSS was much higher than in the other three buffers. This may be due to its relatively high initial pH and low buffer capacity (Section 5.4) which allow it to reach an equilibrium concentration faster than the other systems. HBSS is a sodium bicarbonate based buffer, and in this respect is different to the other three phosphate based buffers, which may also be a reason for its higher 'k' value.

Table 6.9 *Solubility of ketoprofen in PBS 6.8, PBS 7.4, Fagerholm's and HBSS at 37°C, pH values of the buffers and the saturated solutions and buffer osmolarities.*

Buffer System	Solubility C_s (mg ml ⁻¹)	s.d.	k (min ⁻¹)	Initial pH of buffer	Final pH	Osmolarity (milliosmoles/l)
PBS 7.4	9.68	0.08	0.12	7.37	5.91	337.5
PBS 6.8	6.33	0.06	0.11	6.72	5.72	330.6
Fagerholm (pH 6.5)	5.69	0.01	0.11	6.62	5.67	286.8
HBSS	1.54	0.02	0.43	7.19	4.92	310.7

The four buffers used varied in pH over a range of 0.75 pH units from 6.62 to 7.37. The final pH values of the saturated solutions of ketoprofen in each of the systems and the corresponding solubility values were fitted to Equation 5.2 and the pH-solubility profile obtained is shown in Figure 6.10.

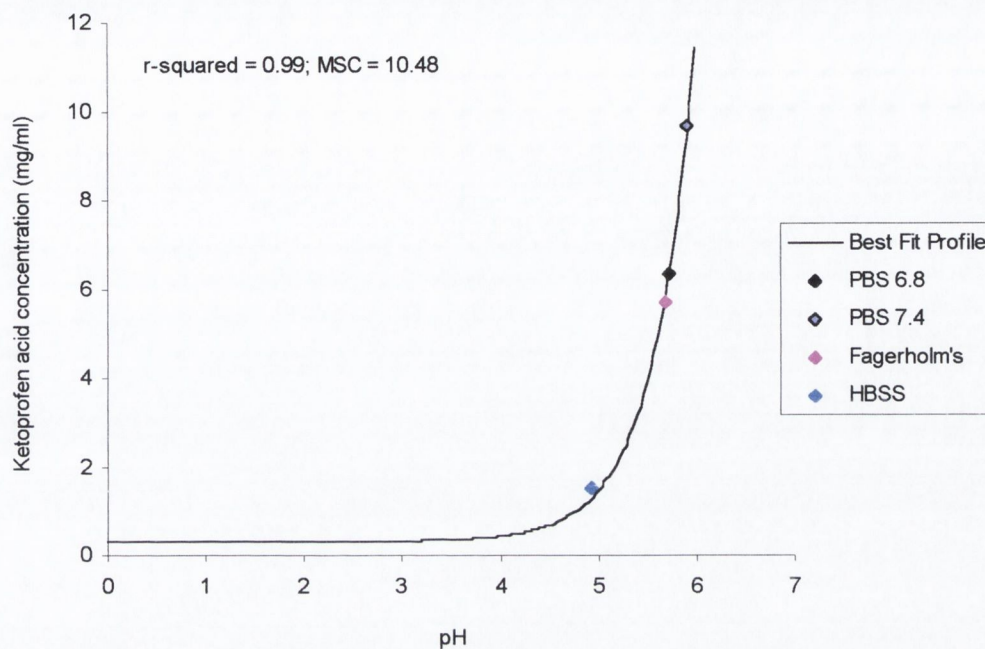


Figure 6.10 pH-solubility profile of ketoprofen.

The best-fit values for C_0 and the pK_a of ketoprofen are 0.32 mg ml^{-1} (1.25 mM) and 4.44 at 37°C respectively. These agree with previously reported values of 0.18 mg ml^{-1} (0.70 mM) and 4.45 by Fini et al. (1995) and Goosen et al. (1998) respectively.

6.3.1.2 Naproxen

Figure 6.11 shows the best-fit solubility profiles (Scientist) of naproxen in each of the buffers.

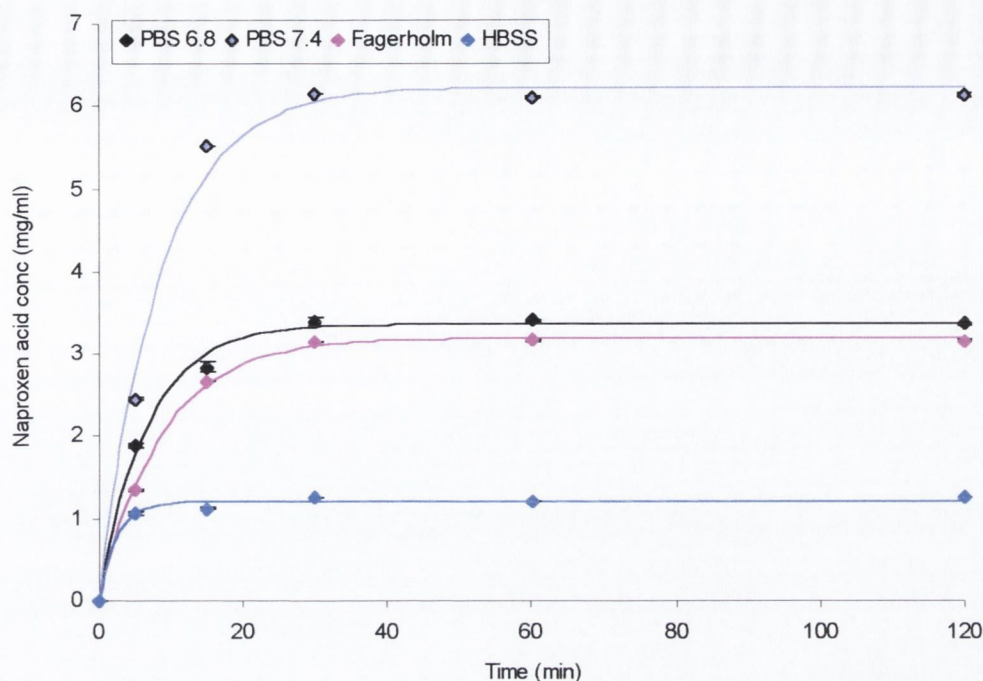


Figure 6.11 Naproxen concentration versus time profiles in PBS 6.8, PBS 7.4, Fagerholm and HBSS.

The solubility of naproxen varied significantly with the buffer, with a five-fold difference between HBSS and PBS 7.4. Saturation appeared to be reached within one hour and the solubility profiles were fitted to Equation 5.1. The 'k' values in Table 6.10 show that, as with ketoprofen, the rate of attainment of steady-state in HBSS was much higher than in the other three buffers and similar reasons may apply as for ketoprofen.

Table 6.10 Solubility of naproxen in PBS 6.8, PBS 7.4, Fagerholm's and HBSS at 37°C, pH values of the buffers and the saturated solutions and buffer osmolarities.

Buffer System	Solubility C_s (mg ml ⁻¹)	s.d.	k (min ⁻¹)	Initial pH of buffer	Final pH	Osmolarity (milliosmoles/L)
PBS 7.4	6.23	0.01	0.12	7.36	6.58	337.5
PBS 6.8	3.36	0.01	0.15	6.72	6.31	330.6
Fagerholm	3.20	0.01	0.12	6.59	6.29	286.8
HBSS	1.22	0.01	0.40	7.19	5.86	310.7

The pH values of the four buffers varied by 0.77 pH units over the range 6.59 to 7.36. The final pH values and the corresponding solubility values of the saturated solutions of naproxen in each of the buffers were fitted to Equation 5.2. The pH-solubility profile obtained is shown in Figure 6.12.

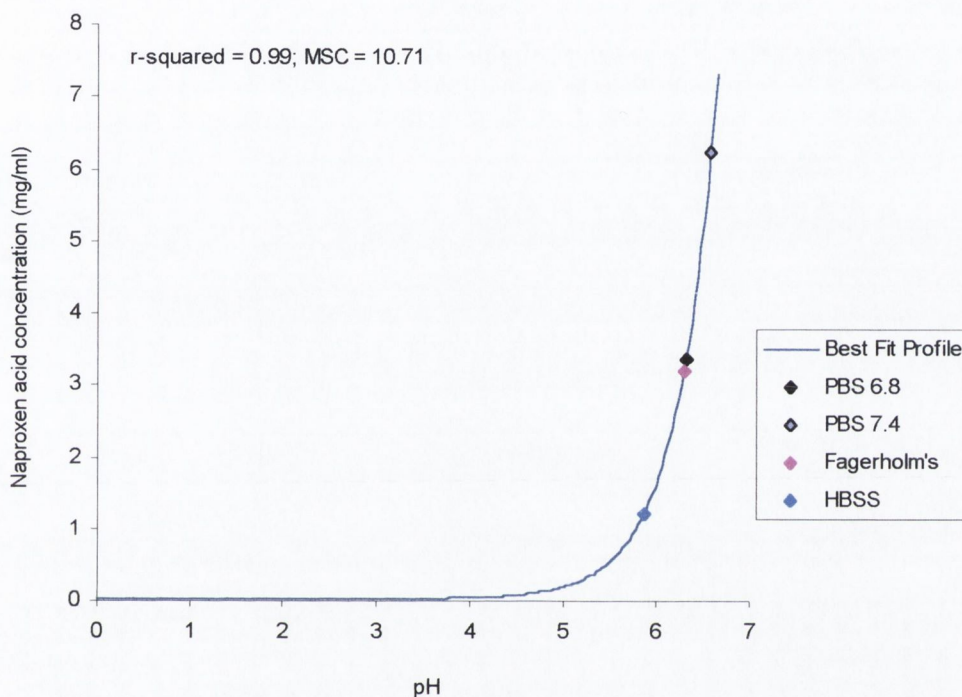


Figure 6.12 pH-solubility profile of naproxen.

The best-fit values for C_0 and the pK_a of ketoprofen are 0.03 mg ml^{-1} (0.14 mM) and 4.29 at 37°C respectively. These agree with the values of 0.02 mg ml^{-1} (0.07 mM) and 4.20 previously reported by Fini et al. (1995) and Clarke and Moffat (1986) respectively.

6.3.1.3 Comparison of solubility results of ibuprofen, ketoprofen and naproxen

Figure 6.13 shows the pH-solubility profiles of ibuprofen, ketoprofen and naproxen. It is apparent from these profiles that ketoprofen has a higher intrinsic solubility and a lower pK_a than ibuprofen and naproxen. Ibuprofen and naproxen have similar values for intrinsic solubility and pK_a as shown in Table 6.11.

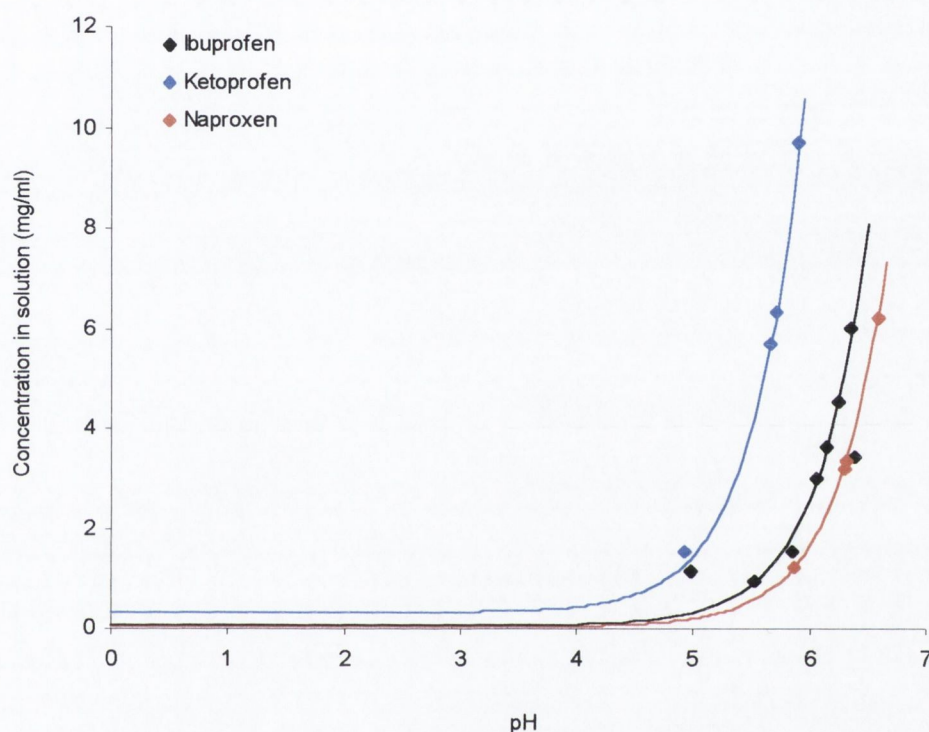


Figure 6.13 pH-solubility profiles of ibuprofen, ketoprofen and naproxen.

Table 6.11 Experimental and literature intrinsic solubility (C_0) and pK_a values, molecular weight (MW), Log P and melting point (MP) values of ibuprofen, ketoprofen and naproxen.

NSAID	Exp. ^c C_0 (mg ml ⁻¹)	Literature ^f C_0 (mg ml ⁻¹)	Exp. ^c pK_a	Lit. ^f pK_a	MW	Log P	MP (°C)
Ibuprofen	0.068	0.078 ^a	4.43	4.55 ^a	206.3 ^c	3.51 ^d	75-78 ^c
Ketoprofen	0.319	0.178 ^a	4.44	4.45 ^b	254.3 ^c	3.12 ^d	93-96 ^c
Naproxen	0.032	0.016 ^a	4.29	4.20 ^c	230.3 ^c	3.34 ^d	156 ^c

^aFini et al., (1995); ^bGoosen et al., (1998); ^cClarke and Moffat (1986); ^dHadgraft et al., (2000); ^edetermined at 37°C; ^fdetermined at 25°C.

The experimental intrinsic solubility (C_0) values of ketoprofen and naproxen are both approximately twice the literature values. This is most likely due to temperature as the literature values were determined by Fini et al. (1995) at room temperature (25°C) while the experimental values were determined at 37°C. Additionally, it may be due to the lack of data points at low pH values ($\text{pH} < 4$) where concentration in solution approximates C_0 . The slight differences between the experimental and literature pK_a values for the three compounds may be due to ionic strength effects as well as temperature.

Ketoprofen showed the highest intrinsic solubility (0.319 mg mL⁻¹), which can be explained by its relatively low Log P value (3.12) compared to ibuprofen (3.51) and naproxen (3.34). The Log P of a compound is a measure of its lipophilicity (Camenisch et al., 1996) and the lower the Log P, the higher aqueous solubility.

Naproxen showed the lowest intrinsic solubility (0.032 mg mL⁻¹) which is due to its melting point (156°C) being significantly higher than ibuprofen (75-78°C) or ketoprofen (93-96°C). This can be explained using Equation 6.2 (Jain and Yalkowsky, 2001) which shows the relationship between the solubility of a solid and its melting point:

$$\text{Log} S_w = 0.5 - 0.01(mp - 25) - \text{Log} P \quad \text{Equation 6.2}$$

where $\text{Log} S_w$ is the logarithm of the molar aqueous solubility of a solid compound, mp is the melting point of the compound in degrees Celcius and $\text{Log} P$ is the logarithm of the octanol:water partition coefficient. For a series of compounds of similar Log P, as their melting points increase their solubilities will decrease.

6.3.2 Absorption profiles

In situ absorption studies were carried out using solutions of naproxen in PBS 6.8 (1 mg mL⁻¹) and ketoprofen in PBS 6.8 (1 mg mL⁻¹) as described in Section 4.4.7. The fraction unabsorbed versus time profiles for these two systems are shown in Figure 6.14 together with the profile of ibuprofen in the same buffer at the same concentration.

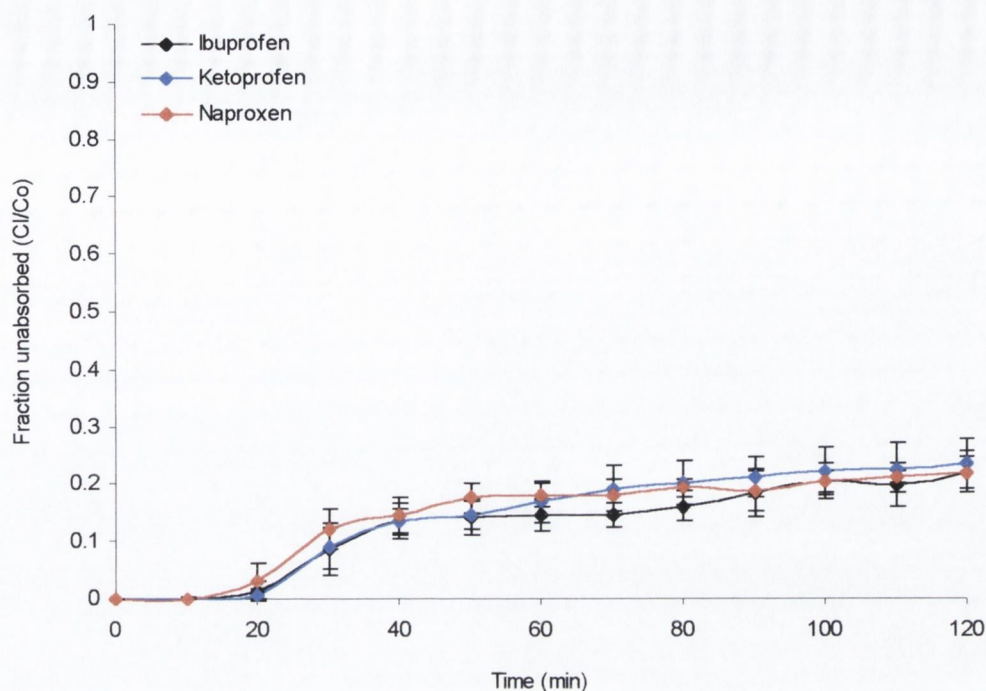


Figure 6.14 Fraction unabsorbed versus time profiles for ibuprofen, ketoprofen and naproxen (all 1 mg ml^{-1}) in PBS 6.8.

The fraction unabsorbed values at the steady-state were corrected for water flux and converted to P_{app} values as described in Section 4.4.12. The steady-state was taken to be between 100–120 minutes. The P_{app} values for each of the systems were corrected for intestinal water flux as described in Section 4.4.13.2 and are given in Table 6.12.

Table 6.12 Permeability coefficients (P_{app}) of ibuprofen, ketoprofen and naproxen present at 1 mg ml^{-1} in PBS 6.8 with their steady-state pH values, literature Log P, calculated Log D values and molecular topological polar surface area (TPSA).

NSAID	P_{app} ($\text{cm sec}^{-1} \times 10^{-4}$)	pH at steady-state	Log P	Log D	TPSA ($\text{\AA}^2$)	n
Ibuprofen	1.40 ± 0.14	6.52 ± 0.10	3.51 ^a	1.42 ^b	37.30 ^c	6
Ketoprofen	1.32 ± 0.15	6.54 ± 0.03	3.12 ^a	1.02 ^b	54.37 ^c	4
Naproxen	1.37 ± 0.07	6.50 ± 0.04	3.34 ^a	1.13 ^b	46.53 ^c	6

^aHadgraft et al., (2000); ^bcalculated from Equation 3.3; ^ccalculated as described in Section 4.4.21.

Based on ANOVA, the P_{app} values in Table 6.12 were not significantly different from each other ($p = 0.49$). This can be explained using the physicochemical properties of the compounds, specifically Log P, pK_a and molecular weight.

As described in Section 3.2.1, Martin (1981) reported that the permeability of the intestinal epithelium increases roughly with the lipophilicity of the drug molecule until it reaches a plateau at a Log P value of approximately two. Drugs with Log P values close to two are generally predicted to be completely absorbed in humans and there is a linear correlation between drug absorption in humans and drug absorption in rats (Fagerholm et al., 1996). However, once the Log P value of a compound rises above four, its intestinal permeability starts to decrease (Wils et al., 1994). Ibuprofen, ketoprofen and naproxen all have Log P values above two (3.51, 3.12 and 3.34 respectively) and so have reached the plateau phase referred to by Martin and have not yet reached the upper limit of four. At this magnitude, any differences in Log P values between the compounds would not be expected to influence their absorption to any significant extent.

However, for ionisable compounds it is more accurate to refer to Log D values than Log P values as, according to the pH-partition hypothesis outlined in Section 3.2.1, the partitioning of a molecule in a membrane is dependent on both its Log P and degree of ionisation, both of which are taken into account when calculating Log D (Equation 3.3). When P_{app} is plotted against Log D in Figure 6.15, there is a slight decrease in P_{app} with decreasing Log D but the changes are not significant ($p > 0.05$).

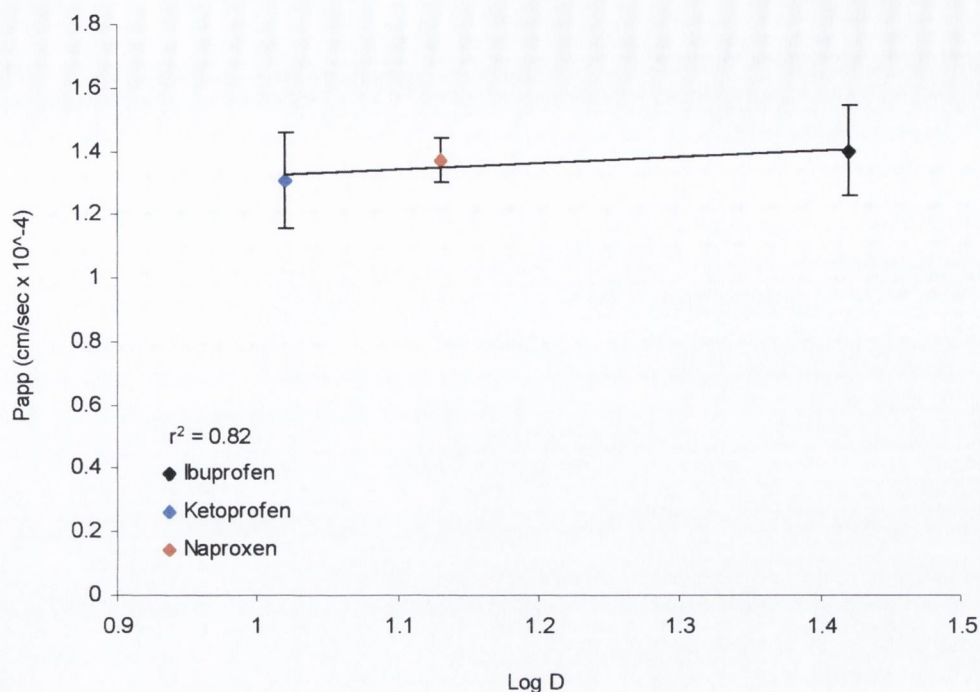


Figure 6.15 Relationship between permeability coefficient (P_{app}) and lipophilicity (Log D).

The pK_a values of ibuprofen, ketoprofen and naproxen (4.43, 4.44 and 4.29 respectively) are such that at a steady-state perfusate pH of approximately 6.5 they are all more than 99% ionised. Any slight differences in pK_a values between the three compounds are not translated into differences in the degree of ionisation.

As described in Section 3.2.3, paracellular transport is essentially limited to compounds with low molecular weight, although the upper limit of molecular weight varies in the literature from 200 (Lennernäs, 1995) to 400-500 (Artursson et al., 1993). The molecular weights of ibuprofen, ketoprofen and naproxen are all between 200-260 and from this it can be assumed that all three compounds should be absorbed to the same extent through the paracellular pores. However, this is based on the assumption that molecular weight is a direct determinant of the more critical parameter, molecular size (Camenisch et al., 1996).

An alternative to molecular weight is the molecular polar surface area (PSA) (Palm et al., 1997), or the more readily calculated topological polar surface area (TPSA) (Ertl et al., 2003) which have both been shown to be good absorption predictors. Palm et al. (1997) have shown that drugs with a $PSA < 63 \text{ \AA}^2$ will be more than 90% absorbed and drugs with a $PSA > 139 \text{ \AA}^2$ will be $< 10\%$ absorbed. Ertl et al. (2003) have shown that a similar relationship exists between TPSA and drug absorption. The

TPSA values for ibuprofen, ketoprofen and naproxen were calculated as described in Section 4.4.21. They are given in Table 6.12 and are all less than $63 \text{ \AA}^2$. Ibuprofen has the lowest TPSA ($37.30 \text{ \AA}^2$) and the highest P_{app} ($1.40 \times 10^{-4} \text{ cm sec}^{-1}$). Conversely, ketoprofen has the highest TPSA ($54.37 \text{ \AA}^2$) and the lowest P_{app} ($1.31 \times 10^{-4} \text{ cm sec}^{-1}$).

6.3.3 pH profiles

The pHs of the perfusate samples were monitored over the course of each experiment and the pH versus time profiles are shown in Figure 6.16. Over the time course of the perfusion experiments, the pH values of the samples gradually change from their initial values towards a median value. This change in pH is significant for the three systems (t-test; $p < 0.05$).

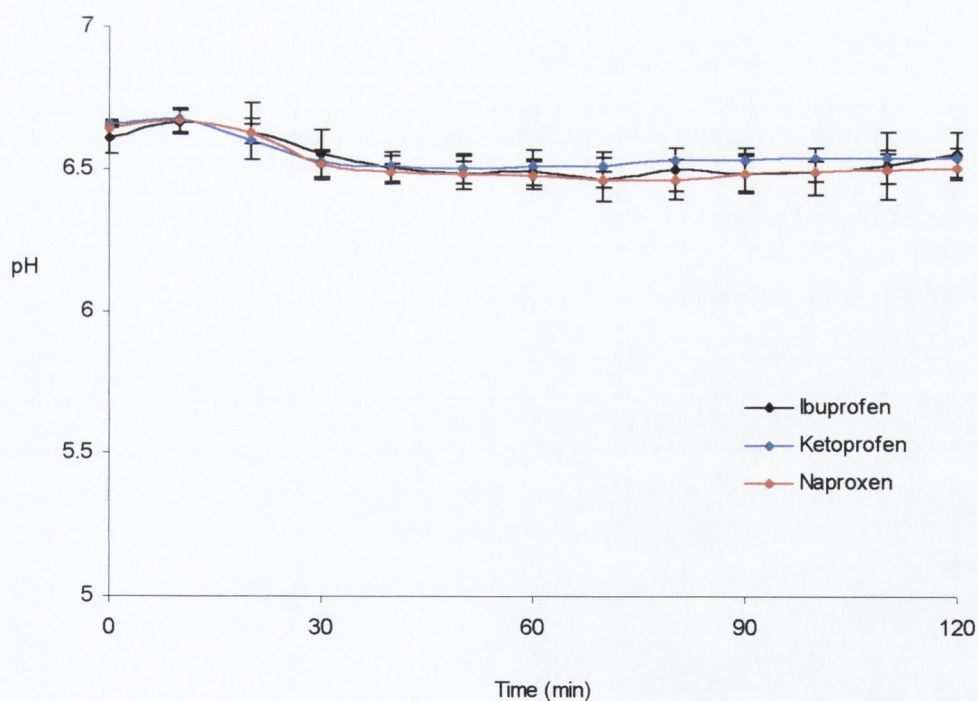


Figure 6.16 pH versus time profiles of the perfusate samples from the ibuprofen, ketoprofen and naproxen absorption studies in PBS 6.8 buffer.

The pHs of the perfusate samples are shifted downwards from their initial values towards a steady-state pH of approximately 6.5. As reported in Section 5.5.2, the shift in perfusate pH is most dramatic over the first 40 minutes, after which there is very little change. Again, as described in Section 6.2.3, the direction of the pH changes is consistent with an intestinal microclimate pH of approximately six as proposed by Ikuma et al. (1996).

6.3.4 PEG 4000 intestinal permeability studies

It is widely accepted that NSAIDs cause gastrointestinal damage through both a local and a systemic action (Cioli et al., 1979; Bundgaard and Nielsen, 1988). They have also been reported to cause intestinal permeability changes in the rat (Davies et al., 1994; Ford et al., 1995). High molecular weight PEG molecules are often used as permeability markers in studies of intestinal water flux (Raoof et al., 1998; Sutton and Rinaldi, 2001) as they are not absorbed from the intestine under normal conditions. However, if the barrier function of the intestine is compromised then some of these marker molecules can be absorbed. For this reason ^{14}C -PEG 4000 was included as a permeability marker in the perfusion solutions of ibuprofen, ketoprofen, naproxen and in blank buffer as described in Section 4.4.8.2 to test for any intestinal permeability changes. The perfusate samples were assayed for PEG 4000 as described in Section 4.4.11.2 and the absorption profiles of PEG 4000 in the three systems and in blank buffer are shown in Figure 6.17.

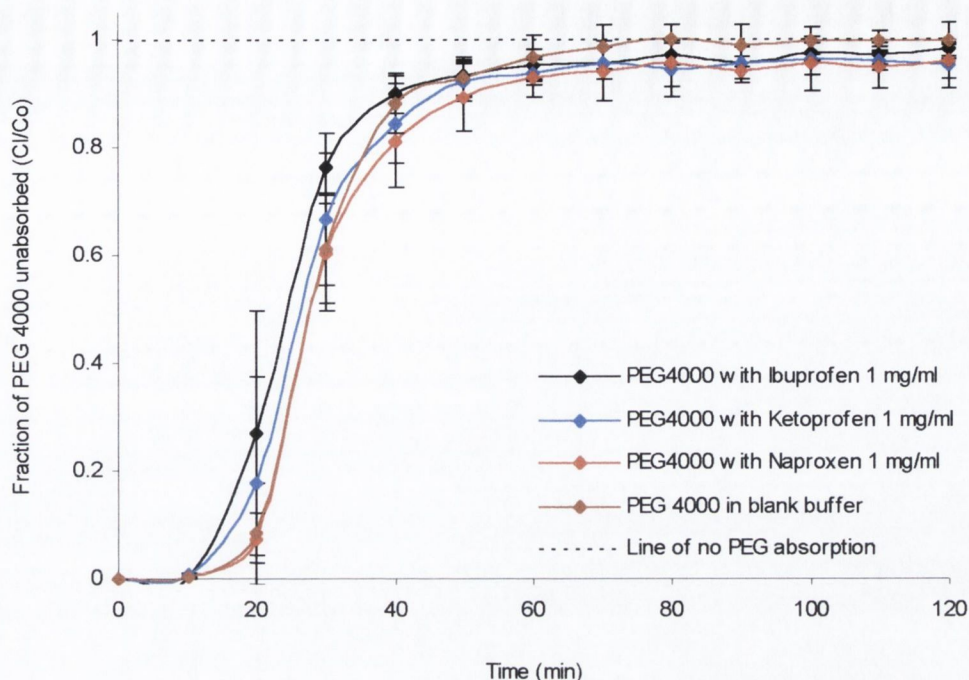


Figure 6.17 Absorption profiles of PEG 4000 in the presence of ibuprofen, ketoprofen and naproxen (all at 1.0 mg ml^{-1}) and in blank PBS 6.8 buffer.

The fraction of PEG 4000 unabsorbed at the steady-state in the ibuprofen, ketoprofen and naproxen systems and in the blank buffer are all between 0.9-1.0, suggesting little or no absorption. These steady-state fraction unabsorbed values were corrected for water flux and converted into P_{app} values as described in Section 4.4.12. The values are given in Table 6.13 together with their 95% confidence intervals.

Table 6.13 P_{app} values of PEG 4000 together with their standard deviations (s.d.), 95% confidence intervals (CI) and sample sizes for ibuprofen, ketoprofen and naproxen in PBS 6.8 and blank buffer.

System	P_{app} ($\text{cm sec}^{-1} \times 10^{-6}$)	s.d.	95% CI	Sample size
PEG 4000 + Ibuprofen	2.68	1.74	1.28-4.07	6
PEG 4000 + Ketoprofen	5.25	2.22	3.07-7.43	4
PEG 4000 + Naproxen	4.34	3.19	1.79-6.90	6
PEG 4000 in blank buffer	0.64	2.47	-1.07-2.35	8

The 95% confidence intervals are represented graphically in Figure 6.18 and there are no significant differences between the PEG 4000 P_{app} values in any of the systems. However, none of the 95% confidence intervals of the NSAID systems encompass zero, while the 95% confidence interval for the blank does. This suggests that NSAIDs appear to promote PEG 4000 absorption.

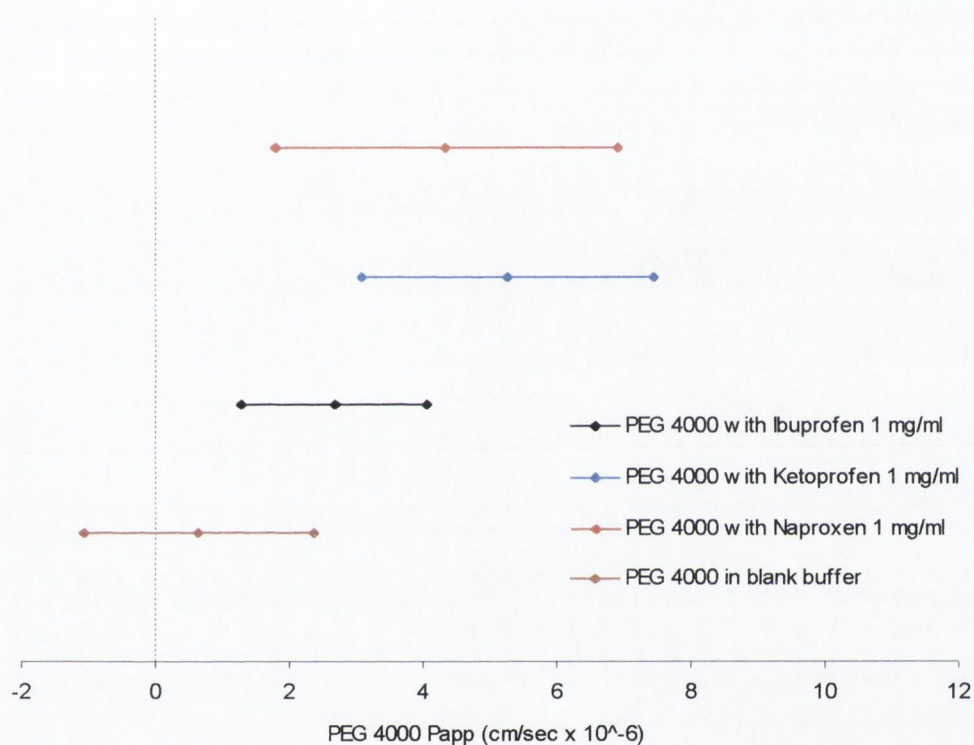


Figure 6.18 PEG 4000 permeability coefficients (P_{app}) with 95% confidence intervals in the presence of ibuprofen, ketoprofen and naproxen (all at 1.0 mg ml⁻¹) and in blank PBS 6.8 buffer.

One-sample t-tests were carried out on the PEG 4000 P_{app} values in the four systems to test if they were significantly different from zero. The values for the three NSAID systems were all significantly different from zero ($p < 0.05$), while the value for the blank was not ($p > 0.05$). These results suggest that ibuprofen, ketoprofen and naproxen enhance the absorption of PEG 4000 relative to blank buffer. This is in agreement with reports by Davies et al. (1994) and Ford et al. (1995) which show that NSAIDs induce intestinal permeability changes in the rat.

6.3.5 Histology

Histological evaluation of the intestinal epithelium was carried out to assess the degree of intestinal mucosal damage induced by the three NSAIDs. Samples of the intestinal segments that were perfused with the drug solutions were prepared and histologically assessed as described in Section 4.4.17. The results are listed in Table 6.14.

Table 6.14 *Histological evaluation of the rat small intestinal mucosa after perfusion with blank buffer, and solutions of ibuprofen, ketoprofen and naproxen (1 mg ml⁻¹).*

	Blank		Ibuprofen 1.0 mg ml ⁻¹		Ketoprofen 1.0 mg ml ⁻¹		Naproxen 1.0 mg ml ⁻¹	
Number of slides assessed	12		8		8		8	
	Score*							
Histological marker	Avg.	s.d.	Avg.	s.d.	Avg.	s.d.	Avg.	s.d.
Mucus / debris	0.92	0.29	1.75	0.89	1.50	0.53	1.75	0.46
Villous shortening	0	0	1.50	1.20	1.50	0.76	1.25	0.46
Erosion	0	0	0.75	0.46	0.88	0.35	1.50	0.53
Swollen epithelial cells	0	0	0	0	0	0	0	0
Flat epithelial cells	0	0	0.75	0.46	0.88	0.64	0.88	0.35
Goblet cells	0	0	0	0	0.13	0.35	0	0
Total score	0.92	0.29	4.75	1.16	4.88	1.13	5.38	1.06

* Scores range from 0 indicating no effect to 3 indicating an extensive effect.

The total scores of mucosal damage caused by ibuprofen, ketoprofen and naproxen are all of the same magnitude and this is clearly seen in Figure 6.19.

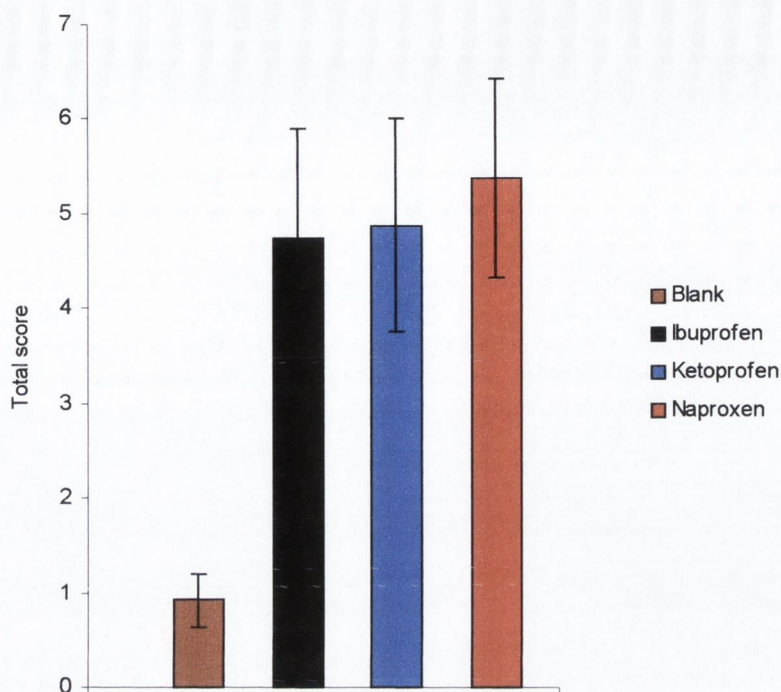


Figure 6.19 Total scores from histological evaluation of the rat small intestinal mucosa after perfusion with blank buffer, and solutions of ibuprofen, ketoprofen and naproxen (1.0 mg ml^{-1}).

The total scores for ibuprofen, ketoprofen and naproxen were all significantly different from the blank buffer (ANOVA; $p < 0.05$) but were not significantly different from each other (ANOVA; $p > 0.05$).

Results from the PEG 4000 and histology studies suggest that ibuprofen, ketoprofen and naproxen alter the barrier properties of the intestinal epithelium to PEG 4000 and cause significant histological damage to the intestinal mucosa relative to blank buffer. This is in line with previous studies (Davies et al., 1994; Ford et al., 1995) which have reported that NSAIDs increase the permeability of the intestine to poorly absorbed marker molecules. Bjarnason et al. (1986) have reported that this disruption of barrier function is probably due to the NSAIDs specifically altering the permeability of the epithelial tight junctions. Additionally, NSAIDs have been shown to have surfactant effects (Fini et al., 1995) and it has been established that surfactants can produce a loss of intestinal mucosal integrity (Oberle et al., 1995).

Also, the direct contact effect of the acidic NSAID molecules with the epithelium and/or a decrease in production/thickness of the protective mucus layer due to the

negative effect of NSAIDs on PG production may also be contributing to this reduction in intestinal barrier properties.

6.3.6 Plasma profiles

Plasma samples were collected at 30 minute intervals over the course of each two hour perfusion for each system, and for two hours after the perfusions had stopped to generate both absorption and elimination data. Samples were assayed for drug content using the methods described in Section 4.4.15.

Ibuprofen, ketoprofen and naproxen have been reported to display two-compartment pharmacokinetics in the rat (Satterwhite and Boudinot, 1992; Satterwhite and Boudinot, 1995; Itoh et al., 1997). Consequently, the plasma concentration versus time profiles for the three compounds were fitted (using the Scientist[®] curve fitting program) to the two compartment model with constant input and first order output as described in Appendix 5. The parameters used in this model are k_{elim} (elimination rate constant), k_{12} (rate constant for transfer from the central to the peripheral compartment), k_{21} (rate constant for transfer from the peripheral to the central compartment) and volume of distribution (V). Dose and t_{iv} (duration of the infusion/perfusion, 120 mins) were set at the experimental values. Dose was calculated from the cumulative amount of drug absorbed over the 2 hour perfusion as described in Section 4.4.14. The input rate constant (k_0) calculated from Equation 6.3:

$$k_0 = \frac{Dose}{Time} \quad \text{Equation 6.3}$$

where *Time* is the duration of the perfusion (120 minutes).

The best-fit profiles together with the experimental data points are shown in Figure 6.20. The pharmacokinetic parameters that describe the best-fit profiles are given in Table 6.15.

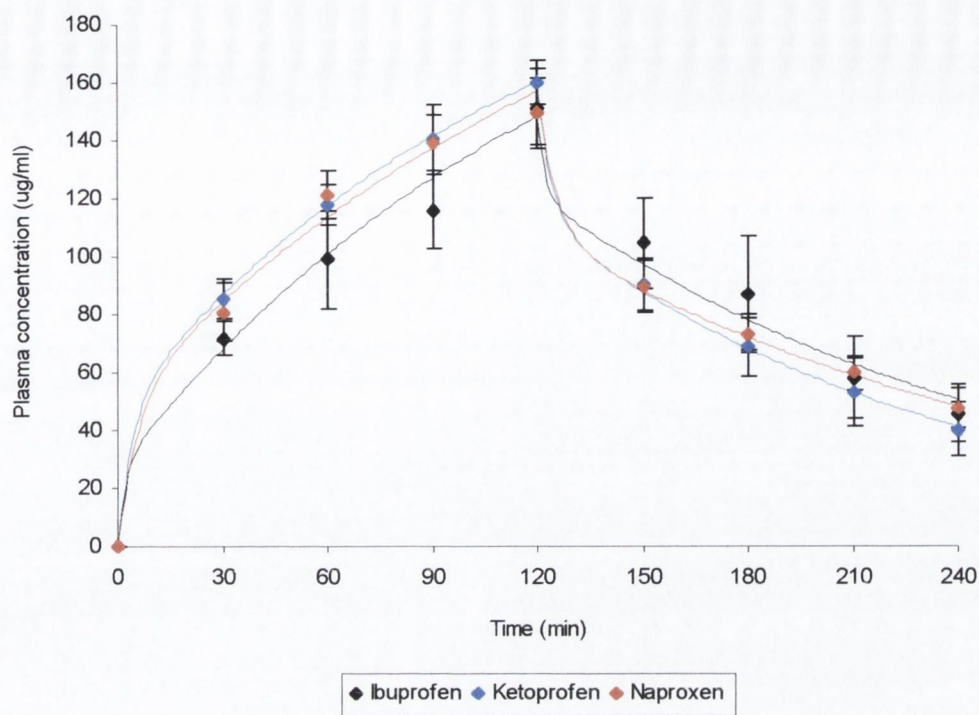


Figure 6.20 The best-fit plasma concentration versus time profiles together with the experimental data points for ibuprofen, ketoprofen and naproxen.

Table 6.15 Results ($\pm$ s.d.) from fitting the plasma data for ibuprofen, ketoprofen and naproxen to the model described above including k_{elim} , k_{12} , k_{21} , dose, k_0 , volume of distribution and goodness of fit (MSC, r^2).

	Ibuprofen 1.0 mg ml ⁻¹	Ketoprofen 1.0 mg ml ⁻¹	Naproxen 1.0 mg ml ⁻¹
k_{elim} (min⁻¹)	0.325 $\pm$ 0.005	0.167 $\pm$ 0.008	0.122 $\pm$ 0.003
k_{12} (min⁻¹)	0.044 $\pm$ 0.016	0.060 $\pm$ 0.007	0.043 $\pm$ 0.002
k_{21} (min⁻¹)	0.081 $\pm$ 0.003	0.035 $\pm$ 0.003	0.029 $\pm$ 0.001
Dose (μg rat⁻¹)	19405	19941	19127
Dose (mg kg⁻¹)	63.87	70.03	61.87
k_0 (μg min⁻¹)	161.71	166.18	159.39
Volume of distribution (ml kg⁻¹)	52.88 $\pm$ 0.42	43.11 $\pm$ 1.37	49.05 $\pm$ 0.42
MSC	4.02	7.19	4.47
r^2	0.99	0.99	0.99

Statistical analysis using ANOVA showed that the values for k_{elim} and volume of distribution for ibuprofen, ketoprofen and naproxen are significantly different from each other ($p < 0.05$). Ibuprofen has the highest (52.88 ml kg^{-1}) and ketoprofen has the lowest (43.11 ml kg^{-1}) volume of distribution. This may be related to their lipophilicities. Ibuprofen has the highest Log D (Table 6.12) of the three NSAIDs. This may allow it to distribute to a greater extent into the peripheral compartment, which involves transfer across lipoidal membranes or distribution into fatty tissue. In contrast, ketoprofen has the lowest Log D value (Table 6.12) and the lowest volume of distribution.

There is almost a three-fold difference in elimination rate constants between naproxen and ibuprofen (Table 6.15). This difference is of a similar magnitude to that seen by Satterwhite and Boudinot (1991 and 1995) who showed a three-fold difference in clearance between naproxen and ibuprofen.

6.3.7 Conclusions

There are no significant differences in the P_{app} values of ibuprofen, ketoprofen and naproxen *in situ* due to the similarities between their physicochemical properties.

Ibuprofen, ketoprofen and naproxen appear to alter the barrier properties of the intestinal mucosa.

There are significant differences between the rates of elimination and volumes of distribution of ibuprofen, ketoprofen and naproxen in the rat.

6.4 ABSORPTION STUDIES AT VARYING CONCENTRATION

Satterwhite and Boudinot (1992) have shown that varying the dose of ketoprofen has a significant effect on the plasma clearance and volume of distribution of the unbound fraction. However, the effect of varying the ketoprofen perfusate concentration on its permeability coefficient is not known. It is of interest to determine if there is any dependency of the intestinal absorption of NSAIDs on their concentration in the gut lumen as it may provide further information on the mechanism of NSAID absorption. *In situ* perfusion studies were carried out as described in Section 4.4.7 using solutions of ketoprofen of three different concentrations (0.5, 1.0 and 2.0 mg ml⁻¹) in PBS 6.8. It has been previously reported that NSAIDs cause intestinal permeability changes (Davies et al., 1994; Ford et al., 1995) and the ability of the varying concentrations of ketoprofen perfusions to enhance intestinal permeability will be investigated using PEG 4000 as an impermeable marker molecule. These perfusion studies may also provide information on the capacity of the rat small intestine to absorb NSAIDs over a range of concentrations. Ketoprofen was chosen for these studies as it has a higher solubility in PBS 6.8 than ibuprofen or naproxen, which would easily allow the preparation of concentrated ketoprofen solutions for perfusion.

6.4.1 Ketoprofen absorption profiles

In situ absorption studies were carried out using the three different ketoprofen systems as described in Section 4.4.7. The fraction unabsorbed versus time profiles are shown in Figure 6.21.

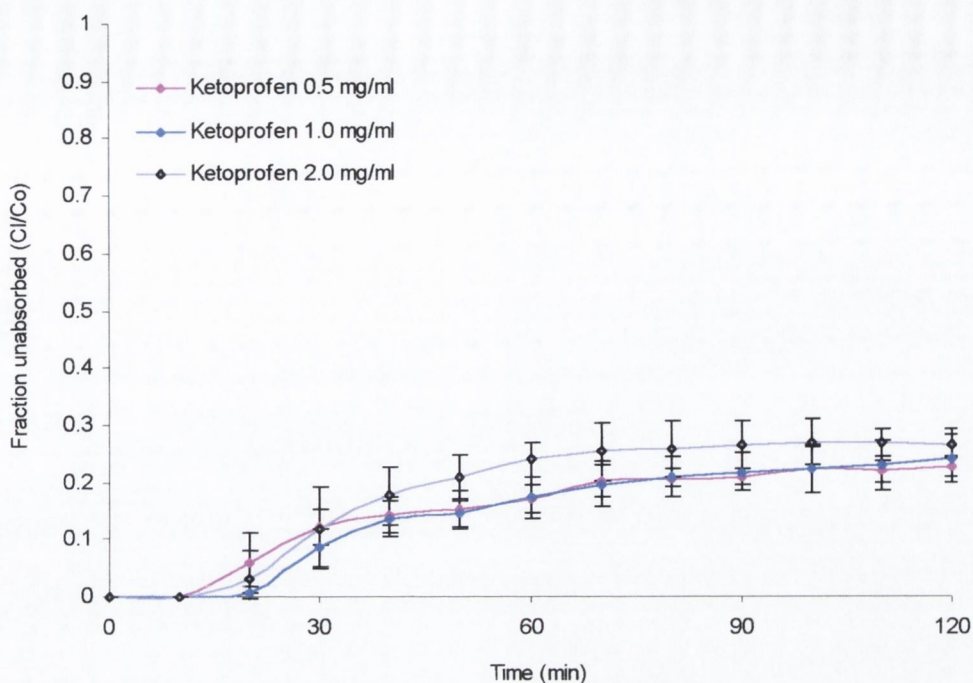


Figure 6.21 Ketoprofen fraction unabsorbed versus time profiles from the studies at three different ketoprofen concentrations (0.5, 1.0 and 2.0 mg ml⁻¹) in PBS 6.8 buffer.

The fraction unabsorbed values were corrected for water flux as described in Section 4.4.13.2 and converted to P_{app} values as described in Section 4.4.12. The P_{app} values were tested for significant differences between them using ANOVA, and are given in Table 6.16 and shown in Figure 6.22.

Table 6.16 Ketoprofen permeability coefficients (P_{app}) at different concentrations in PBS 6.8.

System	n	P_{app} (cm sec ⁻¹ x 10 ⁻⁴)	s.d.
Ketoprofen 0.5 mg ml ⁻¹	5	1.34	0.09
Ketoprofen 1.0 mg ml ⁻¹	4	1.31	0.15
Ketoprofen 2.0 mg ml ⁻¹	7	1.17	0.09

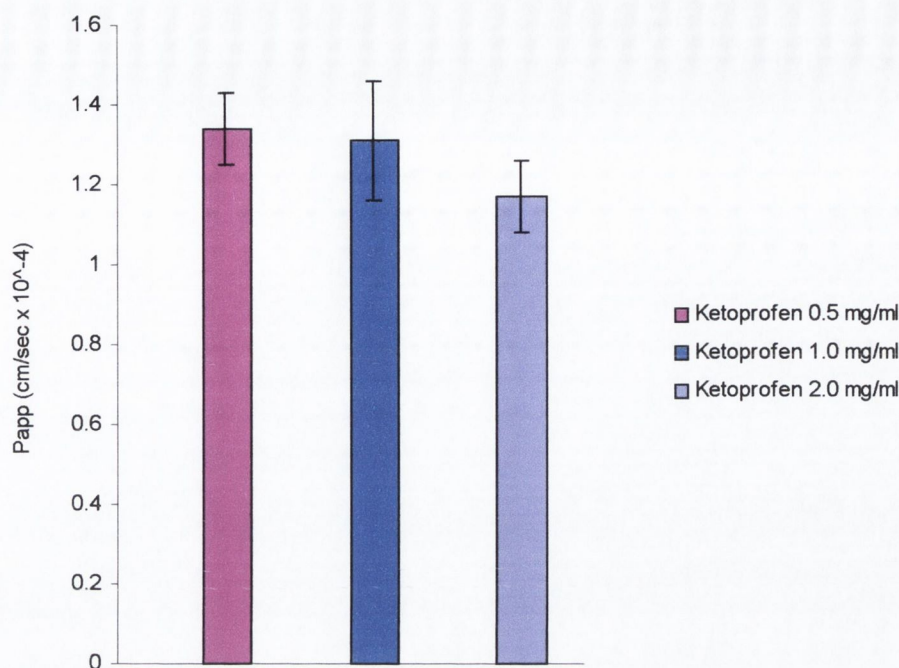


Figure 6.22 Ketoprofen permeability coefficients (P_{app}) at different concentrations of ketoprofen in PBS 6.8.

ANOVA of the P_{app} values of ketoprofen at varying concentrations show that there are no significant differences between them ($p > 0.05$). There is a slight decrease in the P_{app} values as the ketoprofen concentration increases. This reflects a decrease in the fraction unabsorbed with increasing concentration.

6.4.2 Plasma profiles

Plasma samples were collected at 30 minute intervals over the course of each two hour perfusion for each system, and for two hours after the perfusions had stopped to generate both absorption and elimination data. Samples were assayed for drug content using the method described in Section 4.4.15.

Satterwhite and Boudinot (1992) reported that ketoprofen displays two-compartment pharmacokinetics in the rat. The plasma concentration versus time profiles were fitted (using the Scientist[®] curve fitting program) to the two compartment model with constant input and first order output as described in Appendix 5. The parameters used in this model are k_{elim} (elimination rate constant), k_{12} (rate constant for transfer from the central to the peripheral compartment), k_{21} (rate constant for transfer from the

peripheral to the central compartment) and volume of distribution (V). Dose and t_{iv} (duration of the infusion/perfusion, 120 mins) were set at the experimental values. Dose was calculated from the cumulative amount of drug absorbed over the 2 hour perfusion as described in Section 4.4.14. The input rate constant (k_0) calculated from Equation 6.3. The best-fit profiles together with the experimental data points are shown in Figure 6.23.

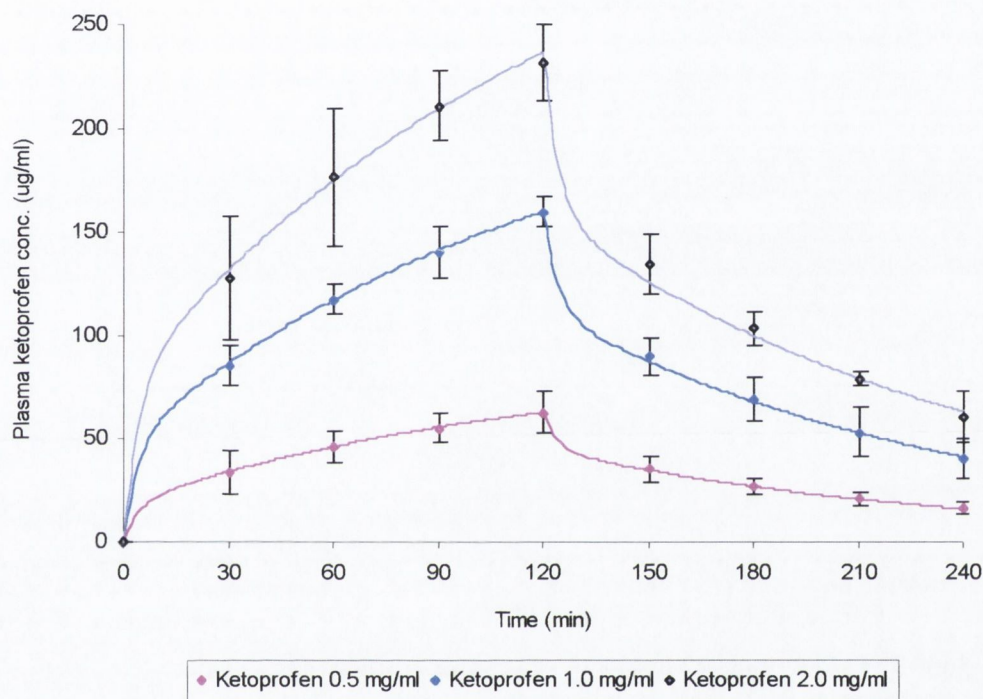


Figure 6.23 The best-fit plasma ketoprofen concentration versus time profiles together with the experimental data points for the three different perfusion concentrations of ketoprofen.

The pharmacokinetic parameters describing the best-fit profiles are given in Table 6.17 together with the goodness of fit to the experimental data points.

Table 6.17 Results ($\pm$ s.d.) from fitting the plasma data for each ketoprofen system to the model described above: k_{elim} , k_{12} , k_{21} , dose, k_0 , volume of distribution, peak plasma concentration and goodness of fit (MSC, r^2).

	Ketoprofen 0.5 mg ml ⁻¹	Ketoprofen 1.0 mg ml ⁻¹	Ketoprofen 2.0 mg ml ⁻¹
k_{elim} (min ⁻¹)	0.175 $\pm$ 0.014	0.167 $\pm$ 0.008	0.154 $\pm$ 0.007
k_{12} (min ⁻¹)	0.061 $\pm$ 0.013	0.060 $\pm$ 0.007	0.060 $\pm$ 0.004
k_{21} (min ⁻¹)	0.038 $\pm$ 0.011	0.035 $\pm$ 0.003	0.031 $\pm$ 0.001
Dose (μ g rat ⁻¹)	7169	19941	30008
Dose (mg kg ⁻¹)	25.90	70.03	105.71
k_0 (μ g min ⁻¹)	59.74	166.18	250.07
Volume of distribution (ml kg ⁻¹)	40.52 $\pm$ 1.95	43.11 $\pm$ 1.37	43.14 $\pm$ 0.93
Peak plasma conc. (μ g ml ⁻¹)	62.71 $\pm$ 10.11	160.07 $\pm$ 7.59	231.69 $\pm$ 18.36
MSC	7.91	7.19	5.57
r^2	0.99	0.99	0.99

Satterwhite and Boudinot (1992) have reported a decrease in both the clearance and volume of distribution of the unbound fraction of ketoprofen in the rat with increasing dose. They suggested that these decreases were due to saturation of elimination pathways and saturable tissue binding/uptake respectively. Statistical analysis using ANOVA of the pharmacokinetic parameters k_{elim} , k_{12} , k_{21} and volume of distribution at the three different ketoprofen concentrations in Table 6.17 showed no significant differences between the systems for all parameters with the exception of k_{elim} . There were no significant differences between the k_{elim} values of the 0.5 and 1.0 mg ml⁻¹ systems or between the 1.0 and 2.0 mg ml⁻¹ systems. However, the k_{elim} values for the 0.5 and 2.0 mg ml⁻¹ systems were significantly different from each other ($p < 0.05$).

The highest dose used by Satterwhite and Boudinot was 10 mg kg⁻¹ administered intravenously over one minute, which produced a peak plasma concentration of approximately 100 μ g ml⁻¹. In this study higher doses were used but were absorbed intestinally over a longer period of time (120 mins.). The lowest dose was 25.9 mg kg⁻¹ and the highest was 105.7 mg kg⁻¹, which corresponded to peak plasma concentrations of 62.71 and 231.69 mg ml⁻¹ respectively. There were no differences in volume of distribution between the three systems studied in this work.

6.4.3 pH monitoring of perfusate

The pHs of the perfusate samples were monitored over the course of each experiment and the pH versus time profiles are shown in Figure 6.24.

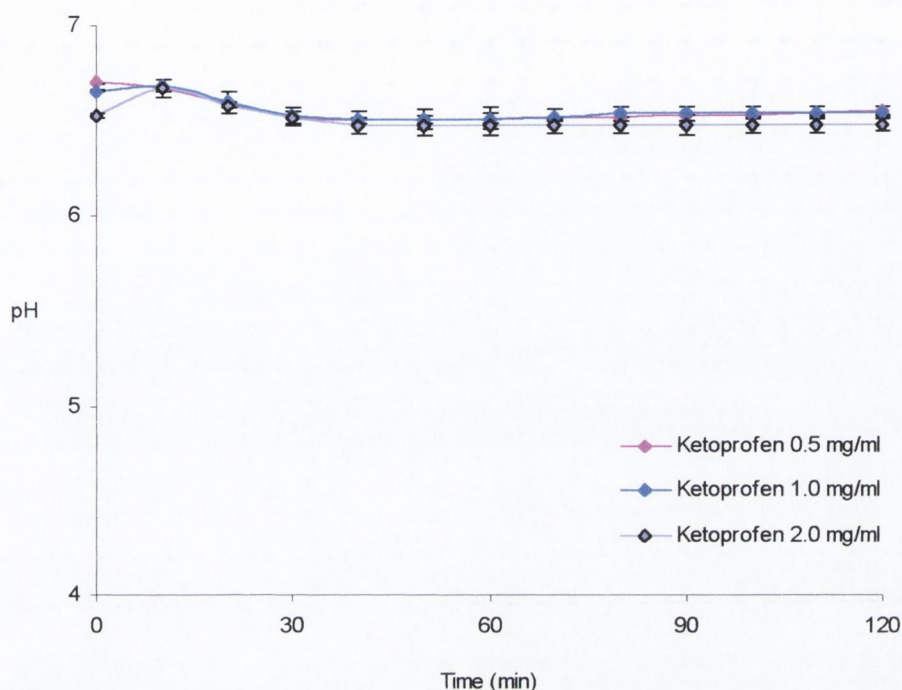


Figure 6.24 pH versus time profiles of the perfusate samples from the ketoprofen absorption studies at three different ketoprofen concentrations (0.5, 1.0 and 2.0 mg ml⁻¹) in PBS 6.8 buffer.

The pHs of the perfusate samples are shifted downwards from their initial values towards a steady-state pH of approximately 6.5. As reported in Section 5.5.2, the shift in perfusate pH is most dramatic over the first 40 minutes. The direction of the pH shift is consistent with an intestinal microclimate pH of approximately 6 as proposed by Ikuma et al. (1996).

6.4.4 PEG 4000 intestinal permeability studies

Section 6.3.4 showed that 1 mg ml⁻¹ perfusion solutions of ibuprofen, ketoprofen and naproxen altered the barrier properties of the intestine through their ability to promote the absorption of the impermeable marker molecule, PEG 4000. In this section the effect of varying the concentration of ketoprofen on the level of PEG 4000 absorption will be investigated. For this reason ¹⁴C-PEG 4000 was included as a permeability marker in the perfusion solutions as described in Section 4.4.8.2. The perfusate samples were assayed for PEG 4000 as described in Section 4.4.11.2 and the absorption profiles of PEG 4000 in three ketoprofen systems (0.5, 1.0 and 2.0 mg ml⁻¹) and in blank buffer are shown in Figure 6.25.

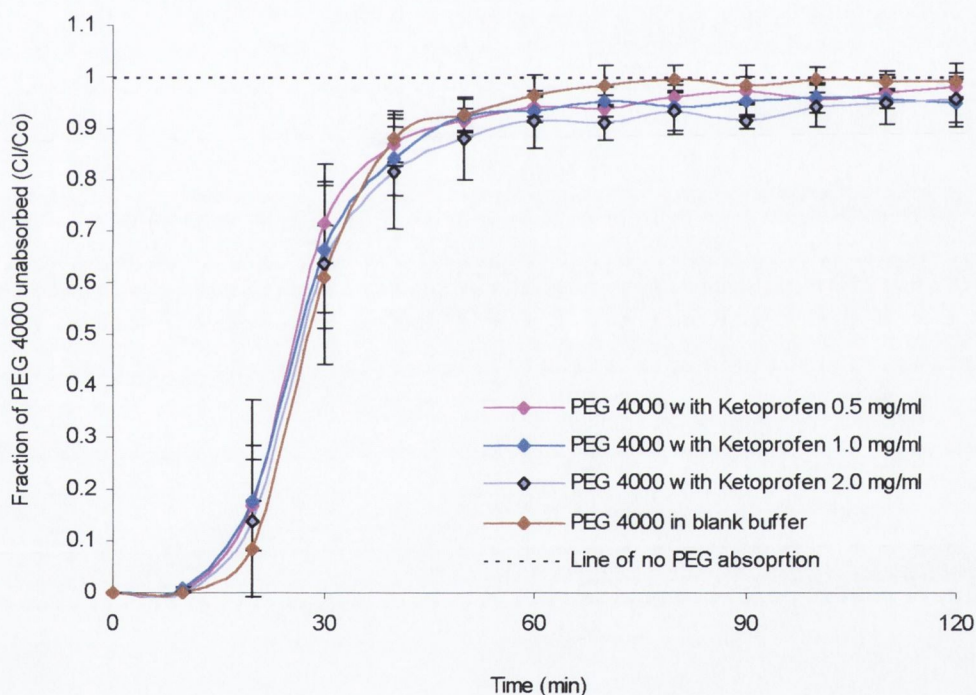


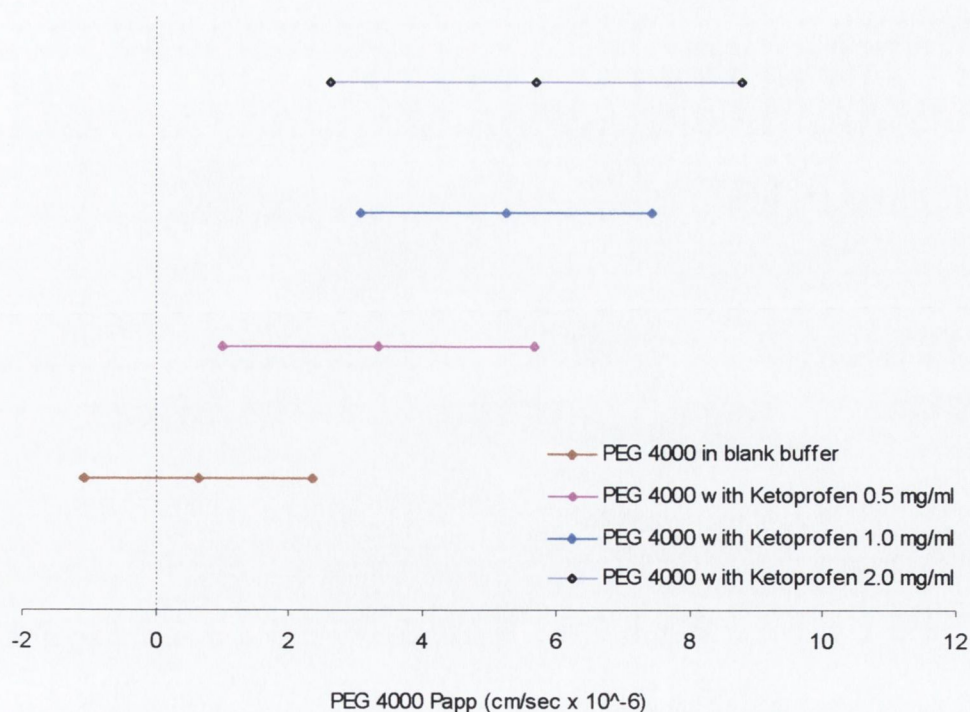
Figure 6.25 Absorption profiles of PEG 4000 in the presence of three different ketoprofen concentrations (0.5, 1.0 and 2.0 mg ml⁻¹) and in blank PBS 6.8 buffer.

The fraction of PEG 4000 unabsorbed at the steady-state in the three ketoprofen systems and in the blank buffer are all between 0.9-1.0, suggesting little or no absorption. These steady-state fraction unabsorbed values were converted to P_{app} values as described in Section 4.4.12 and are given in Table 6.18 together with their 95% confidence intervals.

Table 6.18 P_{app} values of PEG 4000 together with their standard deviations (s.d.), 95% confidence intervals (CI) and sample sizes for the three ketoprofen systems and blank buffer.

System	PEG 4000 P_{app} ($\text{cm sec}^{-1} \times 10^{-6}$)	s.d.	95% CI	Sample size
Ketoprofen 0.5 mg ml^{-1}	3.33	2.66	0.99-5.66	5
Ketoprofen 1.0 mg ml^{-1}	5.25	2.22	3.07-7.43	4
Ketoprofen 2.0 mg ml^{-1}	5.71	3.52	2.63-8.80	5
Blank buffer	0.64	2.47	-1.07-2.35	8

The 95% confidence intervals are represented graphically in Figure 6.26. No significant differences between the PEG 4000 P_{app} values in any of the systems were observed (ANOVA: $p > 0.05$). However, the 95% confidence intervals for the P_{app} values from the ketoprofen systems do not encompass zero, while the 95% confidence interval for the blank does. This suggests that there is some PEG 4000 absorption in the ketoprofen systems but not in the blank.

**Figure 6.26** PEG 4000 permeability coefficients (P_{app}) with 95% confidence intervals in the presence of three different ketoprofen concentrations (0.5, 1.0 and 2.0 mg ml^{-1}) and in blank PBS 6.8 buffer.

One-sample t-tests were carried out on the PEG 4000 P_{app} values in the four systems to test if they were significantly different from zero. The values for the three systems containing ketoprofen were all significantly different from zero ($p = 0.05$), while the value for the blank was not ($p > 0.05$). This is in agreement with the results reported by Davies et al. (1994) and Ford et al. (1995) who showed that NSAIDs induce intestinal permeability changes in the rat. As the concentration of ketoprofen increases, so too does the PEG 4000 P_{app} value. However, as the concentration of ketoprofen increases, the P_{app} of ketoprofen does not. This suggests that ketoprofen enhances the absorption of PEG 4000 but not of ketoprofen itself.

6.4.5 Histology

In addition to using PEG 4000 absorption as a marker of intestinal mucosal damage, histological evaluation of the epithelium was performed to provide a more direct assessment of any intestinal changes induced by the varying concentrations of ketoprofen. Samples of the intestinal segments which were perfused with the ketoprofen solutions were prepared and histologically assessed as described in Section 4.4.17. The results are listed in Table 6.19.

Table 6.19 *Histological evaluation of the rat small intestinal mucosa after perfusion with blank buffer, and solutions of ketoprofen ranging from 0.5 – 2.0 mg ml⁻¹.*

	Blank		Ketoprofen 0.5 mg ml ⁻¹		Ketoprofen 1.0 mg ml ⁻¹		Ketoprofen 2.0 mg ml ⁻¹	
Number of slides assessed	12		6		8		6	
	Score*							
Histological marker	Avg.	s.d.	Avg.	s.d.	Avg.	s.d.	Avg.	s.d.
Mucus / debris	0.92	0.29	1	0	1.50	0.53	2.17	0.41
Villous shortening	0	0	0.83	0.75	1.50	0.76	2.83	0.41
Erosion	0	0	0.83	0.75	0.88	0.35	1.33	0.52
Swollen epithelial cells	0	0	0	0	0	0	0	0
Flat epithelial cells	0	0	0.17	0.41	0.88	0.64	1.50	0.55
Goblet cells	0	0	0	0	0.13	0.35	0	0
Total score	0.92	0.29	2.83	0.75	4.88	1.13	7.83	0.75

* Scores range from 0 indicating no effect to 3 indicating an extensive effect.

It appears that the mucosal damage worsens as the concentration of ketoprofen increases as reflected by the increase in total scores. The histological markers which show the most changes are mucus/debris, villous shortening, erosion of the epithelium and the presence of flat epithelial cells. These changes are characteristic of intestinal damage. The total scores for each system are shown in Figure 6.27.

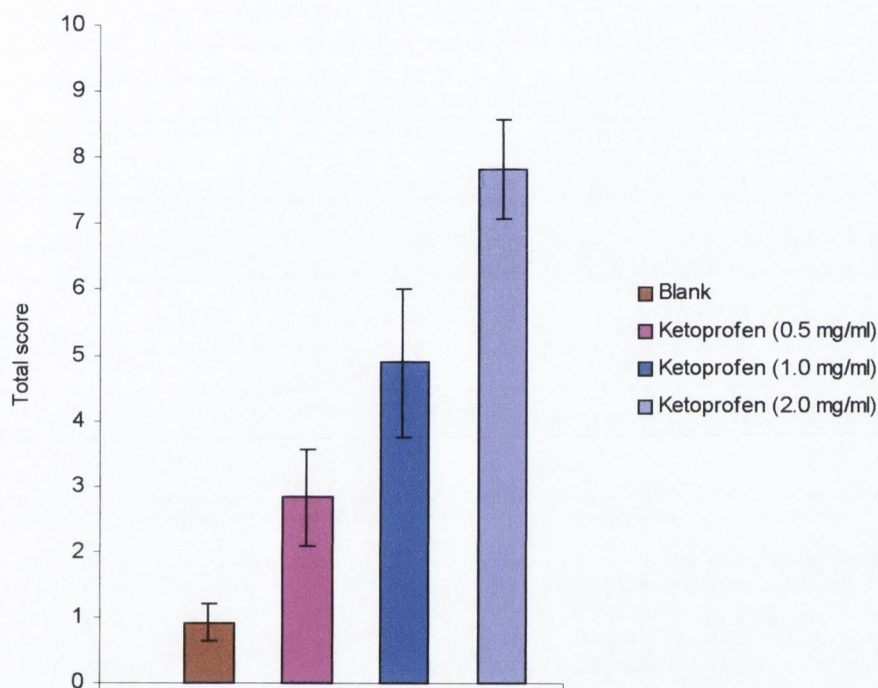


Figure 6.27 Total scores from histological evaluation of the rat small intestinal mucosa after perfusion with blank buffer, and solutions of ketoprofen ranging from 0.5–2.0 mg ml⁻¹.

The total scores from each system were compared for statistical differences using ANOVA and all four systems were significantly different ($p < 0.05$). This implies that ketoprofen causes significant damage to the intestinal mucosa and this damage becomes more severe as the ketoprofen concentration increases. To investigate the relationship between perfusate ketoprofen concentration and intestinal damage, the total score for blank buffer was subtracted from the total scores for the ketoprofen systems, and the resulting values were plotted against ketoprofen concentration. This is shown in Figure 6.28.

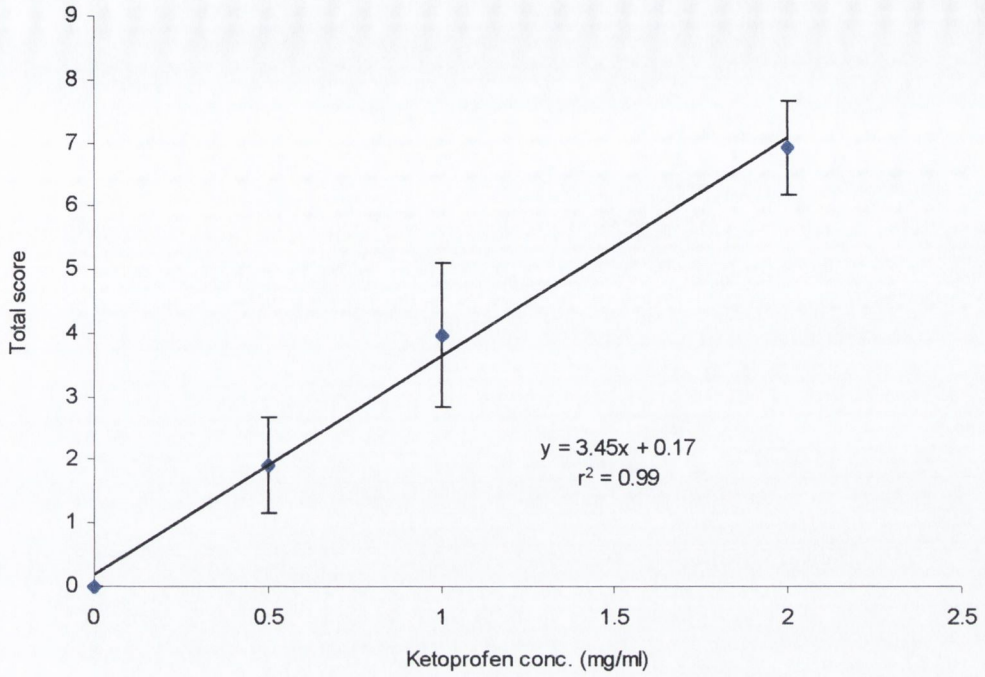


Figure 6.28 Relationship between total scores of intestinal damage against ketoprofen concentration in the perfusion solution.

There is a good correlation between the level of intestinal damage and the concentration of ketoprofen perfused through the intestine. A broader range of concentrations should be studied to determine the exact nature of this relationship.

6.5 CONCLUSIONS

Gly-Sar uptake studies *in situ* indicated that the activity of the transporter proteins involved was influenced by the perfusate pH, indicating proton-dependent activity.

Increasing the concentration of ibuprofen resulted in a reduction in Gly-Sar uptake both *in situ* and in cell culture, indicating that ibuprofen may bind to the same peptide transporters. The reduction in uptake was much more pronounced in cell culture. This may be due to the relatively high concentration of transporters expressed on the cell membrane in cell culture.

No significant differences were observed between the intestinal permeability coefficients of ketoprofen at the three concentrations studied.

For the ketoprofen systems studied, the k_{elim} values for perfusate concentrations of 0.5 and 2.0 mg ml⁻¹ were significantly different from each other.

Ibuprofen, ketoprofen and naproxen alter the barrier properties of the intestinal epithelium to PEG 4000 and produce significant histological damage to the intestinal epithelium relative to blank buffer. There is greater histological damage at higher concentrations of ketoprofen.

Chapter 7

Absorption and pharmacokinetic studies of NSAID derivatives

7.1 INTRODUCTION

Gastrointestinal (GI) side-effects are the most frequent of the adverse reactions associated with NSAID use. These reactions can range in severity from being relatively mild (such as mild gastric discomfort) to being more serious and potentially life-threatening, such as gastrointestinal ulceration and bleeding (Beck et al., 1990; MacDonald et al., 1997). It is generally accepted that this GI toxicity is attributed to direct and/or indirect effects (Cioli et al., 1979; Bundgaard and Nielsen, 1988). The direct effect is due to a local irritation produced by the acidic group of the NSAID and local inhibition of prostaglandin (PG) synthesis in the GI tract. The indirect mechanism is due to a generalised systemic inhibition of PG production occurring after absorption.

Cioli et al. (1979) demonstrated the significance of the direct contact effect in the production of GI lesions by NSAIDs. Their study showed that ibuprofen exerted more pronounced GI toxicity by the oral route than by the intravenous (i.v.) route, yet both routes showed the same anti-inflammatory activity.

As described in Section 2.6, an approach to solving these problems may be derivatisation of the carboxylic acid of the NSAIDs. This derivatisation should ideally prevent the local irritation on the stomach mucosa and be capable of releasing the parent drug spontaneously or enzymatically in the blood following absorption. Several derivatives and structural analogues of NSAIDs have been synthesised and tested for their anti-inflammatory activity and gastrointestinal toxicity. In particular, Shanbhag et al. (1992) synthesised ester and amide derivatives of ibuprofen and naproxen and assessed their GI toxicity. Among the compounds studied was a glycine derivative of ibuprofen which was shown to be significantly less irritating to the GI mucosa than the parent ibuprofen.

As described in Section 1.4.2, nitric oxide (NO) has regulatory/anti-inflammatory effects in the body. Some of these include an important role in gastric cytoprotection, possibly by increasing mucosal blood flow, and mucous/fluid secretion by the gastric epithelial cells. It has been suggested that NO and PGs act synergistically to protect the mucosa (Wallace, 1996). In addition, NO counteracts the thrombotic effects of thromboxane by inhibiting platelet aggregation (Bing et al., 1999). Coupling a NO-releasing moiety to an NSAID might deliver NO to the site of NSAID-induced damage, thereby compensating

for gastric PG reduction induced by NSAIDs, and decreasing gastric toxicity. Palmer et al. (1988) have shown that NO can be released from L-arginine in vascular endothelial cells in cell culture and in rings of rabbit aorta. This suggests that coupling L-arginine to an NSAID may result in a molecule that could act as an NSAID and release the synergistic NO at the same time.

It has been reported that intestinal peptide transporters, and in particular the PepT1 transporter, can be exploited to improve the intestinal absorption of certain drugs (*e.g.* L-dopa) by converting them chemically to di- or tripeptide derivatives (Tsuji, 1999). The PepT1 transporter has a high tolerance for structural diversity and a low level of chemical criteria for substrate recognition (Dantzig and Bergin, 1990; Ganapathy et al., 1995; Ganapathy and Liebach, 1982, 1986; Hidalgo et al., 1993, 1995; Hu et al., 1995; Liang et al., 1995; Zhu et al., 2000). Some of its substrates have been shown to include di- and tripeptides, β -lactam antibiotics, angiotensin-converting enzyme inhibitors, and even compounds without an obvious peptide bond or equivalent, such as δ -amino-levulinic acid (Temple et al., 1998) and ω -amino fatty acids (Döring et al., 1998). This implies that amino acid derivatives of ibuprofen might be candidates for uptake by PepT1 or other peptide transporters.

In Chapter 6 the ability of ibuprofen to bind to peptide transporters was evaluated using the Capan-2 cell culture model. In this section aspects of the absorption and GI toxicity of amino acid derivatives of ibuprofen will be assessed *in situ* and *in vitro*, including their ability to bind to peptide transporters.

7.2 ALANINE, GLYCINE, PHENYLALANINE AND L-ARGININE DERIVATIVES OF IBUPROFEN

Alanine, glycine, phenylalanine and L-arginine derivatives of ibuprofen were synthesised and characterised by Hanlon (2002) and will be referred to as ibu-alanine, ibu-glycine, ibu-phenylalanine and ibu-L-arginine. The amino acid groups were attached to the –OH group of ibuprofen by a dehydration/amidation reaction to produce the derivative. Prior to *in vitro* and *in situ* studies they were purified as described in Section 4.4.6. The molecular structures of these compounds are shown in Figures 7.1 and 7.2.

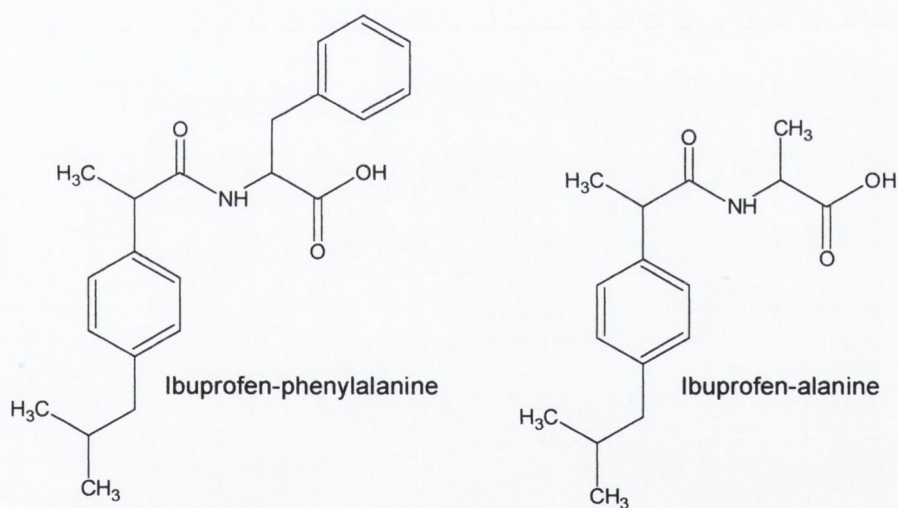


Figure 7.1 Chemical structures of alanine, glycine and phenylalanine derivatives of ibuprofen.

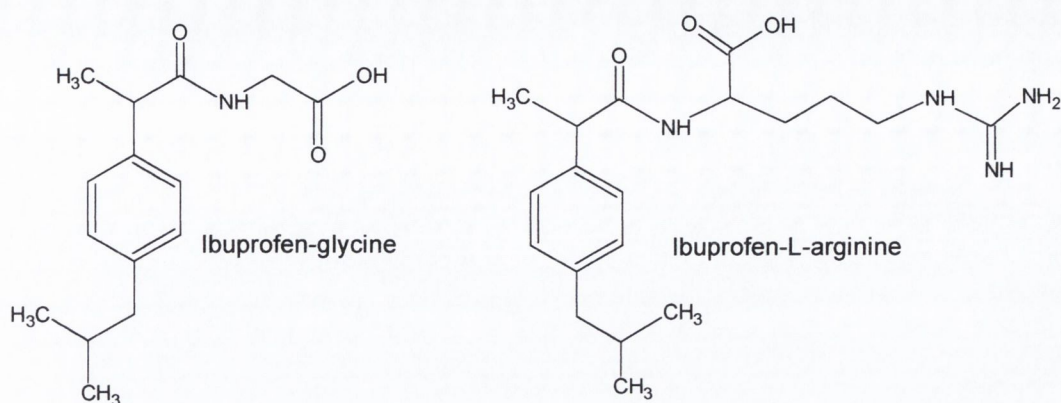


Figure 7.2 Chemical structures of glycine and L-arginine derivatives of ibuprofen.

7.2.1 Physicochemical properties

Before any *in vitro* or *in situ* absorption studies were carried out on the ibu-alanine, ibu-glycine and ibu-phenylalanine derivatives, their pH-solubility profiles were determined. cLog P values (Section 4.4.20) and molecular topological polar surface areas (TPSA) (Section 4.4.21) were determined and are given in Table 7.1. A pH-solubility study was not carried out on the ibu-L-arginine derivative due to the limited availability of the compound. However, its relatively low cLog P value (-0.59) (calculated as described in Section 4.4.20) indicates that the aqueous solubility should be higher than the parent compound ibuprofen (3.68).

7.2.1.1 pH-solubility studies

pH-solubility studies of ibu-alanine, ibu-glycine and ibu-phenylalanine were carried out as described in Section 4.4.2.2. The experimental data points were fitted to Equation 5.2 (Scientist[®]) to generate best-fit pH-solubility profiles, which are shown in Figure 7.3 together with the experimental data points and the profile for ibuprofen. The best-fit pK_a and intrinsic solubility (C₀) values for each compound are given in Table 7.1.

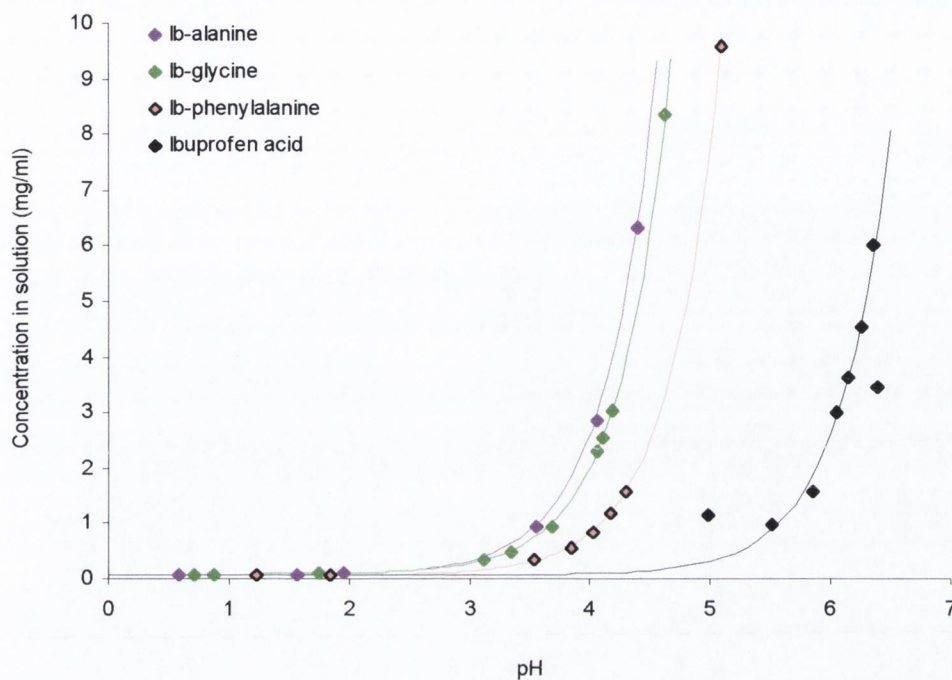


Figure 7.3 pH-solubility profiles of ibu-alanine, ibu-glycine, ibu-phenylalanine and ibuprofen.

Figure 7.3 shows the pH-solubility profiles of the three amino acid derivatives and ibuprofen. It is apparent from these profiles that the three amino acid derivatives have different pK_a values to each other and they are all lower than the pK_a of ibuprofen (Table 7.1). There are also slight differences between the intrinsic solubilities of the four compounds (Table 7.1). pK_a values and intrinsic solubilities were determined at 37°C.

7.2.1.2 Comparison of the physicochemical properties of ibu-alanine, ibu-glycine, ibu-phenylalanine, ibu-L-arginine and ibuprofen

The physicochemical properties of the four amino acid derivatives of ibuprofen are summarised in Table 7.1 and compared to the parent compound ibuprofen.

Table 7.1 pK_a , intrinsic solubility (C_0), $cLog P$, topological polar molecular surface area (TPSA) and molecular weight (MW) values of ibuprofen, ibu-alanine, ibu-glycine, ibu-phenylalanine and ibuprofen.

	pK_a	C_0 ($\mu\text{g ml}^{-1}$)	C_0 (mM)	$cLog P^a$	TPSA ^b ($\text{\AA}^2$)	MW
Ibuprofen	4.43	68.28	0.33	3.68	37.30	206.27
Ibu-alanine	2.45	72.41	0.26	3.35	66.40	277.36
Ibu-glycine	2.64	84.27	0.32	3.04	66.40	263.33
Ibu-phenylalanine	2.89	59.78	0.17	4.77	66.40	353.46
Ibu-L-arginine	--	--	--	-0.59	128.30	362.47

^aCalculated as described in Section 4.4.20; ^bcalculated as described in Section 4.4.21.

The best-fit pK_a values of the three amino acid derivatives are almost two pK_a units smaller than the parent ibuprofen. This indicates that the derivatives are more acidic compounds than ibuprofen. In contrast, the differences between the pK_a values of the three amino acid derivatives are of the order of 0.2 pK_a units. In the context of absorption studies, this means that in the perfusion solutions the amino acid derivatives will be ionized to a greater extent than the parent ibuprofen.

Of the three amino acid derivatives, ibu-phenylalanine has the lowest intrinsic solubility (0.17 mM) and ibu-glycine has the highest (0.32 mM). This can be explained by their $cLog P$ values and molecular weights (MW), as ibu-phenylalanine has the highest $cLog P$ and MW (4.77 and 353.46 respectively) and ibu-glycine has the lowest (3.04 and 263.33).

7.2.2 *In situ* absorption studies

In situ absorption studies were carried out using solutions of ibu-alanine, ibu-glycine, ibu-phenylalanine and ibu-L-arginine in PBS 6.8 as described in Section 4.4.7. The concentrations of the four solutions were 1.34, 1.28, 1.71 and 1.76 mg ml⁻¹ respectively, which were equivalent to 1 mg ml⁻¹ (4.85 mM) of ibuprofen. The fraction unabsorbed versus time profiles for these four systems are shown in Figure 7.4 together with the profile of ibuprofen in the same buffer (1 mg ml⁻¹).

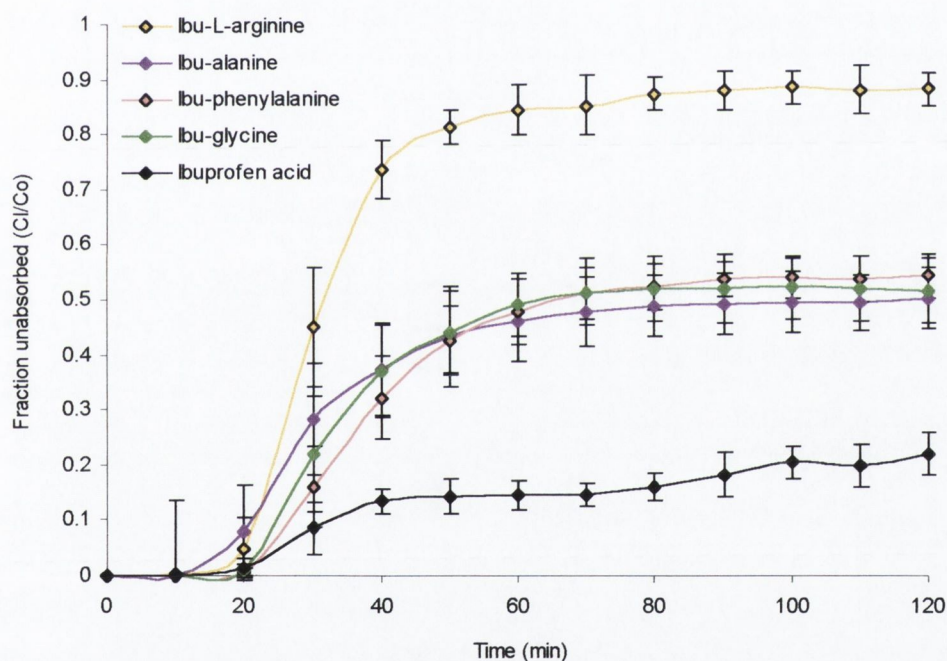


Figure 7.4 Fraction unabsorbed versus time profiles for ibu-alanine, ibu-glycine, ibu-phenylalanine, ibu-L-arginine and ibuprofen in PBS 6.8.

The fraction unabsorbed values at the steady-state (100-120 mins.) were corrected for water flux and converted to P_{app} values as described in Sections 4.4.13.2 and 4.4.12 respectively. The P_{app} values are given in Table 7.2.

Table 7.2 Permeability coefficients ($P_{app} \pm s.d.$) of ibu-alanine, ibu-glycine, ibu-phenylalanine, ibu-L-arginine and ibuprofen with their steady-state pH values, cLog P values, molecular weights (MW) and molecular topological polar surface area (TPSA).

Compound	P_{app} ($\text{cm sec}^{-1} \times 10^{-4}$)	pH at st-state	% unionised	cLog P	MW	TPSA ($\text{\AA}^2$)	n
Ibu-alanine	0.61 ± 0.09	6.47 ± 0.06	0.01	3.35	277.36	66.40	8
Ibu-glycine	0.58 ± 0.11	6.52 ± 0.05	0.01	3.04	263.33	66.40	7
Ibu-phenylalanine	0.55 ± 0.06	6.59 ± 0.09	0.02	4.77	353.46	66.40	7
Ibu-L-arginine	0.11 ± 0.03	6.65 ± 0.06	--	-0.59	362.47	128.30	11
Ibuprofen	1.40 ± 0.14	6.52 ± 0.10	0.81	3.68	206.27	37.30	6

7.2.2.1 Ibu-alanine, ibu-glycine and ibu-phenylalanine

Based on ANOVA, there were no significant differences between the P_{app} values for the ibu-alanine, ibu-glycine and ibu-phenylalanine derivatives ($P > 0.05$). However, the P_{app} values for the three derivatives were all significantly different from the P_{app} value for ibuprofen ($P < 0.05$). Differences in the physicochemical properties of the compounds can explain these results.

As described in Section 3.2.1, Martin (1981) reported that the permeability of the intestinal epithelium increases roughly with the lipophilicity of the drug molecule until it reaches a plateau at a Log P value of approximately two. Drugs with Log P values close to two are generally predicted to be completely absorbed in humans and there is a linear correlation between drug absorption in humans and drug absorption in rats (Fagerholm et al., 1996). The cLog P values of ibu-alanine (3.35), ibu-glycine (3.04) and ibu-phenylalanine (4.77) are all above two and so have reached the plateau phase referred to by Martin and consequently there are no significant differences between their P_{app} values.

As described in Section 3.2.3, paracellular transport is essentially limited to compounds with low molecular weight, although the upper limit of molecular weight varies in the literature from 200 (Lennemäs, 1995) to 400-500 (Artursson et al., 1993). The molecular weights of ibu-alanine, ibu-glycine and ibu-phenylalanine range from 277-353. The MW of ibu-phenylalanine (353) is much higher than that of ibu-alanine (277) and ibu-glycine (263), which may be further responsible for its slightly lower P_{app} than the other two.

Molecular polar surface area (PSA) (Palm et al., 1997), or the more readily calculated topological polar surface area (TPSA) (Ertl et al., 2003) (calculated as described in Section 4.4.21) have both been shown to be good absorption predictors. Palm et al. (1997) have shown that drugs with a $PSA < 63 \text{ \AA}^2$ will be more than 90% absorbed and drugs with a $PSA > 139 \text{ \AA}^2$ will be $< 10\%$ absorbed. Ertl et al. (2003) have shown that a similar relationship exists between TPSA and drug absorption. The TPSA values for ibu-alanine, ibu-glycine and ibu-phenylalanine are all $66.40 \text{ \AA}^2$, which may explain why there are no significant differences between their P_{app} values.

The significant difference between the P_{app} of ibuprofen and the P_{app} values of these three derivatives can be explained by their TPSA values. The TPSA value of ibuprofen ($37.30 \text{ \AA}^2$) is significantly smaller than the TPSA values of the three derivatives ($66.40 \text{ \AA}^2$).

7.2.2.2 Ibu-L-arginine

Based on ANOVA, the P_{app} value for ibu-L-arginine is significantly different from the P_{app} values of the other three amino acid derivatives and of ibuprofen itself ($P < 0.05$). This can be explained in terms of the physicochemical properties of the compound which are related to the larger number of polar groups on the ibu-L-arginine molecule compared to ibuprofen or the other three amino acid derivatives as shown in Figures 7.1 and 7.2. These polar groups are responsible for its lower cLog P value and higher TPSA.

Firstly, the cLog P value of ibu-L-arginine (-0.59) is much lower than that of any of the other compounds and this may explain the low intestinal permeability of ibu-L-arginine. Secondly, the molecular weight (MW) of ibu-L-arginine (362.47) is much higher than any of the other compounds, with the exception of ibu-phenylalanine (353.46). This is close to the previously mentioned upper limit for paracellular transport.

When these two factors, cLog P and MW are considered together it might explain why the P_{app} of ibu-L-arginine is so low. The MW is such that transport through the paracellular route is limited and the main alternative route for absorption is across the cell membrane. However, the cLog P of ibu-L-arginine is such that the molecules may be too hydrophilic to partition effectively into the lipophilic cell membrane. In contrast, ibuprofen has a MW that permits access to the paracellular route and a cLog P that allows absorption across the cell membrane.

The TPSA of ibu-L-arginine is $128.30 \text{ \AA}^2$ which may explain why it is only approximately 12% absorbed at the steady state compared to approximately 80% for ibuprofen which has a TPSA of $37.30 \text{ \AA}^2$.

7.2.2.3 Converting P_{app} values in rats to fraction absorbed in humans

If the assumption is made that these amino acid derivatives are passively absorbed and no active transport processes are involved, then it is possible to convert the rat P_{app} values for these compounds into human P_{app} values and fraction absorbed in humans using Equations 7.1 and 7.2 (Fagerholm et al., 1996):

$$P_{app,man} = 3.6 \cdot P_{app,rat} + 0.03 \cdot 10^{-4} \quad \text{Equation 7.1}$$

where $P_{app,man}$ and $P_{app,rat}$ are the P_{app} values in man and rat respectively, expressed in $\text{cm sec}^{-1} \times 10^{-4}$.

$$FA = 1 - e^{-\left(2 \cdot P_{app,man} \cdot t / r \cdot f\right)} \quad \text{Equation 7.2}$$

where FA is the fraction absorbed in humans, t is the human intestinal residence time expressed in seconds (assumed to be three hours), r is the human intestinal radius (assumed to be 1.75 cm) and f is a correction factor taken to be 2.8.

When Equations 7.1 and 7.2 are applied to the P_{app} values in Table 7.2, the corresponding human P_{app} values and fractions absorbed are obtained. These are given in Table 7.3 below.

Table 7.3 Permeability coefficients ($P_{app} \pm s.d.$) of the four amino acid derivatives and ibuprofen in rats and humans, and their fraction absorbed (FA) in rats and humans.

	Rat P_{app}	Human P_{app}	FA	FA
	($\text{cm sec}^{-1} \times 10^{-4}$)	($\text{cm sec}^{-1} \times 10^{-4}$)	Rats	Humans
Ibu-alanine	0.61 ± 0.09	2.23 ± 0.35	0.50	0.99
Ibu-glycine	0.58 ± 0.11	2.12 ± 0.43	0.48	0.99
Ibu-phenylalanine	0.55 ± 0.06	2.01 ± 0.25	0.46	0.99
Ibu-L-arginine	0.11 ± 0.03	0.43 ± 0.14	0.12	0.77
Ibuprofen	1.40 ± 0.14	5.07 ± 0.53	0.79	1.00

It is apparent from the results in Table 7.3 that the trend in P_{app} values is the same between rats and humans with the same significant differences. However, the pattern of fraction absorbed (FA) values between rats and humans is different. In rats, the fraction absorbed of ibu-alanine, ibu-glycine and ibu-phenylalanine are all similar to each other and are larger than the corresponding value for ibu-L-arginine and smaller than the corresponding value for ibuprofen. When the human fraction absorbed values are compared, there is no difference between ibu-alanine, ibu-glycine, ibu-phenylalanine and ibuprofen, which are all approximately equal to one, indicating complete absorption in humans. Only the corresponding value for ibu-L-arginine is lower, with 77% absorption in humans.

Equations 7.1 and 7.2 only apply if the compounds are passively absorbed. However, membrane transporters are diverse in nature and population along the gastrointestinal tract and previous work by Lane et al., (2003) has suggested that amino acid derivatives of ibuprofen possess some affinity for the PepT1 transporter in vitro.

7.2.2.4 pH profiles

The pH values of the perfusate samples were monitored over the course of each experiment and the pH versus time profiles are shown in Figure 7.5 together with the profile for ibuprofen in PBS 6.8. Over the time course of the perfusion experiments, the pH values of the samples gradually change from their initial values towards a median value. This change in pH is significant for the four ibuprofen derivative systems and for ibuprofen (t-test; $p < 0.05$).

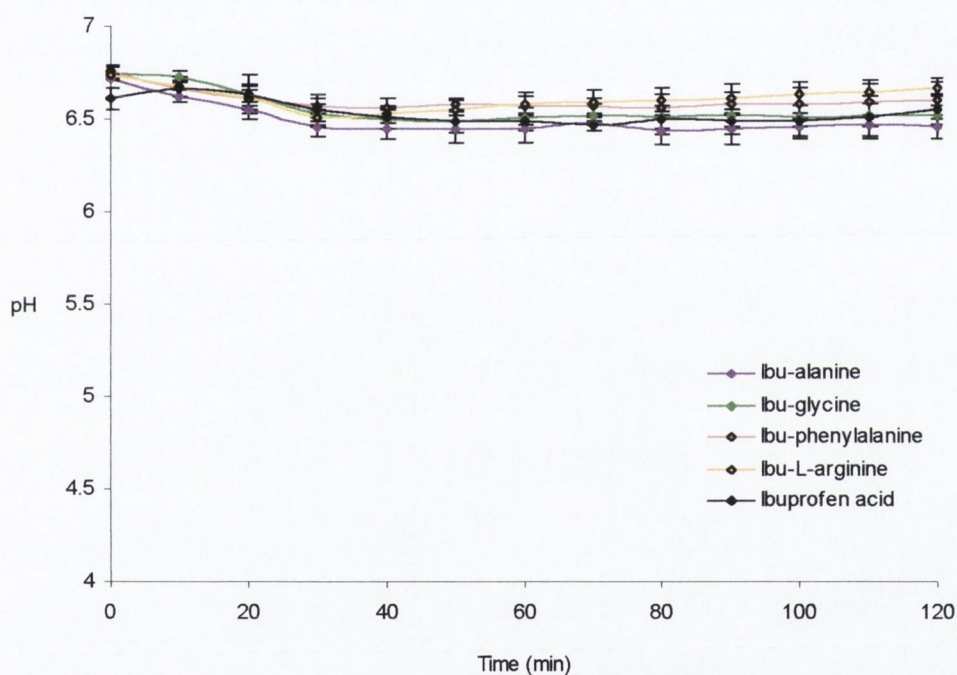


Figure 7.5 pH versus time profiles of the perfusate samples from the ibu-alanine, ibu-glycine, ibu-phenylalanine, ibu-L-arginine and ibuprofen absorption studies in PBS 6.8.

The pH values of the perfusate samples are shifted downwards from their initial values towards a steady-state pH of approximately 6.5. As reported in Section 5.5.2, the shift in perfusate pH is most dramatic over the first 40 minutes, after which there is very little change. The downward shift in pH is consistent with an intestinal microclimate pH of approximately six as proposed by Ikuma et al. (1996). This is supported by the results reported in Section 6.2.3 which showed that perfusion solutions made in PBS 6.8 had

significant downward pH shifts, solutions made in PBS 5.0 had significant upward pH shifts and solutions made in PBS 6.0 showed no significant pH shifts on perfusion through the intestine.

7.2.2.5 PEG 4000 intestinal permeability studies

As described in Section 6.3.2, high molecular weight PEG molecules are often used as permeability markers (Raoof et al., 1998; Sutton and Rinaldi, 2001) as they are not absorbed from the intestine under normal conditions. However, if the barrier function of the intestine is compromised then some of these marker molecules may be absorbed. Results from Section 6.3.4 indicated that ibuprofen, ketoprofen and naproxen enhanced the absorption of PEG 4000 relative to blank buffer. This is in agreement with reports by Davies et al. (1994) and Ford et al. (1995) which show that NSAIDs induce intestinal permeability changes in the rat.

Shanbhag et al. (1992) synthesised a glycine derivative of ibuprofen and assessed it for GI toxicity in the rat. This was done by the daily oral administration of a dose of ibuprofen-glycine that was equivalent to 150 mg kg⁻¹ for four days. Four hours after the last dose the animals were sacrificed and the number of lesions >0.5mm in diameter in the stomach were counted.

The ibuprofen-glycine derivative was shown to be significantly less irritating to the GI mucosa than the parent ibuprofen. Consequently, the ability of the alanine, glycine, phenylalanine and L-arginine derivatives of ibuprofen to enhance PEG 4000 absorption relative to the parent ibuprofen and blank buffer were assessed. For this reason ¹⁴C-PEG 4000 was included as a paracellular permeability marker in the perfusion solutions as described in Section 4.4.8.2. The perfusate samples were assayed for PEG 4000 as described in Section 4.4.11.2 and the absorption profiles of PEG 4000 in the three derivative systems, in ibuprofen and in blank buffer are shown in Figure 7.6.

The fraction of PEG 4000 unabsorbed at the steady-state in the five drug systems and in the blank buffer are all between 0.96-1.00, suggesting little or no absorption. These steady-state fraction unabsorbed values were converted into P_{app} values using Equation 5.5 and are given in Table 7.4 together with their 95% confidence intervals.

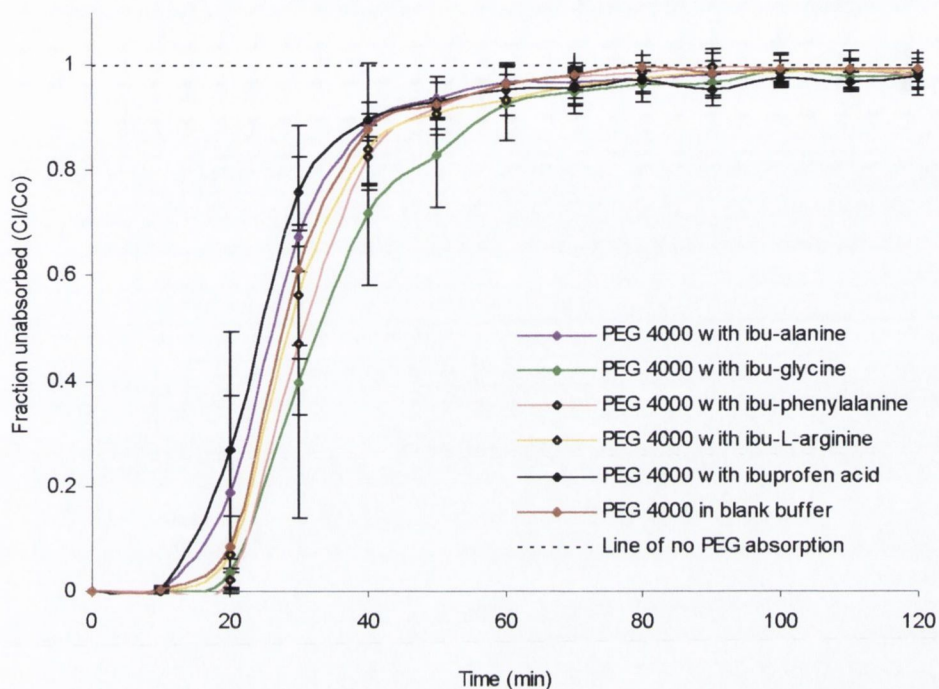


Figure 7.6 Absorption profiles of PEG 4000 in the presence of ibu-alanine, ibu-glycine, ibu-phenylalanine, ibu-L-arginine, ibuprofen and in blank PBS 6.8 buffer.

Table 7.4 P_{app} values of PEG 4000 together with their standard deviations (s.d.), 95% confidence intervals (CI) and sample sizes for ibu-alanine, ibu-glycine, ibu-phenylalanine, ibu-L-arginine and ibuprofen in PBS 6.8 and blank buffer.

System	P_{app} ($\text{cm sec}^{-1} \times 10^{-6}$)	s.d.	95% CI	Sample size
PEG 4000 + Ibu-alanine	1.19	2.64	-0.65-3.02	8
PEG 4000 + Ibu-glycine	1.55	2.86	-0.56-3.67	7
PEG 4000 + Ibu-phenylalanine	1.23	2.09	-0.32-2.78	7
PEG 4000 + Ibu-L-arginine	1.14	2.03	-0.07-2.34	11
PEG 4000 + Ibuprofen	2.68	1.74	1.28-4.07	6
PEG 4000 in blank buffer	0.64	2.47	-1.07-2.35	8

The 95% confidence intervals are represented graphically in Figure 7.7 and there are no significant differences between the PEG 4000 P_{app} values in any of the systems. However, the 95% confidence intervals of the PEG 4000 P_{app} values in the systems containing the ibuprofen derivatives and in the blank all encompass zero, while the 95% confidence interval for the parent ibuprofen does not. This suggests that the ibuprofen derivatives appear to promote less PEG 4000 absorption than the parent ibuprofen.

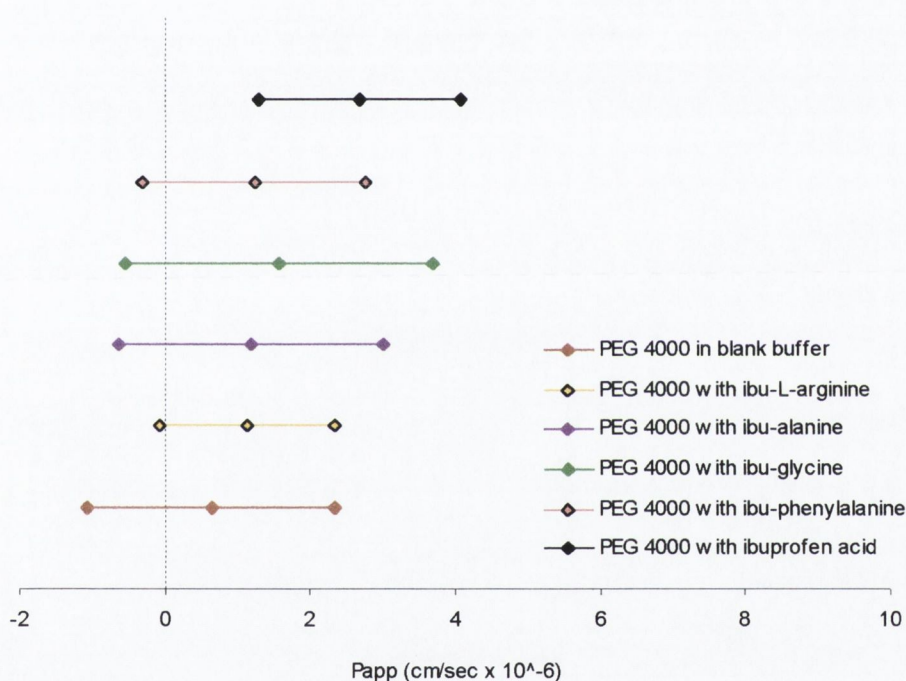


Figure 7.7 PEG 4000 permeability coefficients (P_{app}) with 95% confidence intervals in the presence of ibu-alanine, ibu-glycine, ibu-phenylalanine, ibu-L-arginine, ibuprofen and in blank PBS 6.8 buffer.

One-sample t-tests were carried out on the PEG 4000 P_{app} values in the five systems to test if they were significantly different from zero. The values for the four amino acid derivative systems and the blank were not significantly different from zero ($p > 0.05$), while the value for the ibuprofen system was significantly different from zero ($p < 0.05$). These results suggest that the amino acid derivatives induce less intestinal permeability changes in the rat than the parent compound, ibuprofen.

7.2.2.6 Histology

Histological evaluation of the intestinal epithelium was carried out to assess the degree of intestinal mucosal damage induced by the three NSAIDs. Samples of the intestinal segments that were perfused with the drug solutions were prepared and histologically assessed as described in Section 4.4.17. The results are listed in Tables 7.5 and 7.6.

Table 7.5 *Histological evaluation of the rat small intestinal mucosa after perfusion with blank buffer, and solutions of ibu-alanine, ibu-glycine, ibu-phenylalanine and ibuprofen.*

	Blank		Ibu- alanine		Ibu- glycine		Ibu- phenylalanine		Ibuprofen	
No. slides assessed	12		5		5		6		8	
	Score*									
Histological marker	Avg.	s.d.	Avg.	s.d.	Avg.	s.d.	Avg.	s.d.	Avg.	s.d.
Mucus / debris	0.92	0.29	1	0	1	0	1.17	0.41	1.75	0.89
Villous shortening	0	0	0.60	0.89	0.80	0.45	0.17	0.41	1.50	1.20
Erosion	0	0	0.40	0.55	0.20	0.45	0.67	0.52	0.75	0.46
Swollen epithelial cells	0	0	0	0	0	0	0	0	0	0
Flat epithelial cells	0	0	0	0	0	0	0.17	0.41	0.75	0.46
Goblet cells	0	0	0	0	0	0	0	0	0	0
Total score	0.92	0.29	2	1	2	0.71	2.17	0.75	4.75	1.16

* Scores range from 0 indicating no effect to 3 indicating an extensive effect.

Table 7.6 *Histological evaluation of the rat small intestinal mucosa after perfusion with blank buffer, and solutions of ibu-L-arginine and ibuprofen.*

	Blank		Ibu-L- arginine		Ibuprofen	
Number of slides assessed	12		6		8	
	Score*					
Histological marker	Avg.	s.d.	Avg.	s.d.	Avg.	s.d.
Mucus / debris	0.92	0.29	1.33	0.52	1.75	0.89
Villous shortening	0	0	0.33	0.52	1.50	1.20
Erosion	0	0	0.33	0.52	0.75	0.46
Swollen epithelial cells	0	0	0	0	0	0
Flat epithelial cells	0	0	0	0	0.75	0.46
Goblet cells	0	0	0	0	0	0
Total score	0.92	0.29	2.00	0.89	4.75	1.16

* Scores range from 0 indicating no effect to 3 indicating an extensive effect.

The total scores of mucosal damage caused by ibu-alanine, ibu-glycine, ibu-phenylalanine and ibu-L-arginine are all of the same magnitude and this is clearly seen in Figure 7.8. The histological scores for blank buffer and ibuprofen are included as references.

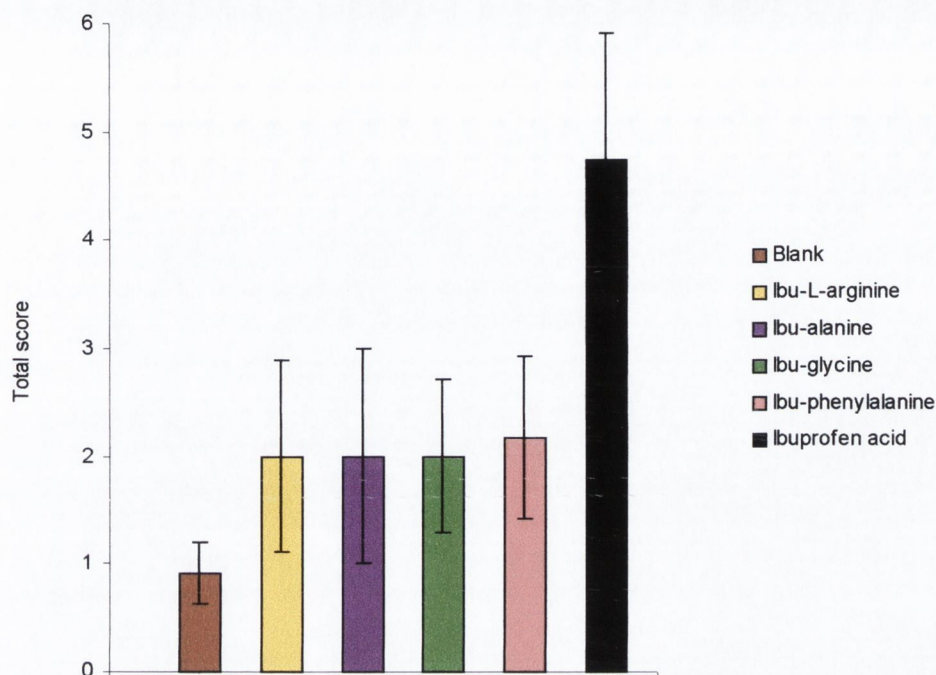


Figure 7.8 Total scores from histological evaluation of the rat small intestinal mucosa after perfusion with blank buffer, and solutions of ibu-alanine, ibu-glycine, ibu-phenylalanine, ibu-L-arginine and ibuprofen.

The total scores for ibu-alanine, ibu-glycine, ibu-phenylalanine and ibu-L-arginine were all significantly larger than the blank and significantly smaller than ibuprofen (ANOVA; $p < 0.05$) but were not significantly different to each other.

Results from the histology studies suggest that the four amino acid derivatives cause significant histological damage to the intestinal mucosa relative to blank buffer. However the extent of this histological damage is significantly less than that caused by the parent compound ibuprofen. This may be due to masking of the acidic function of the NSAID molecule or a reduction in the ability of the NSAID to inhibit the production of PGs involved in the generation of protective intestinal mucus.

7.2.2.7 Conclusions from PEG 4000 and histological data

The pK_a values of ibu-alanine, ibu-glycine and ibu-phenylalanine indicate that they are all stronger acids than ibuprofen itself. Despite this, they have been shown to possess less GI irritation properties compared to ibuprofen.

Elimination or reduction of the role of the direct contact effect of the acidic carbonyl group in GI irritation appears to be the reason for the reduced GI irritation seen with these amino acid derivatives.

The free carbonyl group of ibuprofen has been associated with GI irritation by a direct contact effect on the intestinal mucosa (Cioli et al., 1979). When the amino acid derivatives of ibuprofen are synthesised, this carbonyl group is involved in the linkage between the ibuprofen molecule and the amino acids, and as a result it can no longer function as an acid in the new molecule (Figures 7.1 and 7.2). However, the amino acids themselves have a free carbonyl group but this is not known to be irritant to the GI mucosa. Consequently, by 'tying up' the carbonyl group of ibuprofen, these amino acid derivatives achieve less GI irritation relative to ibuprofen.

The ibu-L-arginine derivative was synthesised as it was speculated that the L-arginine component might act as a source of nitric oxide (NO). It has been suggested that NO and prostaglandins act synergistically to protect the intestinal mucosa (Wallace, 1996). By this rationale, the ibu-L-arginine compound would have been expected to show even less GI irritation than the alanine, glycine and phenylalanine derivatives. No significant differences in histological scores were observed between ibu-L-arginine and the other amino acid derivatives, which may have been due to the stability of the molecule which would prevent the release of NO.

7.2.3 Plasma profiles

Plasma samples were collected at 30 minute intervals over the course of each two hour perfusion for each system, and for two hours after the perfusions had stopped to generate both absorption and elimination data. Samples were assayed for drug content using the methods described in Section 4.4.15.

The plasma concentration versus time profiles for the three amino acid derivatives were fitted (Scientist®) to two pharmacokinetic models: a one compartment model and a two compartment model, both with constant input and first-order output.

The two compartment model showed the best-fit of the absorption and elimination data and this model is described in Appendix 5. This is the same model that was used to fit the ibuprofen data in previous sections.

The parameters used in this model are k_{elim} (elimination rate constant), k_{12} (rate constant for transfer from the central to the peripheral compartment), k_{21} (rate constant for transfer from the peripheral to the central compartment) and volume of distribution (V). Dose and t_v (duration of the infusion/perfusion, 120 mins) were set at the experimental values. Dose was calculated from the cumulative amount of drug absorbed over the 2 hour perfusion as described in Section 4.4.14. The input rate constant (k_0) was calculated from Equation 6.3.

$$k_0 = \frac{\text{Dose}}{\text{Time}} \quad \text{Equation 6.3}$$

where *Time* is the duration of the perfusion (120 minutes).

In previous sections, plasma profiles were plotted using concentration in $\mu\text{g ml}^{-1}$. However, for these compounds plasma profiles were plotted using concentration expressed as both $\mu\text{g ml}^{-1}$ and nanomoles ml^{-1} . The best-fit pharmacokinetic parameters were determined using the nanomoles ml^{-1} versus time data to correct for the large differences in molecular weights between the compounds.

The best-fit profiles together with the experimental data points for the three amino acid derivatives as well as for ibuprofen are shown in Figures 7.9 and 7.10 with concentration

expressed in $\mu\text{g ml}^{-1}$ and nanomoles ml^{-1} respectively. The pharmacokinetic parameters that describe the best-fit profiles are given in Tables 7.7 and 7.8.

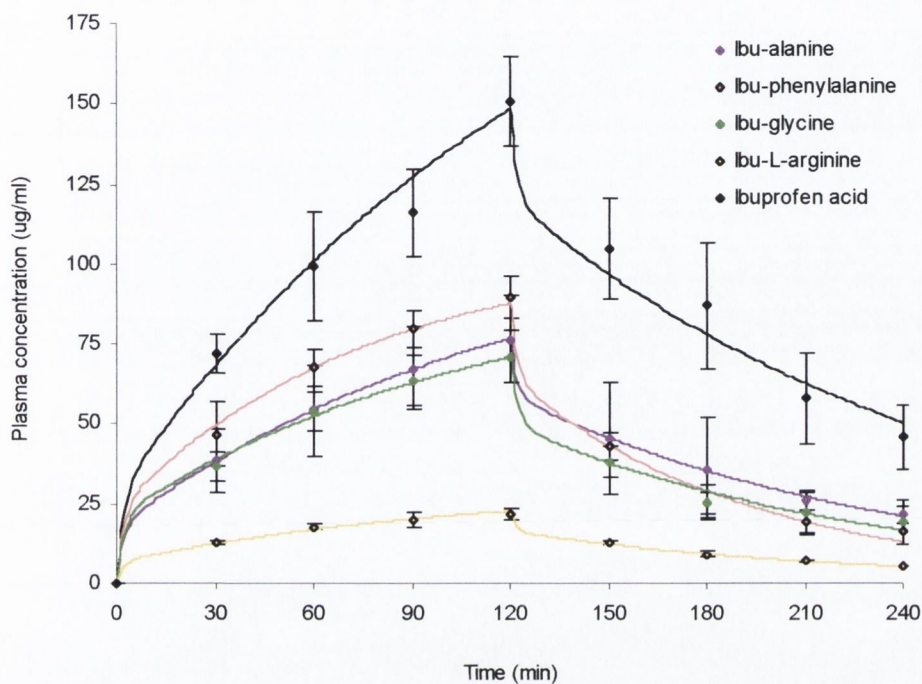


Figure 7.9 The best-fit plasma concentration versus time profiles together with the experimental data points for ibu-alanine, ibu-glycine, ibu-phenylalanine and ibuprofen.

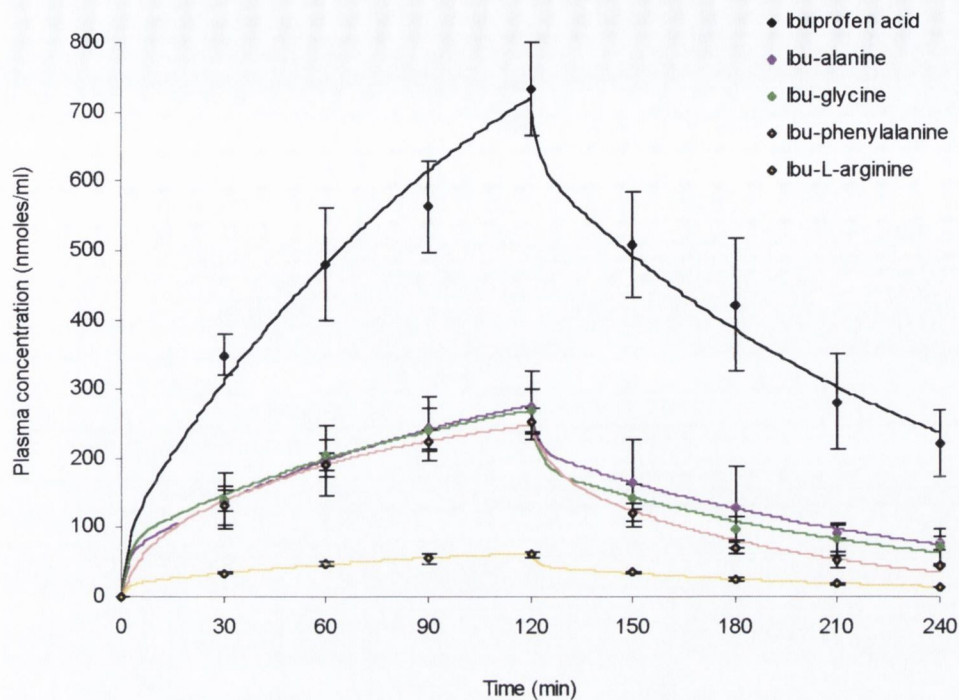


Figure 7.10 The best-fit plasma concentration versus time profiles together with the experimental data points for ibu-alanine, ibu-glycine, ibu-phenylalanine and ibuprofen.

Table 7.7 Results ($\pm$ s.d.) from fitting the plasma data for ibu-alanine and, ibu-glycine to the model described above including k_{elim} , k_{12} , k_{21} , dose, k_0 , volume of distribution (Vol. dist.) and goodness of fit (MSC, r^2). The results for ibuprofen are also included.

	Ibu- alanine	Ibu- glycine	Ibuprofen
$k_{elim} \text{ (min}^{-1}\text{)}$	0.385 ± 0.073	0.295 ± 0.056	0.325 ± 0.005
$k_{12} \text{ (min}^{-1}\text{)}$	0.090 ± 0.001	0.100 ± 0.001	0.044 ± 0.013
$k_{21} \text{ (min}^{-1}\text{)}$	0.050 ± 0.008	0.039 ± 0.007	0.081 ± 0.004
Dose (nmoles rat ⁻¹)	66866	70140	94078
Dose (nmoles kg ⁻¹)	237.23	235.37	309.64
$k_0 \text{ (nmoles min}^{-1}\text{)}$	557.22	584.500	783.98
Vol. dist. (ml kg ⁻¹)	56.04 ± 5.52	53.89 ± 5.31	52.91 ± 0.56
MSC	6.69	4.51	3.35
r^2	0.99	0.99	0.99

Table 7.8 Results ($\pm$ s.d.) from fitting the plasma data for ibu-phenylalanine and ibu-L-arginine to the model described above including k_{elim} , k_{12} , k_{21} , dose, k_0 , volume of distribution (Vol. dist.) and goodness of fit (MSC, r^2). The results for ibuprofen are also included.

	Ibu- phenylalanine	Ibu-L-arginine	Ibuprofen
k_{elim} (min^{-1})	0.087 ± 0.017	0.372 ± 0.071	0.325 ± 0.005
k_{12} (min^{-1})	0.046 ± 0.001	1.19 ± 0.01	0.044 ± 0.013
k_{21} (min^{-1})	0.051 ± 0.009	0.041 ± 0.007	0.081 ± 0.004
Dose (nmoles rat^{-1})	63502	26329	94078
Dose (nmoles kg^{-1})	207.93	91.19	309.64
k_0 (nmoles min^{-1})	529.18	219.41	783.99
Vol. dist. (ml kg^{-1})	128.18 ± 12.63	75.71 ± 7.46	52.91 ± 0.56
MSC	4.10	5.46	3.35
r^2	0.99	0.99	0.99

Statistical analysis using ANOVA of the elimination rate constants (k_{elim}) and volumes of distribution of the four amino acid derivatives and of ibuprofen showed that there were few statistically significant differences between them.

All of these compounds are predominantly ionized at the physiological pH and this may be contributing to the similarity between their volumes of distribution

Ibu-phenylalanine was unique among the compounds in that both its k_{elim} and volume of distribution were significantly different to the corresponding values for the other compounds (ANOVA; $p < 0.05$).

The volume of distribution was significantly higher than the others and this is likely to be related to its relatively high cLog P (4.77, Table 7.2) indicating that the compound may be more lipophilic than the others despite its degree of ionization. This high Log P value may be contributing to the high volume of distribution for two reasons. Firstly, it may allow the compound to more readily distribute into the peripheral tissues in the rat, *e.g.* its lipophilicity would allow it to enter fatty tissue more easily than a less lipophilic

compound. Secondly, as described in Section 3.2.1, Martin (1981) reported that the permeability of the intestinal epithelium increases roughly with the lipophilicity of the drug molecule until it reaches a plateau at a Log P value of approximately two. However, for Log P greater than four, the permeability starts to decrease (Wils et al., 1994) as very lipophilic drugs generally have low aqueous solubilities and, as a result will partition at slower rates from the cell membranes to the aqueous extracellular fluids (Raub et al., 1993). Consequently, they readily leave the gut and enter the enterocytes lining the small intestine at the mucosal side but they will not readily exit them at the serosal side to enter the blood. Since the cLog P of ibu-phenylalanine is above four, this may be contributing to a false estimate of volume of distribution. The 'dose' parameter is calculated from the amount of drug that disappears from the gut as described in Section 4.4.14. The pharmacokinetic model assumes what leaves the gut actually enters the blood. However, this may not be the case as a fraction of the 'dose' may still be in the cell membranes of the enterocytes. To compensate for this, the model calculates the volume of distribution to be larger than what it actually is to justify the lower than expected plasma concentrations on account of the high 'dose'.

Additionally, this theory might also explain why the k_{elim} is significantly lower than the others: the fraction of the dose remaining in the cell membranes of the enterocytes might be acting as a source of ibu-phenylalanine that maintains the plasma levels for longer than expected, thereby giving the impression to the pharmacokinetic model that it is being eliminated from the rat at a lower rate than it actually is.

The volume of distribution of ibu-L-arginine was also significantly different to the other compounds. This result is unusual as it is a hydrophilic compound with a cLog P of -0.59 .

7.2.4 Stability of the amino acid derivatives of ibuprofen

Perfusate and plasma samples from the ibu-alanine, ibu-glycine, ibu-phenylalanine and ibu-L-arginine systems were assayed for ibuprofen to see if it was released from these compounds in the intestine or in the blood. No ibuprofen was detected indicating that these four amino acid derivatives are stable in both the intestinal perfusion solution and in

the blood over the time course of the study. The limit of quantitation of ibuprofen in plasma was $2.35 \mu\text{g ml}^{-1}$ (Appendix 14).

7.2.5 Cell culture studies

In addition to *in situ* absorption studies, the ability of ibu-alanine, ibu-glycine, ibu-phenylalanine and ibu-L-arginine to inhibit Gly-Sar uptake by peptide transporters was studied in cell culture using Capan-2 cells as described in Section 4.4.18. The level of Gly-Sar uptake was assessed in the presence of different concentrations of these compounds and the data was fitted to Equation 6.1 using Sigma Plot[®] 8.0 to calculate best-fit IC_{50} values. IC_{50} values are reported for each compound in Table 7.9.

Table 7.9 *IC_{50} values for ibu-alanine, ibu-glycine, ibu-phenylalanine, ibu-L-arginine and ibuprofen relative to Gly-Sar uptake by peptide transporters.*

Compound	IC_{50} (mM)	s.e.
Ibu-alanine	0.52	0.18
Ibu-glycine	1.03	0.16
Ibu-phenylalanine	0.33	0.10
Ibu-L-arginine	0.07	0.04
Ibuprofen	0.48	0.16

The L-arginine derivative displays the greatest affinity for the population of peptide transporters in the Capan-2 cell line for which Gly-Sar is a substrate. Capan-2 cells possess an array of peptide transporters and it is evident that ibu-glycine appears to demonstrate less binding to these transporters than the parent compound itself. The results for ibu-L-arginine suggest that this compound might be able to utilise peptide transporters for active transport. Within the series of the three amino acid derivatives of ibu-alanine, ibu-glycine and ibu-phenylalanine there appears to be a trend for IC_{50} values

to decrease as the compounds become more hydrophobic as determined by the cLog P values. This is illustrated in Figure 7.11.

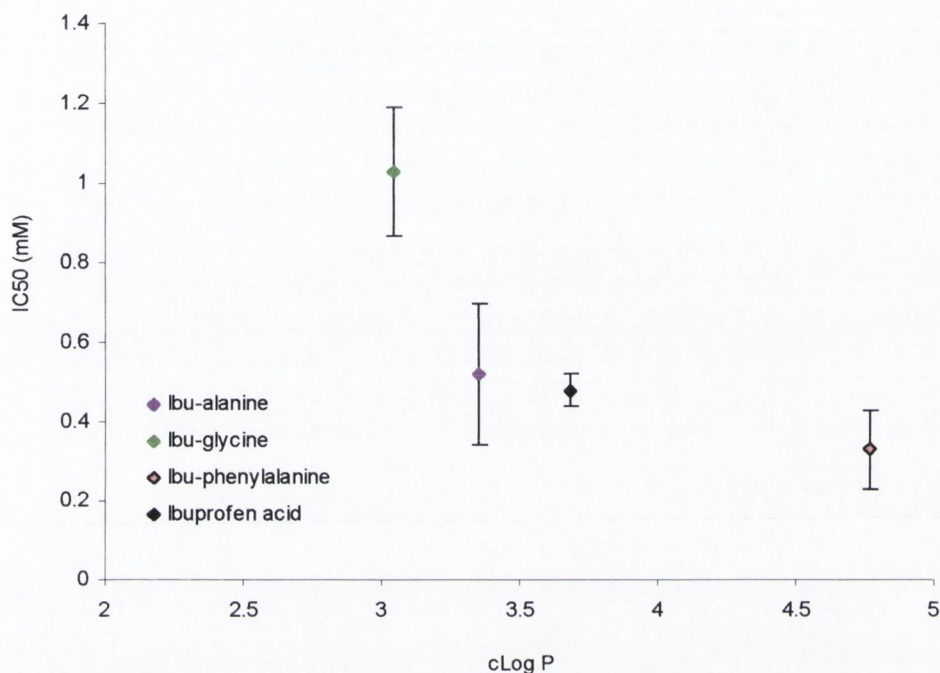


Figure 7.11 Relationship between the IC_{50} and cLog P values of ibu-alanine, ibu-glycine, ibu-phenylalanine and ibuprofen.

This relationship might suggest that these compounds bind via non-specific mechanisms to hydrophobic regions of the relevant peptide transporter with more hydrophobic compounds able to bind more tightly than less hydrophobic compounds. The very low IC_{50} value for the L-arginine derivative relative to other compounds is more difficult to explain, given the more hydrophilic nature of this compound, however binding of ibu-L-arginine to simpler models of the peptide transporter has also been observed. The inhibition of Gly-Sar transport by ibu-L-arginine in HeLa cells transfected with the PepT1 transporter has previously been observed (Lane et al., 2003).

7.3 CONCLUSIONS

Ibu-alanine, ibu-glycine and ibu-phenylalanine have significantly lower P_{app} values than the parent compound, ibuprofen. This may be due to the polar surface areas of the molecules.

The P_{app} of ibu-L-arginine is significantly lower than ibu-alanine, ibu-glycine, ibu-phenylalanine and ibuprofen. This may be due to its relatively high MW and polar surface area combined with its low lipophilicity.

These four ibuprofen derivatives do not appear to alter the barrier properties of the intestinal epithelium to PEG 4000 relative to blank buffer. However, they all produce significant histological damage to the intestinal epithelium relative to blank buffer, but it is significantly less than the damage produced by an equivalent concentration of the parent compound, ibuprofen.

The pH profiles of the perfusate samples are consistent with an intestinal microclimate pH of approximately six.

The ibuprofen derivatives had similar rates of elimination from the plasma and similar volumes of distribution as ibuprofen, with the exception of ibu-phenylalanine which had a significantly higher volume of distribution and a significantly lower rate of elimination than the other compounds. This was attributed to its lipophilicity. Ibu-L-arginine also had a volume of distribution that was significantly higher than ibu-alanine, ibu-glycine and ibuprofen, but this result could not be explained in terms of its physicochemical properties.

Cell culture studies indicate that the four amino acid derivatives of ibuprofen, together with ibuprofen itself, reduce the uptake of Gly-Sar by peptide transporters and the extent to which this occurs, as measured by IC_{50} values, varies with the compound. There may be a relationship between the IC_{50} and the lipophilicity of the compounds, indicating a role of hydrophobic interactions between the drug molecules and the relevant transporter. More specific models are needed to further explore this possibility.

The amide linkage in these four ibuprofen derivatives appears to be stable in both the perfusate solution and in the blood over the time course of the in situ experiments. Their stability in the blood may be of relevance if they are to be investigated as candidates for targeting to selective tissues.

Chapter 8

General discussion

8.1 INTRODUCTION

NSAIDs are one of the most widely used groups of drugs worldwide (Garner, 1992) and ibuprofen in turn is one of the most widely prescribed NSAIDs. Despite the fact that it is so widely used, a literature search indicated that very little is known about the absorption properties of ibuprofen, except that it is well absorbed from the intestine after oral administration (Borne, 1995). The widespread use of ibuprofen, and NSAIDs in general, is complicated by the incidence of gastrointestinal (GI) irritation (Rainsford, 2001; Thomas et al., 2002). A variety of approaches have been taken to overcome this problem including the development of COX-2 selective NSAIDs, nitric oxide-releasing NSAIDs and prodrugs of NSAIDs (Cannon and Breedveld, 2001; Ingram et al., 2001; Rainsford, 2001; Zovko et al., 2001). All of these have been met with limited success and considerable effort is directed at the development of NSAIDs with significantly reduced GI irritation while still having the ability to treat pain and inflammation.

8.1.1 Objectives

The principal objectives of this work were: i) to examine the absorption properties of ibuprofen in a range of different buffer solutions; ii) to study the absorption properties and mechanisms of ibuprofen, ketoprofen and naproxen; and iii) to determine the effect that coupling the ibuprofen molecule to a range of simple amino acids has on its absorption properties and GI irritation.

8.2 IBUPROFEN AND BUFFER COMPOSITION

8.2.1 Solubility of ibuprofen

Initial studies involved the examination of eight different buffers that had been previously used in absorption/perfusion studies as reported in the literature. The solubility of ibuprofen acid in each system was elucidated and was found to vary six-fold over the range of buffers and was related to the buffer pH. Two exceptions to this relationship were observed, Krebs and FeSSIF. The solubility of ibuprofen in Krebs buffer was significantly lower than expected by its pH and this was due to the Ca^{2+}

ions in the buffer forming a poorly soluble salt with the ibuprofen anion. In contrast, the solubility of ibuprofen in FeSSIF was higher than its pH would suggest and this was due to micellisation by the sodium taurocholate in the buffer.

Buffer capacity was observed to vary significantly over the eight systems. The majority of the buffers were phosphate based and as a result were capable of buffering over similar pH ranges. The exception to this was FeSSIF, which was capable of buffering over a lower pH range due to its composition of acetic acid.

The solubility of ibuprofen in a buffer cannot be predicted from the initial pH of the buffer and the pK_a of ibuprofen as these are not fixed values. The pH of the buffer will change as the ibuprofen dissolves, and the extent of this pH change is dependent on the buffer capacity of the buffer. The pK_a of ibuprofen is influenced by the ionic strength of the buffer. Additionally, as demonstrated in the Krebs and FeSSIF systems, ibuprofen may form salts with some of the buffer components or may become solubilised.

8.2.2 Buffer composition and ibuprofen absorption

The *in situ* absorption properties of ibuprofen in each of the buffer systems were examined to investigate if buffer composition could significantly affect the absorption properties of ibuprofen.

8.3 ABSORPTION PROPERTIES OF ALL COMPOUNDS STUDIED

The surface area of the small intestine that is available for paracellular absorption is relatively low compared to the overall surface area available for transcellular absorption, which is extensive (Daugherty and Mrsny, 1999). These two routes of passive absorption are not mutually exclusive as there are no strict criteria that prohibit a drug molecule from using both routes at the same time. However, most molecules are transported predominantly via one route, depending on their physical and chemical properties (Pade and Stavchansky, 1997). In addition to this, some compounds are actively transported across the membrane by carrier mechanisms (Tsuji, 1999; Kim, 2002; Naruhashi et al., 2002).

The permeability coefficients of all of the compounds studied are given in Table 8.1 together with some of their relevant physicochemical properties.

Table 8.1 *Permeability coefficients in the rat ($P_{app} \pm s.d.$), fraction absorbed in humans at the steady-state (FA), molecular weights (MW), Log P and topological polar surface area (TPSA) values of all compounds studied.*

	P_{app} ($\text{cm sec}^{-1} \times 10^{-4}$)	FA (%)	MW	Log P	TPSA ($\text{\AA}^2$)
Ibuprofen	1.40 ± 0.14	100.00	206.3	3.51	37.30
Ketoprofen	1.32 ± 0.15	100.00	254.3	3.12	54.37
Naproxen	1.37 ± 0.07	99.99	230.3	3.34	46.53
Gly-Sar	0.38 ± 0.02	99.95	146.1	-3.07*	83.63
Ibu-alanine	0.61 ± 0.09	99.93	277.4	3.35*	66.40
Ibu-glycine	0.58 ± 0.11	99.90	263.3	3.04*	66.40
Ibu-phenylalanine	0.55 ± 0.06	99.20	353.5	4.77*	66.40
Ibu-L-arginine	0.11 ± 0.03	77.06	362.5	-0.59*	128.3

*cLog P

All of these compounds are more than 90% ionised at the steady-state.

Differences between the permeability coefficients of the compounds listed in Table 8.1 will be discussed further.

8.3.1 *Passive absorption*

Significant differences were observed between the P_{app} values of ibuprofen in Fagerholm, FeSSIF, HBSS, Krebs and PBS 6.8. These differences could be attributed to differences in their osmolarities as it was observed that the buffer with the highest osmolarity (FeSSIF) also showed the lowest ibuprofen P_{app} value while the buffer with one of the lowest osmolarities (HBSS) showed the highest P_{app} value.

Osmolarity has been reported as a driving force for water flux across the intestinal mucosa and this water flux principally occurs through the paracellular route or via the aqueous pores (Lennernas, 1995). The correlation observed between buffer osmolarity and net water flux (Figure 8.1) indicates that this movement of water across the intestinal wall reduces or promotes the absorption of ibuprofen by solvent drag, depending on the direction of the water movement.

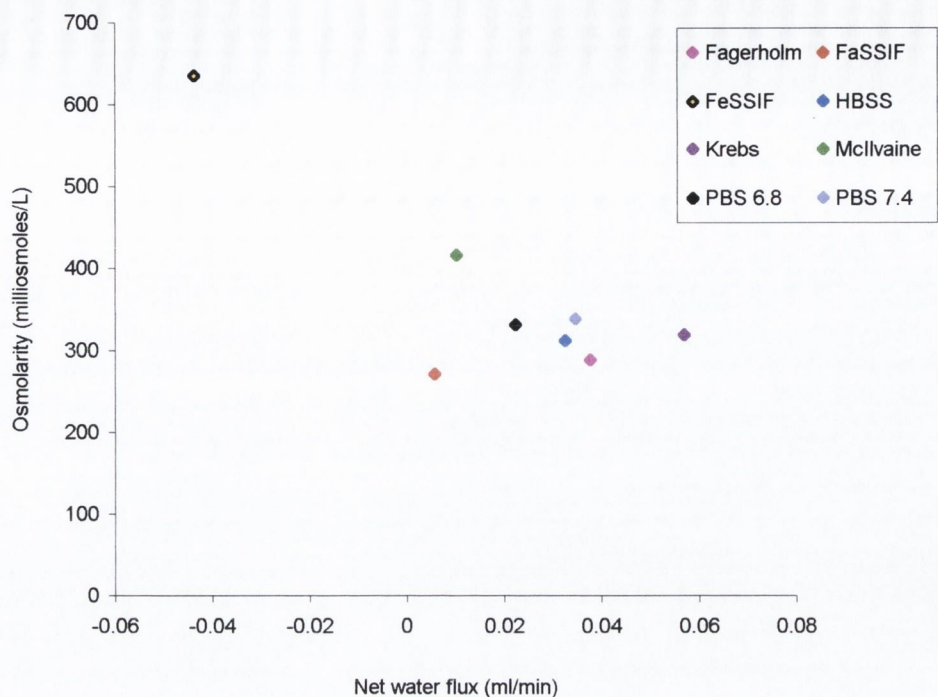


Figure 8.1 Relationship between buffer osmolarity and net water flux across the intestinal mucosa (a negative value for net water flux represents secretion into the lumen and a positive value represents absorption out of the lumen).

This preliminary data suggested that the principal routes of passive absorption available to ibuprofen are the paracellular route or transmembrane transport via the aqueous pores.

No significant differences were seen between the absorption properties of ibuprofen, ketoprofen and naproxen. The absorption properties of ketoprofen were examined at different concentrations and no changes in P_{app} with increasing concentration were observed.

These initial results indicated that the absorption of ibuprofen, ketoprofen and naproxen in the rat is aqueous boundary layer controlled and they are predominantly absorbed via the paracellular or aqueous pore routes. In contrast, the rat intestinal P_{app} of Gly-Sar is sufficiently low and the molecule is sufficiently hydrophilic to suggest that its absorption is membrane controlled. Despite it being a substrate for peptide transporters, a certain amount of it will be passively absorbed and its molecular

weight and Log P are suitable for paracellular transport or passage through the aqueous pores. These physicochemical properties are of such a magnitude that, in addition to uptake by peptide transporters, it would be expected to be absorbed via these routes to the same, if not to a greater, extent as ibuprofen, ketoprofen and naproxen. However, its P_{app} is approximately three-fold smaller than ibuprofen, ketoprofen and naproxen (Table 8.1).

A possible reason for this is that the Log P of Gly-Sar is much lower than for ibuprofen, ketoprofen or naproxen. As a result of its lower Log P, its ability to utilise the transcellular route for passive absorption by partitioning into the lipid structure of the cell membrane is limited, compared to the NSAIDs.

This in turn suggests that the NSAIDs are absorbed to a large extent by the transcellular route by partitioning through the lipid structure. This is despite their degree of ionisation and molecular weights indicating that they are most likely absorbed paracellularly or via the aqueous pores. The much larger surface area available for transmembrane absorption relative to the paracellular route may compensate for the unfavourable degree of ionisation for transmembrane absorption. The net effect of this is that the transmembrane route accounts for a large fraction of the NSAID absorption. In turn, Gly-Sar is ionised to a similar extent, but has a much lower Log P. This reduces its capability of partitioning into the lipid structure of the cell membrane relative to the three NSAIDs and so it is forced to rely more heavily on paracellular transport and the transmembrane aqueous pore routes for passive absorption.

Consequently, what is likely to be occurring is that the three NSAIDs and Gly-Sar are being absorbed at similar rates by the paracellular route but differ in the extent of transmembrane absorption via partitioning across the lipid structure. It is this that accounts for the large difference in values of P_{app} seen between the three NSAIDs and Gly-Sar.

It can be concluded from this the passive absorption of ibuprofen, ketoprofen and naproxen occurs principally by transcellular diffusion, with transport via the paracellular route occurring simultaneously but to a much lesser extent.

The differences in P_{app} values between ibuprofen and ibu-alanine, ibu-glycine and ibu-phenylalanine can be attributed to differences in their TPSA values, as their Log P values are all of a similar order of magnitude.

The differences in P_{app} values seen between ibu-L-arginine and the other three amino acid derivatives of ibuprofen as well as the parent compound, ibuprofen, can be explained by its relatively large TPSA value and relatively low Log P value. The TPSA value of ibu-L-arginine ($128.3 \text{ \AA}^2$) is approximately two-fold greater than the other three amino acid derivatives and approximately four-fold greater than ibuprofen itself. A consequence of this is that the ability of ibu-L-arginine to utilise the paracellular route or pass through the enterocyte cell membrane or aqueous pores is restricted relative to the other compounds. In addition, its relatively low Log P value (-0.59) means that it is a more hydrophilic molecule than the others, and consequently its ability to partition into and traverse the lipid structure of the cell membrane is reduced. The net effect of these two factors is a significant decrease in its overall ability to cross the intestinal mucosa resulting in a relatively low P_{app} value.

Topological polar surface area (TPSA) has been shown to be an effective and rapidly determinable predictor of the intestinal absorption of drug molecules and is defined as the sum of surface contributions of polar atoms (Ertl et al., 2003). Polar surface area has been shown to correlate well with drug transport properties, such as intestinal absorption or blood-brain barrier penetration (Clark, 1999a; 1999b; Kelder et al., 1999). When the fractions unabsorbed of the eight compounds studied are plotted against their TPSA values, the resulting relationship is shown in Figure 8.2.

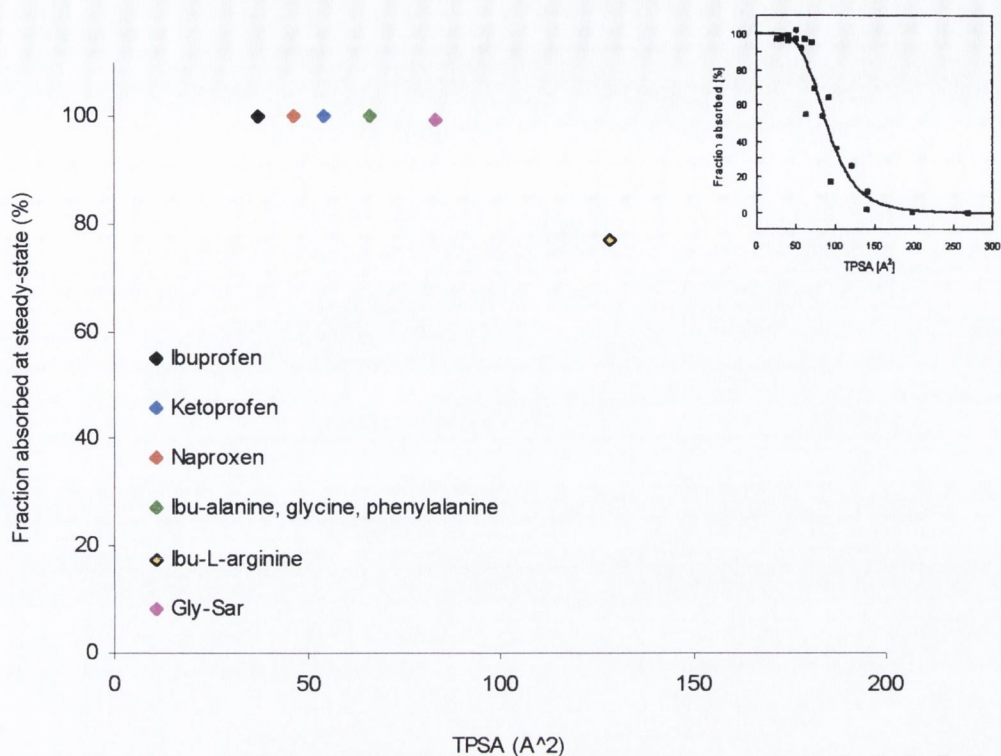


Figure 8.2 Relationship between fraction absorbed in humans at the steady-state (%) and TPSA (Å²). Insert from Ertl et al. (2003).

Figure 8.2 shows that the experimental data forms the beginning of a possible sigmoidal relationship between fraction absorbed and polar surface area. This is in good agreement with similar profiles shown by Palm et al. (1997) and Ertl et al. (2003) (see insert in Figure 8.2), indicating that polar surface area is a useful predictor of intestinal drug absorption.

In terms of the biopharmaceutics drug classification scheme (BCS), ibuprofen, ketoprofen and naproxen are considered as Class II compounds as they have high permeabilities and low aqueous solubilities. The alanine, glycine and phenylalanine derivatives of ibuprofen all have significantly lower P_{app} values than ibuprofen in the rat, yet when converted to fraction absorbed in humans, they are 99% absorbed. Given their lower pK_a values than ibuprofen, they can be expected to be more soluble in the contents of the stomach and the small intestine than ibuprofen. For these reasons it is possible to predict that they belong to Class I of the BCS.

Recently, Legen et al. (2003) have reported a study which suggests that an inwardly directed proton gradient (mucosal to serosal direction) maintained by the acidic microclimate pH at the intestinal surface could be a driving force for the transport of NSAIDs and other monocarboxylic acid type drugs across the intestinal epithelia. At the acidic mucosal surface the lipophilic, unionised form of the drug is quickly absorbed across the cell membrane. Once in the intracellular compartment it dissociates and consequently a high concentration gradient of the unionised species is maintained across the membrane. This mechanism might explain the rapid absorption of NSAIDs and other monocarboxylic acid type drugs from the GI tract.

Additionally, the proton-dependent monocarboxylate transporter, MCT, may also be responsible for the rapid absorption of NSAIDs as monocarboxylate drugs such as aspirin have been reported to be transported by this mechanism in the intestine (Osiecka et al., 1985) and in Caco-2 cells (Takanaga et al., 1994). This is a proton dependent process and, in common with the absorption theory by Legen et al. (2003), would also be promoted by an acidic microclimate pH at the intestinal surface. This involvement of MCT indicates that transporter proteins could play a significant role in the absorption of NSAIDs and might help to further explain why monocarboxylate drugs are generally rapidly absorbed from the GI tract.

8.3.2 The role of peptide transporters

The ability of ibuprofen to bind to intestinal peptide transporters was investigated. Gly-Sar is a dipeptide substrate for intestinal peptide transporters and it was observed that ibuprofen reduced the uptake of Gly-Sar both *in situ* and in cell culture. This indicates that ibuprofen can bind non-specifically to the peptide transporters involved in Gly-Sar uptake. The reduction in Gly-Sar uptake was much more pronounced in cell culture than *in situ*. This may be due to the nature of the *in situ* model, which encompasses a diverse array of transporters and absorption pathways. In contrast, the cell culture model, Capan-2, is derived from a pancreatic adenocarcinoma and although it expresses a range of peptide transporters, it also expresses these at higher levels than the intestine.

Additionally, Gly-Sar uptake by peptide transporters in Capan-2 cells was reduced by the alanine, glycine, phenylalanine and L-arginine derivatives of ibuprofen. This

indicates that all of these compounds are capable of binding to the relevant transporters. Results from the *in situ* absorption studies indicate that the transporters involved are proton dependent as the rate of Gly-Sar uptake appeared to decrease as pH increased. The extent of Gly-Sar uptake inhibition in cell culture varied with the compound, with the L-arginine derivative displaying the lowest IC_{50} and the glycine derivative displaying the highest IC_{50} . This ability of ibu-L-arginine to bind to peptide transporters is significant as it may suggest a potential role of L-arginine in drug derivatisation for the tissue selective delivery involving selective uptake by peptide transporters. For example the newly emerging role of NSAIDs in the treatment of Alzheimer's disease and colon cancer (Katori and Majima, 2000; Aisen, 2002; Kaza et al., 2002) may lead to the need for NSAIDs that specifically target these tissues.

It has been reported that the expression of these transporters at the plasma membrane of Capan-2 cells is sufficiently high so as to show potential as a delivery route for a peptide-like anti-neoplastic agent in cases of pancreatic cancer (Gonzalez et al., 1998). This higher density of peptide transporters at the level of the cell membrane in Capan-2 means that any interaction between ibuprofen and the transporters will be much more apparent in the cell culture model than *in situ*.

From this it can be concluded that in addition to the passive absorption of these compounds, active transport via peptide transporters may be contributing to their absorption.

8.3.3 Pharmacokinetic analysis

A two compartment pharmacokinetic model with constant input and first order output was shown to best describe the ibuprofen plasma data. Best-fit pharmacokinetic parameters for ibuprofen were of a similar order of magnitude to literature values. There was a good correlation between disappearance from the gut and appearance in the plasma. Plasma data for ketoprofen, naproxen, ibu-alanine, ibu-glycine, ibu-phenylalanine and ibu-L-arginine were also fitted to a two compartment pharmacokinetic model with constant input and first order output, and good estimates of fit were obtained.

The rate of elimination of ketoprofen from the blood appeared to decrease with increasing dose although a significant decrease was only seen when comparing the highest and lowest doses.

The rates of elimination and volumes of distribution of ibu-alanine, ibu-glycine and ibuprofen acid were found to be similar. The similarity in volumes of distribution is most likely due to their similar extents of ionisation at physiological pH.

Both the rate of elimination from the blood and the volume of distribution of ibu-phenylalanine were significantly different from ibuprofen and the other amino acid derivatives. Its significantly higher volume of distribution may be related to its lipophilicity as, despite having a similar degree of ionisation at physiological pH, it has a significantly higher cLog P than the other compounds.

Given that the amino acid derivatives were not observed to break down in the plasma and release ibuprofen, further work could be carried out to determine their fate once they enter the blood. This might help to explain why the rate of elimination of ibu-phenylalanine is significantly different to the other amino acid derivatives and to ibuprofen.

8.3.4 *Stability of the amino acid derivatives*

Ibuprofen was not released from these compounds, suggesting that they were not being metabolised by the rat. It is possible they are excreted unchanged. This is in agreement with Shanbhag et al. (1992) and Franssen et al. (1992) who reported on the stability of the amide bond in a range of amino acid derivatives of NSAIDs in a number of different media including buffers (pH 5.0 and 7.0) and human plasma and serum. This level of stability and resistance to hydrolysis of the amide bond in these compounds explains why the parent compound, ibuprofen, was not detected in any of the perfusate or plasma samples in the current work.

8.4 GASTROINTESTINAL TOXICITY

Figure 8.3 compares the GI toxicity of all the compounds studied in terms of their total scores from histological evaluation.

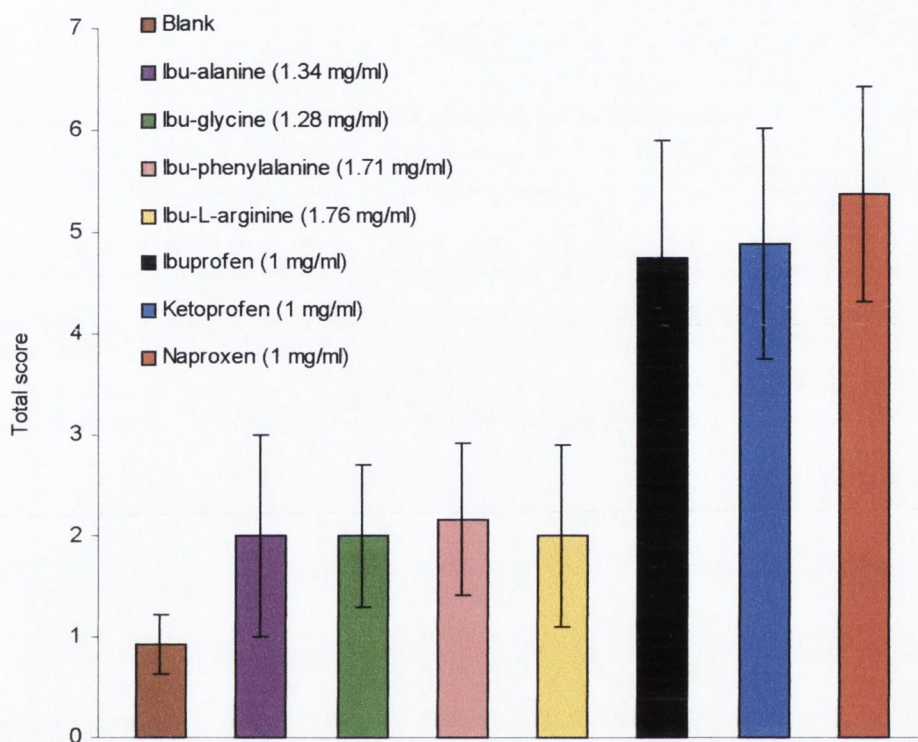


Figure 8.3 Total scores from histological evaluation of the rat small intestinal mucosa after perfusion with a range of different systems.

It is apparent from Figure 8.3 that the amino acid derivatives of ibuprofen are significantly less toxic to the GI mucosa than ibuprofen, ketoprofen and naproxen at similar concentrations (ANOVA; $p < 0.05$). The concentrations of the amino acid derivatives were all equivalent to 1 mg ml^{-1} ibuprofen acid.

When a range of concentrations of ketoprofen were used in the perfusion solutions, the level of intestinal damage increased significantly with each doubling of concentration, within the concentration range of $0.5 - 2.0 \text{ mg ml}^{-1}$. The total score appeared to increase linearly with increasing ketoprofen concentration, but a wider range of concentrations needs to be studied to fully elucidate this relationship.

The pK_a values of ibu-alanine, ibu-glycine and ibu-phenylalanine are all lower than the pK_a of ibuprofen and consequently are more acidic molecules. Despite this, data from PEG 4000 permeability and, in particular, histological studies indicates that they are significantly less irritant to the GI mucosa relative to ibuprofen.

Elimination or reduction of the role of the direct contact effect of the acidic carbonyl group in GI irritation appears to be the reason for the reduced irritation seen with these amino acid derivatives. The free carbonyl group of ibuprofen has been associated with GI irritation by a direct contact effect on the intestinal mucosa (Cioli et al., 1979). When the amino acid derivatives of ibuprofen are synthesised, this carbonyl group is involved in the bond between the ibuprofen molecule and the amino acids. However, the amino acids themselves have a free carbonyl group which could be expected to be irritant to the GI mucosa on account of its lower pK_a . A previous study by Shanbhag et al. (1992), reported that ibu-glycine has similar anti-inflammatory activity to ibuprofen. The reduced histological damage seen with the amino acid derivatives in this work is unlikely due to a reduction in their ability to inhibit prostaglandin production relative to ibuprofen, and is more likely due to the 'tying up' of the carbonyl group of ibuprofen.

It was initially thought that it might be the unionised form of the carboxylic acid that is partly responsible for the GI toxicity. The lower pK_a values of the amino acid derivatives relative to ibuprofen suggest that at any given pH they are ionised to a lesser extent than ibuprofen itself, and hence have a lower fraction of the unionised component. This is supported by Rainsford and Whitehouse (1980), who showed that the co-administration of PBS 6.8 buffer with aspirin had a significant gastro-protective effect relative to aspirin alone. The proposed mechanism for this effect is the generation of a lower hydrogen ion concentration by the buffer in the microenvironment of the aspirin particles which results in an increased ionisation of aspirin (Lanza et al., 1980). However, more recent evidence (Kelly et al., 1996) indicates that buffered aspirin may be equally, if not more, irritant to the GI tract than plain aspirin.

8.5 pH PROFILES OF PERFUSATE SAMPLES

The pH values of the perfusate samples from all of these perfusion studies were measured and plotted in pH versus time profiles. In all cases there was a shift in pH from before the perfusion solution entered the intestinal segment until the steady-state. The direction and extent of this pH change was dependent on the initial pH of the perfusion solution and indicates that the intestinal microclimate buffering system attempts to maintain the intestinal contents at pH six. This is evident in Figure 8.4 which shows the pH versus time profiles for several of the systems studied over the course of this work.

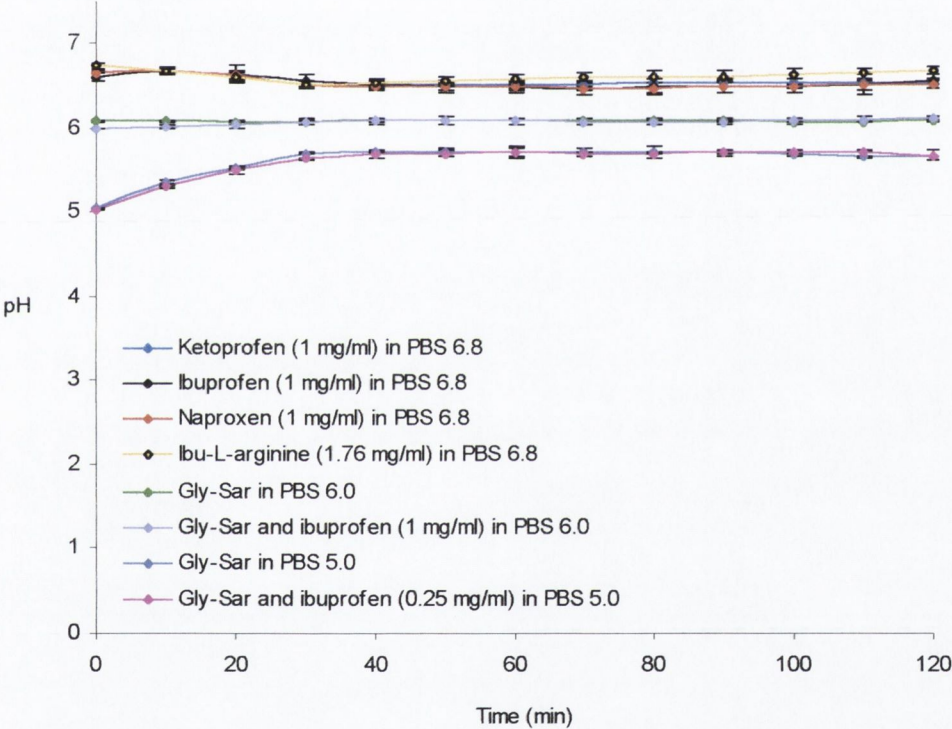


Figure 8.4 pH versus time profiles of the perfusate samples from several of the systems studied.

For most of the systems the shifts in perfusate pH are most dramatic over the first 40 minutes, after which there is very little change. This suggests that the intestinal buffering system reaches a steady-state after 40 minutes. The shift in perfusate pH that it produces is maintained over the course of the perfusion, indicating that there is

no exhaustion of the intestinal buffering system. It also provides evidence of the viability of the model as it indicates that the intestinal segment is functioning normally.

The pH values of the PBS 6.0 samples do not undergo any significant change (t-test: $p>0.05$) and stay at approximately pH 6 over the course of the perfusion. This suggests that the intestinal microclimate pH in the rat is close to pH 6, as proposed by Ikuma et al. (1996).

The relationship between ibuprofen P_{app} and the steady-state pH of the perfusate samples is shown in Figure 8.5.

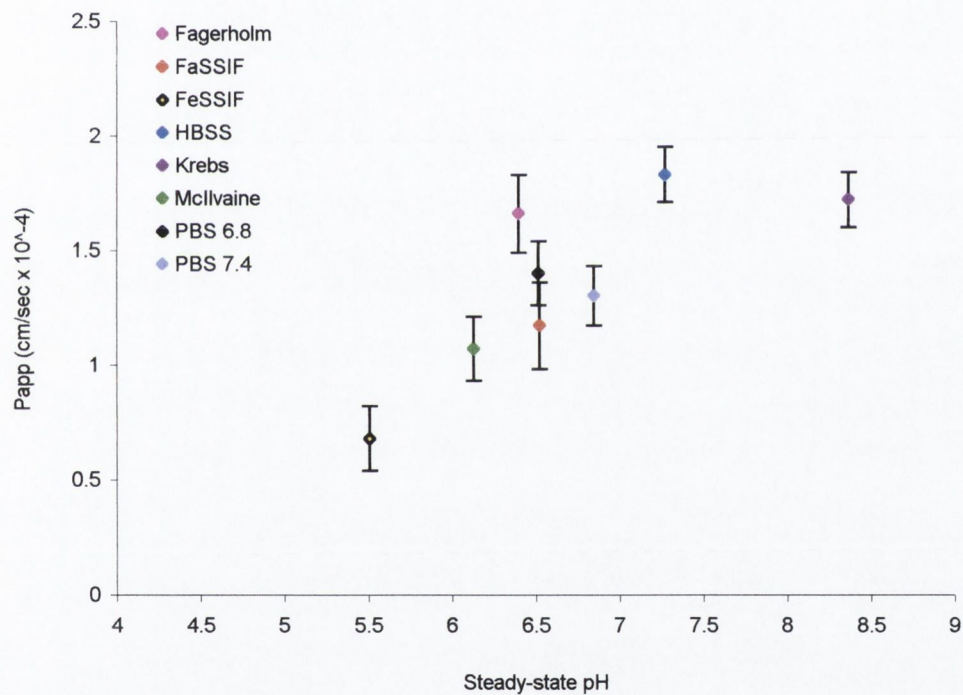


Figure 8.5 Relationship between ibuprofen P_{app} and steady-state pH of the perfusate samples.

It appears that as the steady-state pH increases, so too does the P_{app} value. This result is difficult to explain. The lowest pH is 5.51 and this corresponds to 92.3% of ibuprofen ionised and the highest pH is 8.37 which corresponds to 99.9% ionised. As more of the ibuprofen becomes ionised, it becomes more hydrophilic and

consequently even more suitable for paracellular or aqueous pore transport. However, this relatively small change in percent ionised is not sufficient to account for the two-fold increase in P_{app} . Also, if proton-dependent peptide transporters are involved in ibuprofen uptake, at higher pHs the binding of ibuprofen to the peptide transporter would be expected to decrease due to its proton dependence, and not increase as shown. However, given the significant effect of buffer osmolarity and intestinal water flux on the P_{app} values, the trend between P_{app} and steady-state pH may be ‘artificially’ created due to a relationship between osmolarity/net water flux and pH (Figure 8.6).

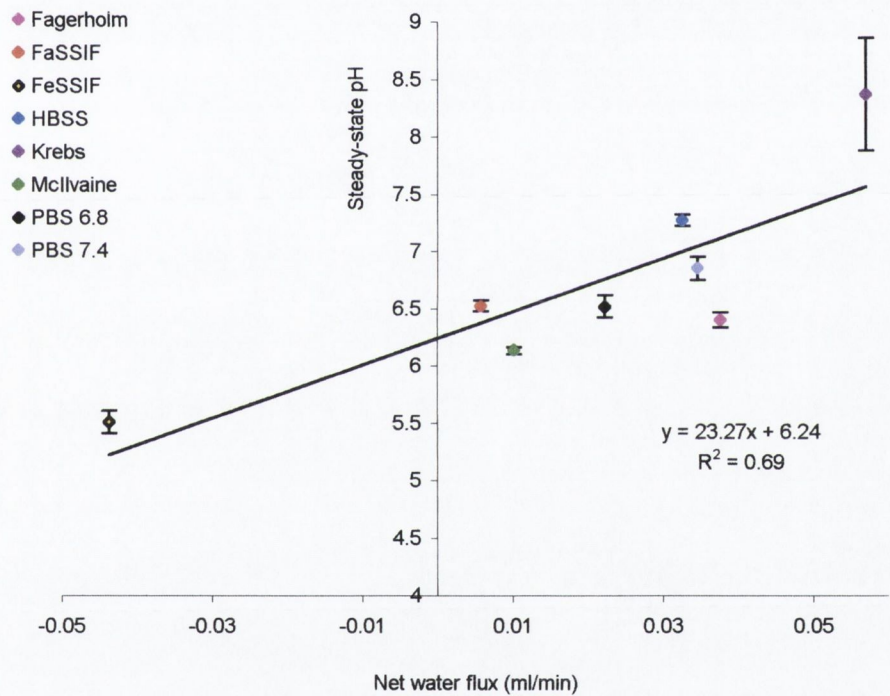


Figure 8.6 Relationship between steady-state pH and net water flux across the intestinal mucosa.

This correlation between net water flux across the intestinal mucosa and steady-state pH of the perfusate may be responsible for the apparent relationship seen between steady-state pH and P_{app} due to the significant effect that net water flux was shown to have on the P_{app} value.

8.3 CONCLUSIONS

The main findings of this work can be summarised as follows:

The composition of the perfusion solution used in *in situ* absorption studies of ibuprofen can have a significant effect on its permeability coefficient. This appears to be primarily due to differences in net water flux across the intestinal mucosa as a consequence of different buffer osmolarities.

When calculating intestinal permeability coefficients, it is important to correct for changes in perfusate drug concentration due to intestinal water flux, as significant differences between systems may be masked by changes in concentration due to water flux which would otherwise be assumed to be due to absorption.

When reporting buffer capacity values it is important to specify the pH of the buffer and whether an acid or a base was used to measure it, as each may produce different results unless the pH is at the pK_a .

The passive absorption of ibuprofen, ketoprofen and naproxen seems to occur principally by transcellular diffusion across the enterocyte cell membrane.

Ibuprofen appears to bind non-specifically to peptide transporters *in situ* and in cell culture and consequently active transport processes by peptide transporters may also be involved in its absorption. This binding is more pronounced in cell culture than *in situ*.

The absorption properties of ketoprofen do not appear to be concentration dependent over the perfusate concentration range of 0.5-2.0 mg ml⁻¹.

Ibu-alanine, ibu-glycine and ibu-phenylalanine are significantly less well absorbed in the rat than their parent compound, ibuprofen. Ibu-L-arginine in turn is significantly less well absorbed than ibu-alanine, ibu-glycine, ibu-phenylalanine and ibuprofen. When their permeability coefficients in rats are converted to fraction absorbed values in humans, there are no significant differences between ibu-alanine, ibu-glycine, ibu-phenylalanine and ibuprofen, and these are all significantly larger than ibu-L-arginine.

Like ibuprofen, the amino acid derivatives are capable of binding to peptide transporters in cell culture, indicating that active transport processes by peptide transporters may also be involved in their absorption. Their ability to bind to peptide

transporters varies with the amino acid derivative. Of the four derivatives, ibu-L-arginine has the highest affinity.

The amino acid derivatives are all significantly less irritating to the GI tract than ibuprofen and are stable in the perfusate and plasma over the time course of the *in situ* perfusion experiments.

The amino acid derivatives produced have favourable GI toxicity relative to ibuprofen and, despite modulated polarity, demonstrate appreciable bioavailability.

8.4 FUTURE WORK

There are a number of areas in which the findings reported in this work could be further investigated. Firstly, there are cell culture models which can be transfected with transporter proteins in contrast to the Capan-2 cell line which has a population of peptide transporters. In this respect they could add to the findings reported in this work by confirming the involvement of specific peptide transporters, such as PepT1, in the uptake of these compounds. This might provide information on any tissue selectivity that these amino acid derivatives might have by investigating their selectivity for transporters that might be found in the cell membranes of specific tissues (*e.g.* colon cancer cells).

Dipeptide derivatives of ibuprofen could be investigated to see if they bind to a greater extent to the peptide transporters.

The amide linkages in the amino acid derivatives of ibuprofen are not cleaved *in vivo* and as a result the NSAID is not released. Approaches to making this linkage more labile and susceptible to esterases after absorption is an area that could be further investigated.

A recent report by Legen et al. (2003) has suggested that an inwardly directed proton gradient maintained by the acidic microclimate pH at the intestinal surface could be considered as a driving force for the transport of monocarboxylic acid type drugs across the intestinal epithelia. This indicates that microclimate pH could be playing a significant role in NSAID absorption and is an area that could be further investigated.

REFERENCES

- Abordo, E.A., Bowden, K., Huntington, A.P. and Powell, S.L., Prodrugs Part 3. 2-formylphenyl esters of indomethacin, ketoprofen and ibuprofen and 6-substituted 2-formyl and 2-acylphenyl esters of aspirin, *Il Farmaco*, 53 (1998) 95-101.
- Abrahamsson, B., Alpsten, M., Jonsson, U.E., Lundberg, P.J., Sandberg, A., Sundgren, M., Svenheden, A. and Tolli, J., Gastrointestinal transit of a multi-unit formulation (metoprolol CR/ZOK) and a non-disintegrating tablet with the emphasis on colon, *Int. J. Pharm.*, 140 (1996) 229-235.
- Adams, S.S., Bresloff, P. and Mason, C.G., Pharmacologic differences between the optical isomers of ibuprofen: evidence for metabolic inversion of the (-)-isomer, *J. Pharm. Pharmacol.*, 28 (1976) 256.
- Aeberhard, E.E., Henderson, S.A., Arabolow, N.S., Griscavage, J.M., Castro, F.E., Barrett, C.T. and Ignarro, L.J., Nonsteroidal anti-inflammatory drugs inhibit expression of the inducible nitric oxide synthase gene, *Biochem. Biophys. Res. Commun.*, 208 (1995) 1053-1059.
- Aisen, P.S., Evaluation of selective COX-2 inhibitors for the treatment of Alzheimer's Disease, *J. Pain and Symptom Management*, 23 (2002) S35-S40.
- Albert, A., Chemical aspects of selective toxicity, *Nature*, 182 (1958) 421-423.
- Allen, A. and Snary, D., The structure and function of gastric mucus, *Gut*, 13 (1972) 666-672.
- Ambs, S., Merriam, W.G., Bennett, W.P., Felley-Bosco, E., Ogunfusika, M.O., Oser, S.M., Klein, S., Shields, P.G., Billiar, T.R. and Harris, C.C., Frequent nitric oxide synthase-2 expression in human colon adenomas: implication for tumor angiogenesis and colon cancer progression, *Cancer Res.*, 58 (1998) 334-341.
- Amidon, G.L., Lennernas, H., Shah, V.P. and Crison, J.R., A theoretical basis for a biopharmaceutic drug classification: the correlation of *in vitro* drug product dissolution and *in vivo* bioavailability, *Pharm. Res.*, 12 (1995) 413-420.

Anderberg E.K., Nystrom C. and Artutsson P., Epithelial transport of drug in cell culture VII: Effects of pharmaceutical surfactant excipients and bile acids on transepithelial permeability in monolayers of human intestinal epithelial (Caco-2) cells, *J. Pharm. Sci.*, 81 (1992) 879-887.

Anderson J.M. and Van Itallie C.M., Tight-junctions and the molecular basis for regulation of paracellular permeability, *Am. J. Physiol.*, 269 (1995) G467-475.

Anderson, K., Launer, L.J., Ott, A., Hoes, A.W., Bretelr, M.M.B. and Hofman, A., Do nonsteroidal anti-inflammatory drugs decrease the risk for Alzheimer's disease? The Rotterdam Study, *Neurology*, 45 (1995) 1441-1445.

Andersson, B. and Ussing, H.H., Solvent drag on non-electrolytes during osmotic flow through isolated toad skin and its response to antidiuretic hormone, *Acta. Physiol. Scand.*, 39 (1957) 228-239.

Andersson, B.W., Levine, A.S., Levitt, D.G., Kneip, J.M. and Levitt, M.D., Physiological measurement of luminal stirring in perfused rat jejunum, *Am. J. Physiol.*, 254 (1988) G843-848.

Arena, B. and del Soltado, P., Nitric esters having a pharmacological activity and a process for their preparation, *United States Patent 5621000* (1997).

Artursson, P., Ungell, A.L. and Lofroth, J.E., Selective paracellular permeability in two models of intestinal absorption: cultured monolayers of human intestinal epithelial cells and rat intestinal segments, *Pharm. Res.*, 10 (1993) 1123-1129.

Artz, J.D., Yang, K., Lock, J., Sanchez, C., Bennett, B.M. and Thatcher, G.R.J., Reactivity of thionitrate esters: putative intermediates in nitrovasodilator activity, *J. Chem. Soc., Chem. Commun.*, (1996) 927-928.

- Asako, H., Kubes, P., Wallace, J., Gaginella, T., Wolf, R.E. and Granger, D.N., Indomethacin-induced leukocyte adhesion in mesenteric venules: role of lipoxygenase products, *Am. J. Physiol.*, 262 (1992) G903-908.
- Aungst, B. and Shen, D.D., *Gastrointestinal absorption of toxic agents*, in: *Gastrointestinal Toxicology*, Rozman, K., Hänninen, O. (Eds.), Elsevier, Amsterdam, (1986) 29-56.
- Aunins, J.G., Southard, M., Myers, R.A., Hemmelstein, K.J. and Stella, V.J., Dissolution of carboxylic acids III: the effect of polyionizable buffers, *J. Pharm. Sci.*, 74 (1985) 1305-1316.
- Avdeef A., pH-metric log P. II: Refinement of partition coefficients and ionization constants of multiprotic substances, *J. Pharm. Sci.*, 82 (1993) 183-190.
- Avdeef, A., *Assessment of distribution-pH profiles*, in: *Lipophilicity in Drug Action and Toxicology*, Pliska, V., Testa, B., van de Waterbeemd, H., Weinheim, V.C.H. (Eds.), (1996) 109-137.
- Azori, M., Polymeric prodrugs, *CRC Reviews in Therapeutic Drug Carrier Systems*, 4 (1987) 39-65.
- Babyatsky, M.W., deBeaumont, M., Thim, L. and Podolsky, D.K., Oral trefoil peptides protect against ethanol- and indomethacin- induced gastric injury in rats, *Gastroenterology*, 110 (1996) 489-497.
- Bak, A.W., McKnight, W., Li, P., Del Soltado, P., Calignano, A., Cirino, G. and Wallace, J.L., Cyclooxygenase-independent chemoprevention with an aspirin derivative in a rat cancer? A model of colonic adenocarcinoma, *Life Sci.*, 62 (1998) PL 367-373.
- Banerjee, P.K. and Amidon, G.L., Physicochemical property modification strategies based on enzyme substrate specificities I: rationale, synthesis and pharmaceutical properties of aspirin derivatives, *J. Pharm. Sci.*, 70 (1981a) 1299-1303.

Banerjee, P.K. and Amidon, G.L., Physicochemical property modification strategies based on enzyme substrate specificities II: α -chymotrypsin hydrolysis of aspirin derivatives, *J. Pharm. Sci.*, 70 (1981b) 1304-1306.

Banerjee, P.K. and Amidon, G.L., Physicochemical property modification strategies based on enzyme substrate specificities III: carboxypeptidase A hydrolysis of aspirin derivatives, *J. Pharm. Sci.*, 70 (1981c) 1307-1309.

Bauer, M.K.A., Lieb, K., Schulze-Osthoff, K., Berger, M., Gebicke-Haerter, P.J., Bauer, J. and Fiebich, B.L., Expression and regulation of cyclooxygenase-2 in rat microglia, *Eur. J. Biochem.*, 243 (1997) 726-731.

Bayly, C.I., Black, W.C., Léger, S., Ouimet, N., Ouellet, M. and Percival, M.D., Structure-based design of COX-2 selectivity into flurbiprofen, *Bioorganic and Medicinal Chemistry Letters*, 9 (1999) 307-312.

Beck, W.S., Schneider, H.T., Dietzel, K., Nuernberg, B. and Brune, K., Gastrointestinal ulcerations induced by anti-inflammatory drugs in rats, *Arc. Toxicol.*, 64 (1990) 210-217.

Beckman, J.S. and Koppenol, W.H., Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and the ugly, *Am. J. Physiol.*, 271 (1996) C1424-1437.

Benet, L.Z., Izumi, T., Zhang, Y., Silverman, J.A. and Wachter, V.J., Intestinal MDR transport proteins and P-450 enzymes as barriers to oral drug delivery, *J. Cont. Rel.*, 62 (1999) 25-31.

Beynon, R.J. and Easterby, J.S., in: *Buffer Solutions*, Oxford University Press, (1996).

Bhat P.G., Flanagan D.R. and Donovan M.D., The limiting role of mucus in drug absorption: drug permeation through mucus solution, *Int. J. Pharm.*, 126 (1995) 179-187.

Bing, R.J., Yamamoto, T., Yamamoto, M., Kakar, N.R. and Cohen, A.M., A new look at myocardial infarction toward a better aspirin, *Cardiovasc. Res.*, 43 (1999) 25-31.

Birnbaum, J.E., Birkhead, N.C., Oronsky, A.L., Dessy, F., Rihoux, J.P. and VanHumbecq, L., Bronchodilator activity of a PGE₂ analog in animals and in man, *Prostaglandins*, 21 (1981) 457-469.

Bisgaard, H., Leukotrienes and prostaglandins in asthma, *Allergy*, 39 (1984) 413-420.

Bjarnason, I., Williams, P., Smethurst, P., Peters, T.J. and Levi, A.J., Effect of non-steroidal anti-inflammatory drugs and prostaglandins on the permeability of the human small intestine, *Gut*, 27 (1986) 1292-1297.

Bloch, E.H. and McCuskey, R.S., *The cardiovascular system*, in: *Histology*, 3rd edition, Greep, R.O., Weiss, L. (Eds.), McGraw-Hill, New York, (1973) 315-338.

Borne, R.F., *Nonsteroidal anti-inflammatory drugs*, in *Principles of Medicinal Chemistry*, 4th edition, Foye, W.O. (Ed.), Williams and Williams, (1995) 535-580.

Boulenc X., Marti E., Joyeux H., Roques C., Berger Y. and Fabre G., Importance of the paracellular pathway for the transport of a new bisphosphonate using the human Caco-2 monolayers model, *Biochem. Pharmacol.*, 46 (1993) 1591-1600.

Brandsch, M., Brandsch, C., Ganapathy, M.E., Chew, C.S., Ganapathy, V. and Leibach, F.H., Influence of proton and essential histidyl residues on the transport kinetics of the H⁺ / peptide cotransport systems in intestine (PEPT1) and kidney (PEPT2), *Biochim. Biophys. Acta.*, 1324 (1997) 251-262.

Brown, A.L.Jr., Microvilli of the human jejunal epithelial cell, *J. Cell Biol.*, 12 (1962) 623-627.

Bundgaard, H. and Nielsen, N.M., Glycolamide esters as a novel biolabile prodrug type for non-steroidal anti-inflammatory carboxylic acid drugs, *Int. J. Pharm.*, 43 (1988) 101-110.

Bundgaard, H., in: *Bioreversible Carriers in Drug Design*, Roche, E.B. (Ed.), Pergamon, New York, (1987) p13.

Bundgaard, H., in: *Design of Prodrugs*, Bundgaard, H. (Ed.), Elsevier, Amsterdam, (1985) p1.

Bundgaard, H., *Trends in design of prodrugs for improved drug delivery*, in: *Medicinal Chemistry for the 21st Century*, Wermuth, C.G., Koga, N., Konig, H. and Metcalf, B.W. (Eds), Blackwell Scientific Publications, (1992) 321-347.

Burne, K., Comparative pharmacology of non-opioid analgesics, *Med. Toxicol. Suppl.*, 1 (1986) 1-9.

Burton P.S., Cornadi R.A., Hilgren A.R. and Ho N.H.F., Evidence of a polarized efflux system for peptides in the apical membrane of Caco-2 cells, *Biochem. Biophys. Res. Commun.*, 190 (1993) 760-766.

Butler, J.N., in: *Ionic Equilibrium*, John Wiley & Sons, New York, (1998).

Buur, A., Trier, L., Magnusson, C. and Artursson, P., Permeability of 5-fluorouracil and prodrugs in Caco-2 cell monolayers, *Int. J. Pharm.*, 129 (1996) 223-231.

Caldwell, J., Hutt, A.J. and Fournel-Gigleux, S., The metabolic chiral inversion and dispositional enantioselectivity of the 2-arylpropionic acids and their biological consequences, *Biochem. Pharmacol.*, 37 (1988) 105-114.

Camenisch, G., Folkers, G. and van de Waterbeemd, H., Review of theoretical drug absorption models: historical background, recent developments and limitations, *Pharm. Acta. Helv.*, 71 (1996) 309-327.

- Campanero, M.A., Calahorra, B., García-Quétglas, E., López-Ocáriz, A. and Honorato, J., Rapid liquid chromatographic assay for the determination of acetaminophen in plasma after propacetamol administration: application to pharmacokinetic studies, *J. Pharm. Biomed. Analysis*, 20 (1999) 327-334.
- Cannon, G.W. and Breedveld, F.C., Efficacy of cyclooxygenase-2-specific inhibitors, *Am. J. Med.*, 110 (2001) 6S-12S.
- Carabaza, A., Cabre, F. and Rotilan, E., Stereoselective inhibition of inducible cyclooxygenase by chiral nonsteroidal antiinflammatory drugs, *J. Clin. Pharmacol.*, 36 (1996) 505-512.
- Chan, O.H. and Stewart, B.H., Physicochemical and drug-delivery considerations for oral drug bioavailability, *Drug Discov. Today*, 1 (1996) 461-473.
- Chang, E.B. and Rao, M.C., *Intestinal water and electrolyte transport: mechanisms of physiological and adaptive responses*, in: *Physiology of the Gastrointestinal Tract*, Johnson, L.R. (Ed.), Raven Press, New York, (1994) 2027-2081.
- Chao, C.C., Hu, S., Molitor, T.W., Shaskan, E.G. and Peterson, P.K., Activated microglia mediate neuronal cell injury via a nitric oxide mechanism, *J. Immun.*, 149 (1992) 2736-2741.
- Chhabra, R.S. and Eastin, Jr., W.C., *Intestinal absorption and metabolism of xenobiotics in laboratory animals*, in: *Intestinal Toxicology*, Schiller, C.M. (Ed.), Raven, New York, (1984) 145-160.
- Child, R.G., Osterberg, A.C., Sloboda, A.D. and Tomcufcik, A.S., Fenbufen, A new anti-inflammatory analgesic: synthesis and structure-activity relationships of analogs, *J. Pharm. Sci.*, 66 (1977) 466-476.
- Chiou W.L. and Kyle L.E., Differential thermal, solubility, and aging studies on various sources of digoxin and digitoxin powder: biopharmaceutical implications *J. Pharm. Sci.*, 68 (1979) 1224-1229.

Chiou, W., Determination of drug permeability in a flat or distended stirred intestine, *Int. J. Clin. Pharmacol. Ther.*, 32 (1994) 474-482.

Cioli, V., Putzolu, S., Rossi, V., Barcellona, P. and Corradino, C., The role of direct tissue contact in the production of gastrointestinal ulcers by anti-inflammatory drugs in rats, *Toxicol. Appl. Pharmacol.*, 50 (1979) 283-289.

Clark, D.E., Rapid calculation of polar molecular surface area and its application to the prediction of transport phenomena.1. Prediction of intestinal absorption, *J. Pharm. Sci.*, 88 (1999a) 807-814.

Clark, D.E., Rapid calculation of polar molecular surface area and its application to the prediction of transport phenomena.2. Prediction of blood-brain barrier penetration, *J. Pharm. Sci.*, 88 (1999b) 815-821.

Clarke, E.G.C. and Moffat, A.C., in: *Clarke's Isolation and Identification of Drugs in Pharmaceuticals, body fluids and post mortem material*, 2nd edition, Moffat, A.C., Jackson, J.V., Moss, M.S. and Widdop, B. (Eds.), Pharmaceutical Press, London, (1986).

Clive, D.M. and Stoff, J.S., Renal syndromes associated with nonsteroidal anti-inflammatory drugs, *New Eng. J. Med.*, 310 (1984) 563-572.

Crofford, L., Cox-1 and Cox-2 tissue expression: implications and predictions, *J. Rheumatol.*, 24 (Suppl. 49) (1997) 15-19.

Crowe, A. and Lemaire, M., In vitro and in situ absorption of SDZ-RAD using a human intestinal cell line (Caco-2) and a single pass perfusion model in rats: comparison with rapamycin, *Pharm. Res.*, 15 (1998) 1666-1671.

Cuzzolin, L., Adami, A., Degan, M., Crivellente, F., Bonapace, S., Minuz, P. and Benoni, G., Effect of single and repeated doses of a new nitroderivative of

acetylsalicylic acid on platelet TXA₂ production in rats, *Life Sci.*, 58 (1996) PL 207-210.

Dantzig, A.H. and Bergin, L., Uptake of cephalosporin, cephalexin, by a dipeptide transport carrier in human intestinal cell line, Caco-2, *Biochim. Biophys. Acta*, 1027 (1990) 211-217.

Daugherty, A.L. and Mersny, R.J., Transcellular uptake mechanisms of the intestinal epithelial barrier Part 1, *Pharm. Sci. Technol. Today*, 2 (1999) 144-151.

Davies, N.M., Wright, M.R. and Jamali, F., Anti-inflammatory drug-induced small intestinal permeability: the rat is a suitable model, *Pharm. Res.*, 11 (1994) 1652-1656.

Davies, N.M., Wright, M.R., Russell, A.S. and Jamali, F., Effect of the enantiomers of flurbiprofen, ibuprofen and ketoprofen on intestinal permeability, *J. Pharm. Sci.*, 85 (1996) 1170-1173.

Dawson, R.M.C., Elliott, D.C., Elliott, W.H. and Jones, K.M. (Eds.), in: *Data for Biochemical Research*, 3rd edition, Oxford Scientific Publications, (1986) 16-26.

De Caprariis, P., Palagiano, F., Bonina, F., Montenegro, L., D'Amico, M. and Rossi, F., Synthesis and pharmacological evaluation of oligoethylene ester derivatives as indomethacin oral prodrugs, *J. Pharm. Sci.*, 83 (1994) 1578-1581.

del Soltado, P., Cirino, G. and Wallace, J.L., *NO-NSAIDs; a novel class of anti-inflammatory drugs with reduced gastrointestinal and renal toxicity*, in: *Cell Injury and Protection in the Gastrointestinal Tract*, Moszik, L., Nagy, A.P. and Rainsford, K.D. (Eds.), Kluwer Academic Publishers, Dordrecht (1997) 25-32.

del Soltado, P., Nitric ester having anti-inflammatory and/or analgesic activity and process for their preparation, *United States Patent 5780495*, (1998).

Desai, K.J., *Biophysical model approach to the study of intestinal transport of drugs*, Ph.D. Thesis, University of Michigan, (1977).

- DeSesso, J.M. and Jacobson, C.F., Anatomical and physiological parameters affecting gastrointestinal absorption in humans and rats, *Food and Chemical Toxicology*, 39 (2001) 209-228.
- Döring, F., Will, J., Amasheh, S., Clauss, W., Ahlbrecht, H. and Daniel, H., Minimal molecular determinants of substrates for recognition by the intestinal peptide transporter, *J. Biol. Chem.*, 273 (1998) 23211-23218.
- Ermak, T.H., Giannasca, P.J., Microparticle targeting to M cells, *Adv. Drug Del. Rev.*, 34 (1998) 261-283.
- Ertl, P., Rohde, B. and Selzer, P., Fast calculation of molecular polar surface area directly from SMILES, www.daylight.com/meetings/emug00/Ertl/, (Accessed 19th May 2003).
- Fagerholm U. and Lennernas H., Experimental estimation of the effective unstirred water layer thickness in the human jejunum and its importance in oral drug absorption, *Eur. J. Pharm. Sci.*, 3 (1995) 247-253.
- Fagerholm, U., Johanssen, M. and Lennernas, H., Comparison between permeability coefficients in rat and human jejunum, *Pharm. Res.*, 13 (1996), 1336-1342.
- Feldman, M., Southwestern Internal Medicine Conference: Prostaglandins and gastric ulcers: from seminal vesicle to misoprostol (Cytotec), *Am. J. Med. Sci.*, 300 (1990) 116-132.
- Filipe, M.I., Mucins in the human gastrointestinal epithelium: a review, *Invest. Cell Pathol.*, 2 (1979) 195-216.
- Fini, A., Fazio, G. and Feroci, G., Solubility and solubilization of non-steroidal anti-inflammatory drugs, *Int. J. Pharm.*, 126 (1995) 95-102.

- Fini, A., Feroci, G. and Fazio, G., Interaction between indomethacin and heavy metal ions in aqueous solution, *Eur. J. Pharm. Sci.*, 13 (2001) 213-217.
- Fiorucci, S., Antonelli, E., Santucci, L., Morelli, O., Miglietti, M., Federici, B., Mannucci, R., Del Soltado, P. and Morelli, A., Gastrointestinal safety of nitro oxide-derived aspirin is related to inhibition of ICE-like cysteine proteases in rats, *Gastroenterology*, 116 (1999) 1089-1106.
- Fleisher, D., Bong, R. and Stewart, B.H., Improved oral drug delivery: solubility limitations overcome by the use of prodrugs, *Adv. Drug Del. Rev.*, 19 (1996) 115-130.
- Florence A. and Atwood D., in: *Physicochemical Principles of Pharmacy*, New York, Chapman and Hall (1988).
- Fogh J., Fogh J.M. and Orfeo T., 127 Cultured human colon cell lines producing tumors in nude mice, *J. Natl. Acad. Sci.*, 59 (1977) 221-226.
- Fojo, A.T., Ueda, K., Slamon, D.J., Poplack, D.G., Gottesman, M.M. and Pastan, I., Expression of a multidrug-resistance gene in human tumors and tissues, *Proc. Natl. Acad. Sci. USA*, 84 (1987) 265-269.
- Ford, J., Martin, S.W. and Houston, J.B., Assessment of intestinal permeability changes induced by nonsteroidal anti-inflammatory drugs in the rat, *J. Pharmacol. Toxicol. Methods*, 34 (1995) 9-16.
- Franks, N.P., Abraham, M.H. and Lieb, W.R., Molecular organisation of liquid n-octanol: An X-ray diffraction analysis, *J. Pharm. Sci.*, 82 (1993) 466-470.
- Franssen, E.J.F., Koiter, J., Kuipers, C.A.M., Bruins, A.P., Moolenaar, F., de Zeeuw, D., Kruizinga, W.H., Kellogg, R.M. and Meijer, D.K.F., Low molecular weight proteins as carriers for renal drug targeting. Preparation of drug-protein conjugates and drug-spacer derivatives and their catabolism in renal cortex homogenates and lysosomal lysates, *J. Med. Chem.*, 35 (1992) 1246-1259.

Fredduzzi, S., Mariucci, G., Tantucci, M., Del Soltado, P. and Ambrosini, M.V., Nitro-aspirin (NCX 4016) reduces brain damage induced by focal cerebral ischemia in the rat, *Neurosci. Lett.*, 302 (2001) 121-124.

Friend, D.R. and Pangburn, S., Site-specific drug delivery, *Med. Res. Rev.*, 7 (1987) 53-106.

Friis, G.J. and Bundgaard, H., *Design and applications of prodrugs*, in: *A Textbook of Drug Design and Development*, Krogsgaard-Larsen, P., Liljefors, T. and Madsen, U. (Eds.), Harwood Academic Publishers, (1996) 351-385.

Gabriel, S.E., Jaakkimainen, R. and Bombardier, C., Risk for serious gastrointestinal complications related to use of nonsteroidal anti-inflammatory drugs. A meta-analysis. *Ann. Intern. Med.*, 115 (1991) 787-796.

Galia, E., Nicolaides, E., Horter, D., Lobenberg, R., Reppas, C. and Dressman, J.B., Evaluation of various dissolution media for predicting in vivo performance of class I and II drugs, *Pharm. Res.*, 15 (1998) 698-705.

Gan L.S., Hsyu P.H., Pritchard J.F. and Thakker D., Mechanism of intestinal absorption of ranitidine and ondansetron: transport across Caco-2 cell monolayers, *Pharm. Res.*, 10 (1993) 1722-1725.

Gan L.S.L. and Thakker D.R., Applications of the Caco-2 model in the design and the development of orally active drugs: elucidation of biochemical and physical barriers posed by the intestinal epithelium, *Adv. Drug Del. Rev.*, 23 (1997) 77-98.

Ganapathy, M.E., Brandsch, M., Prasad, P.D., Ganapathy, V. and Liebach, F.H., Differential recognition of β -lactam antibiotics by intestinal and renal peptide transporters, PEPT1 and PEPT2, *J. Biol. Chem.*, 270 (1995) 25672-25677.

Ganapathy, V. and Liebach, F.H., Carrier-mediated reabsorption of small peptides in renal proximal tubule, *Am. J. Physiol.*, 251 (1986) F945-F953.

Ganapathy, V. and Liebach, F.H., Peptide transport in the intestinal and renal brush border membrane vesicles, *Life Sci.*, 30 (1982) 2137-2146.

Ganong W.F., in: *Review of Medical physiology*, 17th edition, Appleton and Lange (Eds.), Norwalk, Connecticut, (1995) 1-47, 448-477.

Garner, A., Adaptation in the pharmaceutical industry, with particular reference to gastrointestinal drugs and diseases, *Scand. J. Gastroenterol.*, 27 (Suppl. 193) (1992) 83.

Garvey, D.S., Letts, L.G., Renfroe, H.B. and Tam, S.W., Nitroso esters of beta-oxo-amides and aryl propionic acid derivatives of non-steroidal anti-inflammatory drugs, *United States Patent 6043232*, (2000).

Geiss, G.S., Hubbard, R.C. and Callison, D.A., Safety and efficacy of celecoxib, a specific COX-2 inhibitor, *Rheumatol. Eur.*, 27 (suppl. 1) (1998) 118.

Ghose, T.I. and Blair, A.H., The design of cytotoxic-agent-antibody conjugates, *CRC Critical Reviews in Therapeutic Drug Carrier Systems*, 3 (1987) 262-361.

Ghosh, M.K. and Mitra, A.K., Effects of 5'-ester modification on the physicochemical properties and plasma protein binding of 5-iodo-2'-deoxyuridine, *Pharm. Res.*, 8 (1991) 771-775.

Gonzalez, D.E., Covitz, K.-M. Y., Sadée, W. and Mrsny, R.J., An oligopeptide transporter is expressed at high levels in the pancreatic carcinoma cell lines AsPc-1 and Capan-2, *Cancer Res.*, 58 (1998) 519-525.

Goosen, C., du Pleiss, J., Müller, D.G. and Janse van Rensburg, L.F., Correlation between physicochemical characteristics, pharmacokinetic properties and transdermal absorption of NSAIDs, *Int. J. Pharm.*, 163 (1998) 203-209.

- Gottesman, M.M. and Pastan, I., Biochemistry of multidrug resistance mediated by the multidrug transporter, *Annu. Rev. Biochem.*, 62 (1993) 385-427.
- Granger, D.N., Barrownam, J.A. and Kvietys, P.R., in: *Clinical Gastrointestinal Physiology*, W.B. Saunders, Philadelphia (1985).
- Gretzer, B., Ehrlich, K., Maricic, N., Lambrecht, N., Respondek, M. and Peskar, B.M., Selective cyclooxygenase-2 inhibitors and their influence on the protective effect of a mild irritant in the rat stomach, *Br. J. Pharmacol.*, 123 (1998) 927-935.
- Grisham, M.B., Jourdain, D. and Wink, A., Nitric oxide I. Physiological chemistry of nitric oxide and its metabolites: implications in inflammation, *Am. J. Physiol.*, 276 (1999) G315-321.
- Grisham, M.B., Specian, R.D. and Zimmerman, T.E., Effects of nitric oxide synthase inhibition on the pathophysiology observed in a model of chronic granulomatous colitis, *J. Pharmacol. Exp. Ther.*, 271 (1994) 1114-1121.
- Gruber P., Longer M. and Robinson J., Some biological issues in oral controlled drug delivery, *Adv. Drug. Del. Rev.*, 1 (1987) 1-18.
- Ha, H.R., Follath, F., Bloemhard, Y. and Krähenbühl, S., Determination of saquinavir in human plasma by high-performance liquid chromatography, *J. Chromatogr. B*, 694 (1997) 427-433.
- Hadgraft, J., du Pleiss, J. and Goosen, C., The selection of non-steroidal anti-inflammatory agents for dermal delivery, *Int. J. Pharm.*, 207 (2000) 31-37.
- Halliday, G., Robinson, S.R., Shepherd, C. and Kril, J., Alzheimer's disease and inflammation: a review of cellular and therapeutic mechanisms, *Clin. Exp. Pharmacol. Physiol.*, 27 (2000) 1-8.

Halter, F., Rainsford, K.D., Sirko, S.P. and Schmassmann, A., NSAID-induced mucosal injury: analysis of gastric toxicity of new generation NSAIDs: ulcerogenicity compared with ulcer healing, *Yale J. Biol. Med.*, 70 (1997) 33-43.

Hamor, G.H., in *Principles of Medicinal Chemistry*, 3rd edition, Foye, W.O. (Ed.), Philadelphia, Lea and Febiger, (1989) 503.

Hanahan, D. and Folkman, J., Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis, *Cell*, 86 (1996) 353-364.

Hanby, A.M., Poulsom, R. and Elia, G., The expression of the trefoil peptide pS2 and human spasmolytic polypeptide (hSP) in gastric metaplasia of the proximal duodenum: implications for the nature of gastric metaplasia, *J. Pathol.*, 169 (1993) 355-360.

Hanlon, M., *Personal communication*, (2002).

Hardy, J.G., Wilson, C.G. and Wood, E., Drug delivery to the proximal colon, *J. Pharm. Pharmacol.*, 37 (1985) 874-877.

Harper, N.J., Drug Latentiation, *J. Med. Pharm. Chem.*, 1 (1959) 467-500.

Harris, R.C., McKanna, J.A., Akai, Y., Jacobson, H.R., Dubois, R.N. and Breyer, M.D., Cyclooxygenase-2 is associated with the macula densa of rat kidney and increases with salt restriction, *J. Clin. Invest.*, 93 (1994) 2504-2510.

Healy, A.M. and Corrigan, O.I., Predicting the dissolution rate of ibuprofen-acidic excipient compressed mixtures in reactive media, *Int. J. Pharm.*, 84 (1992) 167-173.

Helliwell, M., The use of bioadhesives in targeted delivery within the gastrointestinal tract, *Adv. Drug Del. Rev.*, 11 (1993) 221-251.

Henry, D., Lim, L.L., Rodriguez, G.L.A., Gutthann, P.S., Carson, J.L., Griffin, M., Savage, R., Logan, R., Moride, Y., Hawkey, C., Hill, S.L. and Fries, J.T., Variability

in risk of gastrointestinal complications with individual non-steroidal anti-inflammatory drugs: results of a collaborative meta-analysis, *Br. Med. J.*, 312 (1996) 1563-1566.

Hidalgo, I.J., Bhatnagar, P., Lee, C.-P., Miller, J., Cucullino, G. and Smith, P.L., Structural requirements for interaction with the oligopeptide transporter in Caco-2 cells, *Pharm. Res.*, 12 (1995) 317-319.

Hidalgo, I.J., Ryan, F.M., Marks, G.J. and Smith, P.L., pH-dependent transepithelial transport of cephalexin in rabbit intestinal mucosa, *Int. J. Pharm.*, 98 (1993) 83-92.

Higuchi W.L., Parrott D., Wurster D. and Higuchi T., Investigation of drug release from solids II: Theoretical and experimental study of influences of bases and buffers on rates of dissolution of acidic solids, *J. Am. Pharm. Assoc.*, 47 (1958) 376-383.

Ho N.F.H., Park J.Y., Ni P.F. and Higuchi W.L., in: *Animal models for oral drug delivery in man*, Crouthamel, W. and Sarapu, A.C. (Eds.), Am. Pharm. Assoc. Washington DC, (1983) 27-106.

Hoensch, H.P. and Schwenk, M., *Intestinal absorption and metabolism of xenobiotics in humans*, in: *Intestinal Toxicology*, Schiller, C.M. (Ed.), Raven, New York, (1984) 169-192.

Hogben C.A.M., Tocco D.J., Brodie B.B. and Schanker L.S., On the mechanism of intestinal absorption of drugs, *J. Pharmacol. Exp. Ther.*, 125 (1959) 275-282.

Hovgaard L. and Bronstead H., Drug delivery studies in Caco-2 monolayers IV: Absorption enhance effects of cyclodextrins, *Pharm. Res.*, 12 (1995) 1328-1332.

Hu, M., Zheng, L., Chen, J., Liu, L., Zhu, J., Dantzig, A.H. and Stratford, Jr.R.E., Mechanism of transport of quinapril in Caco-2 cell monolayers: comparison with cephalexin, *Pharm. Res.*, 12 (1995) 1120-1125.

- Hui, Y., Kolars, J., Hu, Z. and Fleisher, D., Intestinal clearance of H₂-antagonists, *Biochem. Pharmacol.*, 48 (1994) 229-231.
- Hung, Q.Q., Harvey, C.M., Paterson, A.R.P., Cass, C.E. and Young, J.D., Functional expression of Na⁺-dependent nucleoside transport system of rat intestine in isolated oocytes of *Xenopus laevis*, *J. Biol. Chem.*, 268 (1993) 20613-20619.
- Hunter J., Hirst B.H. and Simmons N.L., Drug absorption limited by P-glycoprotein-mediated secretory drug transport in human intestinal epithelial Caco-2 cell layers, *Pharm. Res.*, 10 (1993) 743-749.
- Ikuma, M., Hanai, H., Kaneko, E., Hayashi, H. and Hoshi, T., Effects of aging on the microclimate pH of the rat jejunum, *Biochim. Biophys. Acta*, 1280 (1996) 19-26.
- Inagaki, M., Tsuru, T., Jyoyama, H., Ono, T., Yamada, K., Kobayashi, M., Hori, Y., Arimura, A., Yasui, K., Ohno, K., Kakudo, S., Koizami, K., Suzuki, R., Kato, M., Kawai, S. and Matsumoto, S., Novel antiarthritic agents with 1,2-isothiazolidine-1,1-dioxide (γ -sultam) skeleton: phytokine suppressive dual inhibitors of cyclooxygenase-2 and 5-lipoxygenase, *J. Med. Chem.*, 43 (2000) 2040-2048.
- Ingram, M.J., Moynihan, H.A. and Rostron, C., Synthesis and nitration of ibuprofen 1-monoglyceride, *J. Pharm. Pharmacol.*, 50 (1998) Suppl. 237.
- Ingram, M.J., Moynihan, H.A., Powell, M.W. and Rostron, C., Synthesis and hydrolytic behaviour of glycerol-1,2-diibuprofenate-3-nitrate, a putative pro-drug of ibuprofen and glycerol-1-nitrate, *J. Pharm. Pharmacol.*, 53 (2001) 345-350.
- Inui K.I., Yamamoto M. and Saitoh H., Transepithelial transport of oral cephalosporine by monolayers of intestinal epithelial cell line Caco-2: specific transport systems in apical and basolateral membranes, *J. Pharmacol. Exp. Ther.*, 261 (1992) 195-201.

- Itoh, T., Maruyama, J., Tsuda, Y. and Yamada, H., Stereoselective pharmacokinetics of ibuprofen in rats: effect of enantiomer-enantiomer interaction in plasma protein binding, *Chirality*, 9 (1997) 354-361.
- Iwanaga, K., Ono, S., Narioka, K., Kakemi, M., Morimoto, K., Yamashita, S., Namba, Y. and Oku, N., Application of surface coated liposomes for oral delivery of peptide: Effects of coating the liposomes surface on the GI transit of insulin, *J. Pharm. Sci.*, 88 (1999) 248-252.
- Iwatsubo, T., Yamazaki, M., Sugiyama, Y., Suzuk, H., Yanai, S., Chool Kim, D., Satoh, H., Miyamoto, Y., Iga, T. and Hanano, M., Epidermal growth factor as a regulatory hormone maintaining a low pH microclimate in the rat small intestine, *J. Pharm. Sci.*, 78 (1989) 457-459.
- Jain, N. and Yalkowsky, S.H., Estimation of the aqueous solubility I: Application to organic nonelectrolytes, *J. Pharm. Sci.*, 90 (2001) 234-252.
- Jakšić, P., Mlinaric-Majerski, K., Zorc, B. and Dumić, M., Macromolecular prodrugs. VI. Kinetic study of poly($[\alpha, \beta$ -(N-2-hydroxyethyl-DL-aspartamide)])-ketoprofen hydrolysis, *Int. J. Pharm.*, 135 (1996) 177-182.
- Jeffrey, P., Tucker, G.T., Bye, A., Crewe, H.K. and Wright, P.A., The site of inversion of R(-)-ibuprofen: studies using rat in-situ isolated perfused intestine/liver preparations, *J. Pharm. Pharmacol.*, 43 (1991) 715-720.
- Jodal, M. and Lundgren, O., Countercurrent mechanisms in the mamallian gastrointestinal tract, *Gastroenterology*, 91 (1986) 225-241.
- Johnson, P.M. and Rainsford, K.D., The physical properties of mucins: preliminary observations on the sedimentation behaviour of porcine gastric mucin, *Biochim. Biophys. Acta*, 286 (1972) 72-78.
- Kaiser, D.G., Vangiessen, G.J., Reischer, R.J. and Wechter, W.J., Isomeric inversion of ibuprofen R-enantiomer in humans, *J. Pharm. Sci.*, 65 (1976) 269-273.

- Kaminsky, L.S. and Fasco, M.J., Small intestinal cytochromes P450, *Crit. Rev. Toxicol.*, 21 (1992) 407-422.
- Katori, M. and Majima, M., Cyclooxygenase-2: its rich diversity of roles and possible application of its selective inhibitors, *Inflamm. Res.*, 49 (2000) 367-392.
- Kaza, C.S., Kashfi, K. and Rigas, B., Colon cancer prevention with NO-releasing NSAIDs, *Prostaglandins and other Lipid Mediators*, 67 (2002) 107-120.
- Kedem, O. and Katchalsky, A., Thermodynamics of flow processes in biological systems, *Biophys. J. Suppl.*, (1958) 53-78.
- Kelder, J., Grootenhuis, P.D.J., Bayada, D.M., Delbressine, L.P.C. and Ploemen, J.P., Polar molecular surface as a dominating determinant for oral absorption and brain penetration of drugs, *Pharm. Res.*, 16 (1999) 1514-1519.
- Kelly, J.P., Kaufman, D.W., Jurgelon, J.M., Sheehan, J., Koff, R.S. and Shapiro, S., Risk of aspirin-associated major upper-gastrointestinal bleeding with enteric-coated or buffered product, *Lancet*, 348 (1996) 1413-1416.
- Kim, J.S., Oberle, R.L., Krummel, D.A., Dressman, J.B. and Fleisher, D., Absorption of ACE inhibitors from small intestine and colon, *J. Pharm. Sci.*, 83 (1994) 1350-1356.
- Kim, R.B., Transporters and xenobiotic disposition, *Toxicology*, 181-182 (2002) 291-297.
- Kitazawa, S., Ito, H. and Sezaki, H., Transmucosal fluid movement and its effect on drug absorption, *Chem. Pharm. Bull.*, 23 (1975) 1856-1865.
- Kitazawa, S., Ito, H., Johno, I., Takahashi, T. and Takenada, H., Generality in effects of transmucosal fluid movement and glucose on drug absorption from the rat small intestine, *Chem. Pharm. Bull.*, 26 (1978) 915-924.

Klaassen, C.D. and Watkins, III J.B., Mechanisms of bile formation, hepatic uptake, and biliary excretion, *Pharmacol. Rev.*, 36 (1984) 1-67.

Knowles, R.J. and Moncada, S., Nitric oxide synthases in mammals, *Biochem. J.*, 298 (1994) 249-258.

Komiya, I., Park, J.Y., Kamani, A., Ho, N.H.F. and Higuchi, W.I., Quantitative mechanistic studies in simultaneous fluid flow and intestinal absorption using steroids as model solutes, *Int. J. Pharm.*, 4 (1980) 249-262.

Krämer S.D., Absorption prediction from physicochemical parameters, *Pharm. Sci. Technol. Today*, 12 (1999) 373-380.

Krugliak, P., Hollander, D., Ma, T.Y., Tran, D., Dadafulza, V.D. and Katz, K.D., Mechanisms of polyethylene glycol 400 permeability of perfused rat intestine, *Gastroenterology*, 97 (1989) 1164-1170.

Kuel, F.A., Daugherty, H.W. and Ham, E.A., Interaction between prostaglandins and leukotrienes, *Biochem. Pharmacol.*, 33 (1984) 1-5.

Kurumbail, R.G., Stevens, A.M., Gierse, J.K., McDonald, J.J., Stegeman, R.A., Pak, J.Y., Gildehaus, D., Miyashiro, J.M., Penning, T.D., Seibert, K., Isakson, P. and Stallings, W.C., Structural basis for selective inhibition of cyclooxygenase-2 by anti-inflammatory agents, *Nature*, 384 (1996) 644-648.

Lalande, M., Wilson, D.L. and McGilveray, I.J., Rapid high-performance liquid chromatographic determination of ibuprofen in human plasma, *J. Chromatogr. B*, 337 (1986) 410-414.

Lane, M.E., *Personal communication*, (2002).

Lane, M.E., Hanlon, M., Levis, K.A., Gilmer, J.F., Landowski, C.P., Corrigan, O.I. and Amidon, G.L., Synthesis and characterization of amino acid derivatives of

ibuprofen, *Proceed. Cont. Rel. Soc. 30th Annual Meeting and Exposition*, In Press, (2003).

Lane, M.E., O'Driscoll, C.M. and Corrigan, O.I., The relationship between rat intestinal permeability and hydrophilic probe size, *Pharm. Res.*, 13 (10) (1996) 1554-1558.

Lanza, F.L., Royer, G.L.Jr. and Nelson, R.S., Endoscopic evaluation of the effects of aspirin, buffered aspirin, and enteric-coated aspirin on gastric and duodenal mucosa, *N. Eng. J. Med.*, 303 (1980) 136-138.

Larsen, C. and Johansen, M., Dextran as carriers for drug compounds – realized and potential applications, *Arch. Pharm. Chemi.*, 92 (1985) 809-830.

Larsen, C., Macromolecular prodrugs. XII. Kinetics of release of naproxen from various polysaccharide ester prodrugs in neutral and alkaline solution, *Int. J. Pharm.*, 51 (1989) 233-240.

Lauterbach, F., Intestinal permeation of nonquaternary amines: a study with telenzepine and pirenzepine in the isolated mucosa of guinea pig jejunum and colon, *J. Pharmacol. Exp. Ther.*, 243 (1987) 1121-1130.

Le Corre, P., Dollo, G., Chevanne, F. and Le Verge, R., Influence of hydroxypropyl- β -cyclodextrin and dimethyl- β -cyclodextrin on diphenhydramine intestinal absorption in a rat in situ model, *Int. J. Pharm.*, 169 (1998) 221-228.

Leahy, D.E., Lynch, J. and Taylor, D.C., *Mechanisms of absorption of small molecules*, in: *Novel Drug Delivery and its Therapeutic Application*, Prescott, L.F. and Nimmo, W.S. (Eds.), Wiley, New York, (1989) 33-44.

Lee C.-O., de Vruet R.L.A. and Smith P.L., Selection of development candidates based on in vitro permeability measurements, *Adv. Drug Del. Rev.*, 23 (1997) 47-62.

Lefer, A.M. and Lefer, D.J., Nitric oxide II. Nitric oxide protects in intestinal inflammation, *Am. J. Physiol.*, 276 (1999) G572-575.

Legen, I., Žakelj, S. and Kristl, A., Polarised transport of monocarboxylic acid type drugs across rat jejunum in vitro: the effect of mucolysis and ATP-depletion, *Int. J. Pharm.*, 7419 (2003) 1-6.

Lennernas H., Gastrointestinal absorption mechanisms: a comparison between animal and human models, *Eur. J. Pharm. Sci.*, 2 (1994) 39-43.

Lennernas, H., Does fluid flow across the intestinal mucosa affect quantitative oral drug absorption? Is it time for a reevaluation?, *Pharm. Res.*, 12 (1995) 1573-1582.

Leone-Bay, A., Leipold, H., Sarubbi, D., Variano, B., Rivera, T. and Baughman, R.A., Oral delivery of sodium cromolyn: preliminary studies in vivo and in vitro, *Pharm. Res.*, 13 (1996) 222-226.

Levitt, M.D., Aufderheide, T., Fetzer, C.A., Bond, J.H. and Levitt, D.G., Use of carbon monoxide to measure luminal stirring in the rat gut, *J. Clin. Invest.*, 74 (1984) 2056-2064.

Levitt, M.D., Fetzer, C.A., Kneip, J.M., Dond, J.H. and Levitt, D.G., Quantitative assessment of luminal stirring in the perfused small intestine of the rat, *Am. J. Physiol.*, 252 (1987) G325-332.

Levitt, M.D., Furne, J.K. and Levitt, D.G., Shaking of the intact rat and intestinal angulation diminish the jejunal unstirred layer, *Am. J. Gastroenterol.*, 103 (1992b) 1460-1466.

Levitt, M.D., Furne, J.K., Strocchi, A., Andersson, B.W. and Levitt D.G., Physiological measurements of luminal stirring in the dog and human small bowel, *J. Clin. Invest.*, 86 (1990) 1540-1547.

Levitt, M.D., Strocchi, A. and Levitt, D.G., Human jejunal unstirred layer: evidence for extremely efficient luminal stirring, *Am. J. Physiol.*, 262 (1992a) G593-596.

Lewis, R.A., Austen, K.F. and Soberman, R.J., Leukotrienes and other products of the 5-lipoxygenase pathway. Biochemistry and relation to pathobiology in human diseases, *New Eng. J. Med.*, 323 (1990) 645-655.

Liang, R., Fei, Y.J., Prasad, P.D., Ramamoorthy, S., Han, H., Yang-Feng, T.L., Hediger, M.A., Ganapathy, V. and Leibach, F.H., Human intestinal H⁺ / peptide cotransporter: Cloning, functional expression and chromosomal localization, *J. Biol. Chem.*, 270 (1995) 6456-6463.

Lichtenberger, L.M., Ulloa, C. and Romero, J.J., Nonsteroidal anti-inflammatory drug and phospholipid prodrugs: combination therapy with antisecretory agents in rats, *Gastroenterology*, 111 (1996) 990-995.

Lichtenberger, L.M., Wang, Z.-M. and Romero, J.J., Nonsteroidal anti-inflammatory drugs (NSAIDs) associate with zwitterionic phospholipids: insight into the mechanism and reversal of NSAID-induced gastrointestinal injury, *Natl. Med.*, 1 (1995) 154-158.

Lichtenberger, L.M., Where is the evidence that cyclooxygenase inhibition is the primary cause of nonsteroidal anti-inflammatory drug (NSAID)-induced gastrointestinal injury? Topical injury revisited, *Biochem. Pharmacol.*, 61 (2001) 631-637.

Lipsky, P.E., The clinical potential of cyclooxygenase-2-specific inhibitors, *Am. J. Med.*, 106 (5B) (1999) 51S-57S.

Lister, N., Bailey, P.D., Collier, I.D., Boyd, C.A.R. and Bronk, J.R., The influence of luminal pH on transport of neutral and charged dipeptides by rat small intestine, in vitro, *Biochim. Biophys. Acta*, 1324 (1997) 245-250.

Liversidge, G.G. and Conzentino, P., Drug particle size reduction for decreasing gastric irritancy and enhancing absorption of naproxen in rats, *Int. J. Pharm.*, 125 (1995) 309-313.

Lockwood, G.F. and Wagner, J.G., High-performance liquid chromatographic determination of ibuprofen and its major metabolites in biological fluids, *J. Chromatogr.*, 231 (1982) 335-343.

Longer, M.A., Ch'ng, H.S. and Robinson, J.R., Bioadhesive polymers as platforms for oral controlled drug delivery. III. Oral delivery of chlorothiazide using a bioadhesive polymer, *J. Pharm. Sci.*, 74 (1985) 406-411.

Lu H-H., Thomas J., Tukker J. and Fleisher D., Intestinal water and solute absorption studies: comparison of in situ perfusion with chronic isolated loops in rats, *Pharm. Res.*, 9 (1992) 894-900.

Lucas, M.L. and Blair, J.A., The magnitude and the distribution of the acid microclimate in proximal jejunum and its relation to luminal acidification, *Proc. Royal Society A*, 200 (1978) 27-41.

Lucas, M.L., Lei, F.H. and Blair, J.A., The influence of buffer pH, glucose and sodium ion concentration on the acid microclimate in rat proximal jejunum *in vitro*, *Pflügers Archiv*, 385 (1980) 137-142.

Lucas, M.L., Shineider, W., Haberich, F.J. and Blair, J.A., Direct measurement by pH-microelectrode of the pH microclimate in rat proximal jejunum, *Proc. Royal Society B*, 192 (1975) 39-48.

MacDonald, T.M., Morant, S.V., Robinson, G.C., Shield, M.J., McGilchrist, M.M., Murray, F.E. and McDevitt, D.G., Association of upper gastrointestinal toxicity of non-steroidal anti-inflammatory drugs with continued exposure: cohort study, *Br. Med. J.*, 315 (1997) 1333-1337.

Macheras, P., Reppas, C. and Dressman, J.B., *Uptake of drug by the gastrointestinal mucosa*, in: *Biopharmaceutics of Orally Administered Drugs*, Rubinstein, M.H. (Ed.), Ellis Horwood, UK, (1995) 125-151.

Madara J.L. and Pappenheimer J.R., Structural basis for physiological regulation of paracellular pathways in intestinal epithelia, *J. Membr. Biol.*, 100 (1987) 149-164.

Mahfouz, N.M., Omar, F.A. and Aboul-Fadl, T., Cyclic amide derivatives as potential prodrugs II. N-hydroxymethylsuccinimide-/isatin esters of some NSAIDs as prodrugs with an improved therapeutic index, *Eur. J. Med. Chem.*, 34 (1999) 551-562.

Mangan, F.R., *Nabumetone*, in *Non-Steroidal Anti-Inflammatory Drugs. Mechanisms and Clinical Use*, Lewis, A.J., Furst, D.E. (Eds.), Marcel Dekker, New York, (1987) 439.

Martin, Y.C., A practitioner's perspective of the role of quantitative structure-activity analysis in medicinal chemistry, *J. Med. Chem.*, 24 (1981) 229-237.

Martindale, 27th edition, Wade, A. (Ed.), The Pharmaceutical Press, London, (1977).

McNamara, D. and Amidon, G., Reaction plane approach for estimating the effects of buffers on the dissolution rate of acidic drugs, *J. Pharm. Sci.*, 77 (6) (1988) 511-517.

Miller, M.J., Sadowska-Krowicka, H., Chotinaruemol, S., Kakkis, J.L. and Clark, D.A., Amelioration of chronic ileitis by nitric oxide synthase inhibition, *J. Pharmacol. Exp. Ther.*, 264 (1993) 11-16.

Minuz, P., Lechi, C., Tommasoli, R., Gaino, S., Degan, M., Zuliani, V., Bonapace, S., Benoni, G., Adami, A., Cuzzolin, L. and Lechi, A., Antiaggregating and vasodilatory effects of a new nitro derivative of acetylsalicylic acid, *Thromb. Res.*, 80 (1995) 367-376.

Mitchell, J.A., Akarasereenont, P. and Thiemermann, C., Selectivity of nonsteroidal anti-inflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase, *Proc. Natl. Acad. Sci. USA*, 90 (1993) 11693-11697.

Momi, S., Emerson, M., Paul, W., Leone, M., Mezzasoma, A.M., Del Soltado, P., Page, C.P. and Gresele, P., Prevention of pulmonary thromboembolism by NCX 4016, a nitric oxide-releasing aspirin, *Eur. J. Pharmacol.*, 397 (2000) 177-185.

Mooney, K.G., Mintun, M.A., Hemmelstein, K.J. and Stella, V.J., Dissolution kinetics of Carboxylic Acids II: Effect of Buffers. *J. Pharm. Sci.*, 70 (1981) 22-32.

Morham, S.G., Langenbach, R., Loftin, C.D., Tiano, H.F., Vouloumanos, N., Jenette, J.C., Mahler, J.F., Kluckman, K.D., Ledford, A. and Lee, C.A., Prostaglandin synthase 2 gene description causes severe renal pathology in the mouse, *Cell*, 83 (1995) 473-482.

Moseley, R.H., Smit, H., van Solkema, B.G.H., Wang, W. and Meijer, D.K.F., Mechanisms for the hepatic uptake and biliary excretion of tributylmethylammonium: studies with rat liver plasma membrane vesicles, *J. Pharmacol. Exp. Ther.*, 276 (1996) 561-567.

Mukherjee, D., Nissen, S.E. and Topol, E.J., Risk of cardiovascular events associated with selective COX-2 inhibitors, *JAMA*, 286 (2001) 954-959.

Naruhashi, K., Sai, Y., Tamai, I., Suzuki, N., Tsuji, A., PepT1 mRNA expression is induced by starvation and its level correlates with absorptive transport of cefadroxil longitudinally in the rat intestine, *Pharm. Res.*, 19 (2002) 1417-1423.

Navia, M.A. and Chaturvedi, P.R., Design principles for orally bioavailable drugs, *Drug Discov. Today*, 1 (1996) 179-189.

Needleman, P. and Isakson, P.C., The discovery and function of Cox-2, *J. Rheumatol.*, 24 (Suppl. 49) (1997) 6-8.

Ni P.F., Ho N.H.F., Fox J.L., Leuenberger H. and Higuchi W.I., Theoretical model studies of intestinal drug absorption v. non-steady-state fluid flow and absorption, *Int. J. Pharm.*, 5 (1980) 33-47.

Nielsen, N.M. and Bundgaard, H., Evaluation of glycolamide esters and various other esters of aspirin as true aspirin prodrugs, *J. Med. Chem.*, 32 (1989) 727.

Niibuchi, J.-J., Aramaki, Y. and Tsuchiya, S., Binding of antibiotics to rat intestinal mucin, *Int. J. Pharm.*, 30 (1986) 181-187.

Nilsson, D., Fagerholm, U. and Lennernas, H., The influence of net water absorption on the permeability of antipyrine and levodopa in the human jejunum, *Pharm. Res.*, 11 (1994) 1541-1545.

Nunn, B. and Chamberlain, P.D., effect of nabumetone (BRL 14777), a new antiinflammatory drug, on human platelet reactivity ex vivo: comparison with naproxen, *J. Pharm. Pharmacol.*, 34 (1982) 576-579.

Oates, M.D., Rosbottom, A.C. and Schrager, J., Further investigations into the structure of human gastric mucin: the structural configuration of the oligosaccharide chains, *Carbohydrate Res.*, 34 (1974) 115-137.

Oberle, R.L., Moore, T.J. and Krummel, D.A.P., Evaluation of mucosal damage of surfactants in rat jejunum and colon, *J. Pharmacol. Toxicol. Methods*, 33 (1995) 75-81.

Ogihara, H., Suzuki, T., Nagamachi, Y., Inui, K. and Takata, K., Pepide transporter in the rat small intestine: ultrastructural localization and the effect of starvation and administration of amino acids, *Histochem. J.*, 31 (1999) 169-174.

Oguri, S., Kumazaki, M., Kitou, R., Nonoyama, H. and Tooda, N., Elucidation of intestinal absorption of D,L-amino acid enantiomers and aging in rats, *Biochim. Biophys. Acta – General Subjects*, 1472 (1999) 107-114.

- Ohmukai, O., Lipo-NSAID preparation, *Adv. Drug Del. Rev.*, 20 (1996) 203-207.
- Oikawa, T., Kusuhara, M., Ishikawa, S., Hitomi, J., Kono, A., Iwanaga, T. and Yamaguchi, K., Production of endothelin-1 and thrombomodulin by human pancreatic cancer cells, *Br. J. Cancer*, 69 (1994) 1059-1064.
- Omar, F.A., Cyclic amide derivatives as potential prodrugs. Synthesis and evaluation of N-hydroxymethylphthalimide esters of some non-steroidal anti-inflammatory carboxylic acid drugs, *Eur. J. Med. Chem.*, 33 (1998) 123-131.
- Osiecka, I., Porter, P.A., Borchardt, R.T., Fix, J.A. and Gardner, C.R., In vitro drug absorption models. I. Brush-border membrane vesicles, isolated mucosal cells and everted intestinal rings: characterisation and salicylate accumulation, *Pharm. Res.*, 2 (1985) 284-293.
- Ott, R.J., Hui, A.C., Yaun, G. and Giacomini, K.M., Organic cation transport in human renal brush-border membrane vesicles, *Am. J. Physiol.*, 261 (1991) F443-F451.
- Ozturk S., Palsson B. and Dressman J., Dissolution of ionisable drugs in buffered and unbuffered solutions, *Pharm. Res.*, 5(5) (1988) 272-282.
- Pade, V. and Stavchansky, S., Estimation of the relative contribution of the transcellular and paracellular pathway to the transport of passively absorbed drugs in the Caco-2 cell culture model, *Pharm. Res.*, 14 (1997) 1210-1215.
- Paino, T.C. and Moore, A.D., Determination of the LOD and LOQ of an HPLC method using four different techniques, *Pharmaceutical Technology*, October (1999) 86-90.
- Pairat, M., van Ryn, J. and Mauz, A., *Differential inhibition of COX-1 and COX-2 by NSAIDs: a summary of results obtained using various test systems*, in *Selective COX-2 Inhibitors: Pharmacology, Clinical Effects and Therapeutic Potential*, Vane, J., Botting, J. (Eds.), Kluwer Academic Publishers (1998) 27-46.

Palm, K., Stenberg, P., Luthman, K. and Artursson, P., Polar molecular surface properties predict the intestinal absorption of drugs in humans, *Pharm. Res.*, 14 (1997) 568-571.

Palmer, R.M.J., Rees, D.D., Ashton, D.S. and Moncada, S., L-arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation, *Biomed. Biophys. Res. Commun.*, 153 (1988) 1251-1256.

Pappenheimer, J.R. and Reiss, K.Z., Contribution of solvent drag through intercellular junctions to absorption of nutrients by the small intestine of the rat, *J. Membr. Biol.*, 100 (1987) 123-136.

Park G-B. and Mitra A.K., Mechanism and site dependency of intestinal mucosal transport and metabolism of thymidine analogues, *Pharm. Res.*, 9 (1992) 326-331.

Parrott, K.A. and Christensen, M., Influence of cimetidine on the disposition of ibuprofen in the rat, *Res. Commun. Chem. Pathol. Pharmacol.*, 43 (1984) 369-380.

Payne, J.W., Payne, G.M., Gupta, S., Marshall, N.J. and Grail, B.M., Conformational limitations of glycylsarcosine as a prototypic substrate for peptide transporters, *Biochim. Biophys. Acta*, 1514 (2001) 65-75.

Peréz, M.A.C., Díaz, H.G., Teruel, C.F., Plá-Delfina, J. and Sanz, M.B., A novel approach to determining physicochemical and absorption properties of 6-fluoroquinolone derivatives: experimental assessment, *Eur. J. Pharm. Biopharm.*, 53 (2002) 317-325.

Persico, F.J., Pritchard, J.F., Fisher, M.C., Yorgey, K., Wong, S. and Carson, J., Effect of tolmetin glycineamide (McN-4366), a prodrug of tolmetin sodium, on adjuvant arthritis in the rat, *J. Pharmacol. Exp. Ther.*, 247 (1988) 889-896.

Pharmaceutical Codex, 12th edition, Lund, W. (Ed.), Pharmaceutical Press, London, (1994).

Pharmaceutical Handbook, 19th edition, Wade, A. (Ed.), The Pharmaceutical Press, London, (1980).

Picot, D., Loll, P.J. and Garavito, M., The X-ray crystal structure of the membrane protein prostaglandin H₂ synthase-1, *Nature*, 367 (1994) 243-249.

Pipili-Synetos, E., Papageorgiou, A., Sakkoula, E., Sotiropoulou, G., Fotsis, T., Karakiulakis, G. and Maragoudakis, M.E., Inhibition of angiogenesis, tumor growth and metastasis by the NO-releasing vasodilators, isosorbide mononitrate and dinitrate, *Br. J. Pharmacol.*, 116 (1995) 1829-1834.

Poelma, F.G.J., Tukker, J.T. and Crommelin, D.J.A., The role of bile salts in the intestinal absorption of drugs, *Acta. Pharm. Technol.*, 36 (1990) 43-52.

Powell D.W., Barrier function of epithelia, *Am. J. Physiol.*, 241 (1981) G275-88.

Powell D.W., *Intestinal water and electrolyte transport*, in: *Physiology of the gastrointestinal tract*, Johnson L.R. (Ed.), Raven Press, New York, (1987) 1267-1305.

Prasad, K., Shah, V.P., Knight, P., Malinowski, H., Cabana, B.E. and Meyer, M.C., Importance of Media Selection in establishment of in Vitro-in Vivo relationships for Quinidine Gluconate, *Int. J. Pharm.*, 13 (1983) 1-8.

Qiao, L., Rigas, B. and Knudson, C.M., Nitric oxide-releasing sulindac inhibits pancreatic cancer cell growth and induces their apoptosis, *Gastroenterology*, 120 (2001) Abstract 3606.

Rainsford, K.D. and Quadir, M., Gastrointestinal damage and bleeding from non-steroidal anti-inflammatory drugs. I. Clinical and epidemiological aspects, *Inflammopharmacol.*, 3 (1995) 169-190.

Rainsford, K.D. and Whitehouse, M.W., Biochemical gastroprotection from acute ulceration induced by aspirin and related drugs, *Biochem. Pharmacol.*, 29 (1980) 1281-1289.

Rainsford, K.D. and Whitehouse, M.W., Gastric irritancy of aspirin and its congeners: anti-inflammatory activity without this side-effect, *J. Pharm. Pharmacol.*, 28 (1976) 599-610.

Rainsford, K.D., *History and development of ibuprofen*, in *Ibuprofen: a critical bibliographic review*, Rainsford, K.D. (Ed.), Taylor and Francis, London, (1999) 1-24.

Rainsford, K.D., The ever-emerging anti-inflammatories. Have there been any real advances?, *J. Physiol. – Paris*, 95 (2001) 11-19.

Ramtoola, Z. and Corrigan, O.I., Influence of the Buffering Capacity of the Medium on the Dissolution of Drug-Excipient Mixtures, *Drug Dev. Ind. Pharm.*, 15 (1989) 2359-74.

Raoof, A.A., Butler, J. and Devane, J.G., Assessment of regional differences in intestinal fluid movement in the rat using a modified *in situ* single pass perfusion model, *Pharm. Res.*, 15 (8) (1998) 1314-1316.

Raoof, A.A., *Personal communication*, (2001).

Rathbone, M.J. and Hadgraft, J., Absorption of drugs from the human oral cavity, *Int. J. Pharm.*, 74 (1991) 9-24.

Raub, T.J., Barsuhn, C.L., Williams, L.R., Deckers, D.E., Sawada, G.A. and Ho, N.H.F., Use of a biophysical-kinetic model to understand the roles of protein binding and membrane partitioning on passive diffusion of highly lipophilic molecules across cellular barriers, *J. Drug Targeting*, 1 (1993) 269-286.

Reddy, B.S., Hirose, Y., Lubet, R., Steele, V., Kelloff, G., Paulson, S., Seibert, K. and Rao, C.V., Chemoprevention of colon cancer by specific cyclooxygenase-2 inhibitor, celecoxib, administered during different stages of carcinogenesis, *Cancer Res.*, 60 (2000)293-297.

Reuss, L., Tight junction permeability to ions and water, In: Tight Junctions, Ed., Cereijido, M., Boca Raton, CRC Press, (1991) 49-66.

Reynolds, W.F., Polar Substituent Effects, *Prog. Phys. Org. Chem.*, 14 (1983) 165-203.

Rossoni, G., Berti, M., Colonna, V.D., Bernareggi, M., Del Soltado, P. and Berti, F., Myocardial protection by the nitroderivative of aspirin, NCX 4016: in vitro and in vivo experiments in the rabbit, *Italian Heart J.*, 1 (2000) 146-155.

Roth, G.J. and Calverley, D.C., Aspirin, platelets and thrombosis: theory and practice, *Blood*, 83 (1994) 885-898.

Rozman, K.K. and Klaassen, C.D., *Absorption, distribution, and excretion of toxicants*, in: *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 5th edition, Klaassen, C.D. (Ed.), McGraw-Hill, New York, (1996) 91-112.

Rubas W. and Cromwell M.E.M., The effect of chemical modifications on octanol/water partition (Log D) and permeabilities across Caco-2 monolayers, *Adv. Drug Del. Rev.*, 23 (1997) 157-162.

Saavedra, J.E., Booth, M.N., Hrabie, J.A., Davies, K.M. and Keefer, L.K., Piperazine as a linker for incorporating the nitric-oxide releasing diazeniumdiolate group into other biomedically relevant functional molecules, *J. Org. Chem.*, 64 (1999) 5124-5131.

Samuelsson, B., Dahlen, S.-E. and Lindgren, J.A., Leukotrienes and lipoxins: structures, biosynthesis, and biological effects, *Science*, 237 (1987) 1171-1176.

- Sandoval, M., Liu, X., Mannick, E.E., Clark, D.A. and Miller, M.J.S., Peroxynitrite-induced apoptosis in human intestinal epithelial cells is attenuated by mesalamine, *Gastroenterology*, 113 (1997) 1480-1488.
- Sattari, S. and Jamali, F., Evidence of absorption rate dependency of ibuprofen inversion in the rat, *Chirality*, 6 (1994) 435-439.
- Satterwhite, J.H. and Boudinot, F.D., Effects of age and dose on the pharmacokinetics of ibuprofen in the rat, *Drug Metab. Disp.*, 19 (1991) 61-67.
- Satterwhite, J.H. and Boudinot, F.D., Effects of age on the pharmacodynamics of naproxen in the rat, *Exp. Gerontology*, 30 (1995) 505-515.
- Satterwhite, J.H. and Boudinot, F.D., Pharmacokinetics of ketoprofen in rats: effect of age and dose, *Biopharm. Drug Disp.*, 13 (1992) 197-212.
- Scherrer, R.A. and Howard, S.M., Use of distribution coefficients in quantitative structure-activity relationships, *J. Med. Chem.*, 20 (1977) 53-58.
- Schiff, M.H. and Whelton, A., Renal toxicity associated with disease-modifying antirheumatic drugs used for the treatment of rheumatoid arthritis, *Seminars in Arthritis and Rheumatism*, 30 (2000) 196-208.
- Scientist Handbook, Micromath Scientific Software, Utah, USA, (1995).
- Shah, A. and Jung, D., Dose-dependent pharmacokinetics of ibuprofen in the rat, *Drug Metab. Disp.*, 15 (2) (1987) 151-154.
- Shanbhag, R., Crider, A.M., Gokhale, R., Harpalani, A. and Dick, M., Ester and amide prodrugs of ibuprofen and naproxen: synthesis, anti-inflammatory activity, and gastrointestinal toxicity, *J. Pharm. Sci.*, 81 (1992) 149-154.
- Sheha, M., Khedr, A. and Elsherief, H., Biological and metabolic study of naproxen-propyphenazone mutual prodrug, *Eur. J. Pharm. Sci.*, 17 (2002) 121-130.

Shen, T.Y. and Winter, C.A., Chemical and biological studies on indomethacin, sulindac and their analogs, *Adv. Drug Res.*, 12 (1977) 89-295.

Shen, T.Y., Ellis, R.L., Windholz, T.B., Matzuk, A.R., Rosegay, A., Lucas, S., Witzel, B.E., Stammer, C.H., Wilson, A.N., Holly, F.W., Willett, J.D., Sareti, L.H., Holtz, W.J., Risley, E.A., Nuss, G.W. and Winter, C.A., Non-steroidal anti-inflammatory agents, *J. Am. Chem. Soc.*, 85 (1963) 488-489.

Shiau, Y.F., Fernandez, P., Jackson, M.J. and MacMonagle, S., Mechanisms maintaining a low-pH microclimate in the intestine, *Am. J. Physiol.*, 248 (1985) G608-617.

Shimada, T., Factors affecting the microclimate pH in rat jejunum, *J. Physiol. (Lond.)*, 392 (1987) 113-127.

Sietsema, W.K., The absolute oral bioavailability of selected drugs, *Int. J. Clin. Pharmacol. Ther. Toxicol.*, 27 (1989) 179-211.

Sigma Plot, www.statsol.ie/sigmaplot/equations.htm, (Accessed May 2003)

Silverman, R.B., *Prodrugs and Drug Delivery Systems*, in: *The Organic Chemistry of Drug Design and Drug Action*, Silverman, R.B. (Ed.), Academic Press, San Diego, (1992) 352-401.

Sinkula, A.A. and Yalkowsky, S.H., Rationale for design of biologically reversible drug derivatives: prodrugs, *J. Pharm. Sci.*, 64 (1975) 181-210.

Sinkula, A.A., Morozowich, W. and Rowe, E.L., Chemical Modification of Clindamycin: Synthesis and evaluation of selected esters, *J. Pharm. Sci.*, 62 (1973) 1106-1111.

Skelly, J.P., Yau, M.K., Elkins, J.S., Yamamoto, L.A., Shah, V.P. and Barr, W.H., In Vitro Topographical Characterisation as a Predictor of in Vivo Controlled Release Quinidine Gluconate Bioavailability, *Drug Dev. Ind. Pharm.*, 12 (1986) 1177-1201.

- Smith P.L., Eddy E.P., Lee C.-P. and Wilson G., Exploitation of the intestinal oligopeptide transporter to enhance drug absorption, *Drug Deliv.*, 1 (1993) 103-111.
- Snyder, W.S., Cook, M.J., Nasset, E.S., Karhausen, L.R., Howells, G.P. and Tipton, I.H. (Eds.), in: *Report of the Task Group on Reference Man*, Pergamon, New York, (1975).
- Staggers, J.E., Hernell, O., Stafford, R.J. and Carey, M.C., Physical-chemical behaviour of dietary and biliary lipids during intestinal digestion and absorption. 1. Phase behaviour and aggregation states of model lipid systems patterned after aqueous duodenal contents of healthy adult human beings, *Biochemistry*, 29 (1990) 2028-2040.
- Steffansen, B., Lepist, E.-I., Taub, M.E., Larsen, B.D., Frokjaer, S. and Lennernäs, H., Stability, metabolism and transport of D-Asp(Obzl)-Ala-a model prodrug with affinity for the oligopeptide transporter, *Eur. J. Pharm. Sci.*, 8 (1999) 67-73.
- Steinman R.M., Brodie S.E. and Cohn Z.A., Membrane flow during pinocytosis. A stereologic analysis, *J. Cell Biol.*, 68 (1976) 665-687.
- Stewart, B.H., Chan, H.O., Jezyk, N. and Fleisher, D., Discrimination between drug candidates using models for evaluation of intestinal absorption, *Adv. Drug Del. Rev.*, 23 (1997) 27-45.
- Stratman, N.C., Carter, D.B. and Sethy, V.H., Ibuprofen: effect on inducible nitric oxide synthase, *Molecular Brain Research*, 50 (1997) 107-112.
- Sutton, S.C. and Rinaldi, M.T.S., Comparison of the gravimetric, phenol red, and ^{14}C -PEG-3350 methods to determine water absorption in the rat single-pass intestinal perfusion model, *AAPS Pharm. Sci.*, 3 (2001) Article 25.
- Swenson E.C. and Curatolo W.J., Intestinal permeability enhancement for proteins, peptides and other polar drugs: mechanisms and potential toxicity, *Adv. Drug Del. Rev.*, 8 (1992) 39-92.

Swenson, E.S., Milisen, W.B. and Curatolo, W., Intestinal permeability enhancement: efficacy, acute local toxicity and reversibility, *Pharm. Res.*, 11 (1994) 1132-1142.

Takanaga, H., Tamai, I. and Tsuji, A., pH-dependent and carrier-mediated transport of salicylic acid across Caco-2 cells, *J. Pharm. Pharmacol.*, 46 (1994) 567-570.

Tamai, I., Takanaga, H., Maeda, H., Sal, Y., Ogihara, T., Higashida, H. and Tsuji, A., Participation of a proton-cotransporter, MCT1, in the intestinal transport of monocarboxylic acids, *Biochem. Biophys. Res. Commun.*, 214 (1995) 482-489.

Tammara, V.J., Narurkar, M.M., Crider, A.M. and Khan, M.A., Morpholinoalkyl ester prodrugs of diclofenac: synthesis, in vitro, and in vivo evaluation, *J. Pharm. Sci.*, 83 (1994) 644-648.

Tammara, V.J., Narurkar, M.M., Crider, A.M. and Khan, M.A., Synthesis and evaluation of morpholinoalkyl ester prodrugs of indomethacin and naproxen, *Pharm. Res.*, 10 (1993) 1191-1199.

Tanaka, H., Miyamoto, K.I., Morita, K., Haga, H., Segawa, H., Shiraga, T., Fujioka, A., Kouda, T., Taketani, Y., Hisano, S., Kukui, Y., Kitagawa, K. and Takeda, E., Regulation of the PepT1 peptide transporter in the rat small intestine in response to 5-fluorouracil induced injury, *Gastroenterology*, 114 (1998) 714-723.

Taylor, D.C., Pownall, R. and Burke, W., The absorption of β -adrenoceptor antagonists in rat in-situ small intestine; the effect of lipophilicity, *J. Pharm. Pharmacol.*, 37 (1985) 280-283.

Temple, C.S., Stewart, A.K., Meredith, D., Lister, N.A., Morgan, K.M., Collier, I.D., Vaughan-Jones, R.D., Boyd, C.A.R., Bailey, P.D. and Bronk, J.R., Peptide mimics as substrates for the intestinal peptide transporter, *J. Biol. Chem.*, 273 (1998) 20-22.

Testa, B. and Caldwell, J., Prodrugs revisited: the ad hoc approach as a complement to ligand design, *Med. Res. Rev.*, 16 (1996) 233-241.

Testa, B. and Seiler, P., Steric and lipophilic components of the hydrophobic fragmental constant, *Arzneim. Forsch. (Drug Res.)*, 31 (1981) 1053-1058.

Testa, B., Drug Metabolism, in: *Burger's Medicinal Chemistry and Drug Discovery*, Vol. 1, 5th edition, Wolff, M.E. (Ed.), Wiley-Interscience, New York, (1995) 129-180.

Thomas, G., *Drug Metabolism*, in: *Medicinal Chemistry- an introduction*, Thomas, G. (Ed.), John Wiley and Sons, England, (2000) 327-374.

Thomas, J., Straus, W.L. and Bloom, B.S., Over-the-counter nonsteroidal anti-inflammatory drugs and risk of gastrointestinal symptoms, *Am. J. Gastroenterol.*, 97 (2002) 2215-2219.

Thomas, N.W., Palin, K.J. and Waller, D.A., The application of an in situ loop technique to the study of rectal absorption, *Int. J. Pharm.*, 20 (1984) 163-169.

Thomson A.B.R. and Dietschy J.M., *The role of the unstirred water layer in intestinal permeation*, in: *Pharmacology of intestinal permeation II*, Csaky, T.Z. (Ed.), Springer, Berlin, (1984) Ch.21.

Thomson, A.B. and Dietschy, J.M., Derivation of the kinetics that describe the effects of unstirred water layers on the kinetic parameters of active transport processes in the intestine, *J. Theor. Biol.*, 64 (1977) 277-294.

Tocco, G., Freire-Moar, J. and Schreiber, S.S., *Exp. Neurol.*, 144 (1997) 339-349.

Trier J.S., The surface coat of gastrointestinal epithelial cells, *Gastroenterology*, 56 (1969) 618-622.

Tripathi, S. and Boulpaep, E.L., Mechanisms of water transport by epithelial cells, *Quarterly J. Exp. Physiol.*, 74 (1989) 385-417.

Tsuji A. and Tamai I., Carrier-mediated intestinal transport of drugs, *Pharm. Res.*, 13 (1996) 963-977.

Tsuji, A., Takanaga, H., Tamai, I. and Terasaki, T., Transcellular transport mechanism of benzoic acid across Caco-2 cells by a pH-dependent and carrier-mediated transport mechanism, *Pharm. Res.*, 11 (1994) 30-37.

Tsuji, A., Tissue selective drug delivery utilizing carrier-mediated transport systems, *J. Cont. Rel.*, 62 (1999) 239-244.

Tsunematsu, H., Yoshida, S., Horie, K. and Yamamoto, M., Synthesis and stereoselective enzymatic hydrolysis of flurbiprofen-basic amino acid ethyl esters, *J. Drug Targetting*, 2 (1995) 517-525.

Uhing M.R. and Kimura R.E., The effect of surgical bowel manipulation and anaesthesia on intestinal glucose absorption in rats, *J. Clin. Invest.*, 95 (1995) 2790-2798.

Ussing H.H. and Zerahn K., Active transport of sodium as the source of electric current in the short-circuited frog skin, *Acta Physiol. Scand.*, 23 (1951) 110-127.

Van de Waterbeemd, H. and Testa, B., Parametrization of lipophilicity and other structural properties in drug design, *Adv. Drug Res.*, 16 (1987) 85-225.

Van Os, C.H., Deen, P.M.T. and Dempster, J.A., Aquaporins: water selective channels in biological membranes. Molecular structure and tissue distribution, *Biochim. Biophys. Acta*, 1197 (1994) 291-309.

Van Slyke, D.D., On the measurement of buffer values and on the relationship of buffer value to the dissociation constant of the buffer and the concentration and reaction of the buffer solution, *J. Biol. Chem.*, 52 (1922) 525-536.

Vane, J., Towards a better aspirin, *Nature*, 367 (1994) 215-216.

Vane, J.R. and Botting, R.M., in *Aspirin and other salicylates*, Chapman and Hall Medical, London, (1992) 3-16.

Vane, J.R., Flower, R.J. and Botting, R.M., History of aspirin, *Stroke*, 21 (Suppl. 4) (1990) 12-23.

Vane, J.R., Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs, *Nature*, 231 (1971) 232-235.

Vane, J.R., Mitchell, J.A. and Appleton, I., Inducible isoforms of cyclooxygenase and nitric-oxide synthase in inflammation, *Proc. Natl. Acad. Sci. USA*, 91 (1994) 2046-2050.

Vijaylakshmi, D. and Belt, J.A., Sodium-dependent nucleoside transport in mouse intestinal epithelial cells, *J. Biol. Chem.*, 263 (1988) 19419-19423.

Wallace, J.L., Cooperative modulation of gastrointestinal mucosal defence by prostaglandins and nitric oxide, *Clin. Invest. Med.*, 19 (1996) 346-351.

Wallace, J.L., Distribution and expression of cyclooxygenase (COX) isoenzymes, their physiological roles, and the categorisation of nonsteroidal anti-inflammatory drugs (NSAIDs), *Am. J. Med.*, 107 (1999) 11S-17S.

Wallace, J.L., Muscara, M.N., McKnight, W., Dicay, M., Del Soltado, P. and Cirino, G., In vivo antithrombotic effects of nitric-oxide releasing aspirin derivative, NCX-4016, *Thromb. Res.*, 93 (1999) 43-50.

Wallace, J.L., Reuter, B., Cicala, C., McKnight, W., Grishan, M.B. and Cirino, G., Novel nonsteroidal anti-inflammatory drug derivatives with markedly reduced ulcerogenic properties in the rat, *Gastroenterology*, 107 (1994) 173-179.

Walter, E., Kissel, T. and Amidon, G.L., The intestinal peptide carrier: a potential transport system for small peptide derived drugs, *Adv. Drug Del. Rev.*, 20 (1996) 33-58.

Warwick, R. and Williams, P.L., in: *Gray's Anatomy*, 35th edition, W.B. Saunders, Philadelphia, (1973).

Weiss, L., *Lymphatic vessels and lymph nodes*, in: *Histology*, 3rd edition, Greep, R.O., Weiss, L. (Eds.), McGraw-Hill, New York, (1973) 423-444.

Wermuth, C.G., Gagnault, J.C. and Marchandeu, C., *Designing prodrugs and bioprecursors. I. Carrier prodrugs*, in: *The Practice of Medicinal Chemistry*, Wermuth, C.G. (Ed.), Academic Press, (1996) 671-696.

Wermuth, C.G., in: *Drug Metabolism and Drug Design: Quo Vadis?*, Briot, M., Cautreels, W., Roncucci, R. (Eds.), Sanofi-Clin-Midy, Montpellier, France, (1983) 253.

Whitehouse, M.W. and Rainsford, K.D., Esterification of acidic anti-inflammatory drugs suppresses their gastrototoxicity without adversely affecting their anti-inflammatory activity in rats, *J. Pharm. Pharmacol.*, 32 (1980) 795-796.

Wikman-Larhed, A., Artursson, P., Grasjo, J. and Bjork, E., Diffusion of drugs in native and purified gastrointestinal mucus, *J. Pharm. Sci.*, 86 (1997) 660-665.

Williams, J.L., Simona, B., Hasan, I., Castillo, E., Traganos, F. and Rigas, B., Nitric oxide-releasing nonsteroidal anti-inflammatory drugs (NSAIDs) alter the kinetics of human colon cancer cell lines more effectively than traditional NSAIDs: implications for colon cancer chemoprevention, *Cancer Res.*, 61 (2001) 3285-3289.

Williams, T.C. and Jarvis, S.M., Multiple sodium-dependent nucleoside transport systems in bovine renal brush-border membrane vesicles, *Biochem. J.*, 274 (1991) 27-33.

Wils, P., Warnery, A., Phung-Ba, V., Legrain, S. and Scherman, D., High lipophilicity decreases drug transport across intestinal epithelial cells, *J. Pharmacol. Exp. Ther.*, 269 (1994) 654-658.

- Wilson T.H. and Wiseman G., The use of sacs of everted small intestine for the study of the transference of substances from the mucosal to the serosal surface, *J. Physiol.*, 123 (1954) 116-125.
- Wink, D.A., Vodovotz, Y., Laval, J., Laval, F., Dewhirst, M.W. and Mitchell, J.B., The multifaceted roles of nitric oxide in cancer, *Carcinogenesis*, 19 (1998) 711-721.
- Winne D., Closed rat jejunal segment in situ: role of pre-epithelial diffusion resistance (unstirred layer) in the absorption process and model analysis, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 335 (1987) 204-215.
- Winne D., Rat jejunum perfused in situ: effect of perfusion rate and intraluminal radius on absorption rate and effective unstirred layer thickness. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 307 (1979) 265-274.
- Winne D., *Unstirred layer as a diffusion barrier in vitro and in vivo*, in: *Intestinal Absorption and Secretion*, Skadhauge, E. and Heintze, K. (Eds.), MTP Press, Lancaster, (1984) 21-38.
- Xiang, J., Fang, X. and Li, X., Transbuccal delivery of 2',3'-dideoxycytidine: in vitro permeation study and histological investigation, *Int. J. Pharm.*, 231 (2002) 57-66.
- Xiang, T.X. and Andersson, B.D., The relationship between permeant size and permeability in lipid bilayer membranes, *J. Membr. Biol.*, 140 (1994) 111-122.
- Xie, W., Chipman, J.G. and Robertson, D.L., Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing, *Proc. Natl. Acad. Sci. USA*, 96 (1999) 7563-7568.
- Yamagata, K., Andreasson, K.I. and Kaufmann, W.E., Expression of a mitogen-inducible cyclooxygenase in brain neurons: regulation by synaptic activity and glucocorticoids, *Neuron*, 1192 (1993) 371-386.

Yuasa H., Matsuda K. and Watanabe J., The influence of anaesthetic regimens on intestinal absorption in rats, *Pharm. Res.*, 10 (1993) 884-888.

Yuen, K.H., Deshmukh, A.A., Newton, J.M., Short, M. and Melchor, R., Gastrointestinal transit and absorption of theophylline from a multiparticulate controlled release formulation, *Int. J. Pharm.*, 97 (1993) 61-77.

Zhu, T., Chen, X.Z., Steel, A., Hediger, M.A. and Smith, D.E., Differential recognition of ACE inhibitors in *Xenopus laevis* oocytes expressing rat PepT1 and PepT2, *Pharm. Res.*, 17 (2000) 523-532.

Zovko, M., Zorc, B., Lovrek, M. and Boneschans, B., Macromolecular prodrugs. IX. Synthesis of polymer-fenoprofen conjugates, *Int. J. Pharm.*, 228 (2001) 129-138.

APPENDICES

APPENDIX 1

Mathematical model to describe the variation in buffer capacity with pH when a monoprotic acid is used as the buffer species.

Independent Variable: PH

Dependent Variable: BETA

Parameters: PKHA, ATOT

$$KHA = 10^{(-PKHA)}$$

$$KW = 10^{(-14)}$$

$$H = 10^{(-PH)}$$

$$OH = KW/H$$

$$HAA = H/KHA$$

$$FH = 1 + HAA$$

$$A = ATOT/FH$$

$$HA = HAA * A$$

$$PBAL = H - OH + HA$$

$$BETA = -DERIV(PBAL, PH)$$

APPENDIX 2

Mathematical model to describe the variation in buffer capacity with pH when a triprotic acid is used as the buffer species.

Independent Variable: PH

Dependent Variable: BETA

Parameters: PKHA, PKH2A, PKH3A, ATOT

```

KHA = 10^(-PKHA)
KH2A = 10^(-PKH2A)
KH3A = 10^(-PKH3A)
KW = 10^(-14)
H = 10^(-PH)
OH = KW/H
HAA = H/KHA
H2AA = H*HAA/KH2A
H3AA = H*H2AA/KH3A
FH = 1+HAA+H2AA+H3AA
A = ATOT/FH
HA = HAA*A
H2A = H2AA*A
H3A = H3AA*A
PBAL = H-OH+3*H3A+2*H2A+HA
BETA = -DERIV(PBAL, PH)
***

```


APPENDIX 3

Mathematical model to describe the variation in buffer capacity with pH when a diprotic and a triprotic acid are used as the buffer species.

Independent Variables: PH

Dependent Variables: BETA

Parameters: PKHA, PKH2A, PKH3A, PKHB, PKH2B, ATOT, BTOT

```

KHA = 10^(-PKHA)
KH2A = 10^(-PKH2A)
KH3A = 10^(-PKH3A)
KHB = 10^(-PKHB)
KH2B = 10^(-PKH2B)
KW = 10^(-14)
H = 10^(-PH)
OH = KW/H
HAA = H/KHA
H2AA = H*HAA/KH2A
H3AA = H*H2AA/KH3A
HBB = H/KHB
H2BB = H*HBB/KH2B
FHA = 1+HAA+H2AA+H3AA
FHB = 1+HBB+H2BB
A = ATOT/FHA
B = BTOT/FHB
HA = HAA*A
H2A = H2AA*A
H3A = H3AA*A
HB = HBB*B
H2B = H2BB*B
PBAL = H-OH+3*H3A+2*H2A+HA+HB+2*H2B
BETA = -DERIV(PBAL, PH)
***

```

APPENDIX 4

Mathematical model to describe the variation in buffer capacity with pH when two triprotic acids are used as the buffer species.

Independent Variables: PH

Dependent Variables: BETA

Parameters: PKHA, PKH2A, PKH3A, PKHB, PKH2B, PKH3B, ATOT, BTOT

$$KHA = 10^{(-PKHA)}$$

$$KH2A = 10^{(-PKH2A)}$$

$$KH3A = 10^{(-PKH3A)}$$

$$KHB = 10^{(-PKHB)}$$

$$KH2B = 10^{(-PKH2B)}$$

$$KH3B = 10^{(-PKH3B)}$$

$$KW = 10^{(-14)}$$

$$H = 10^{(-PH)}$$

$$OH = KW/H$$

$$HAA = H/KHA$$

$$H2AA = H*HAA/KH2A$$

$$H3AA = H*H2AA/KH3A$$

$$HBB = H/KHB$$

$$H2BB = H*HBB/KH2B$$

$$H3BB = H*H2BB/KH3B$$

$$FHA = 1 + HAA + H2AA + H3AA$$

$$FHB = 1 + HBB + H2BB + H3BB$$

$$A = ATOT/FHA$$

$$B = BTOT/FHB$$

$$HA = HAA*A$$

$$H2A = H2AA*A$$

$$H3A = H3AA*A$$

$$HB = HBB*B$$

$$H2B = H2BB*B$$

$$H3B = H3BB*B$$

$$PBAL = H - OH + 3*H3A + 2*H2A + HA + 3*H3B + 2*H2B + HB$$

$$BETA = -DERIV(PBAL, PH)$$

APPENDIX 5

Two compartment pharmacokinetic model with constant input and first order output.

Independent Variables: TIME

Dependent Variables: CONCENTRATION

Parameters: KELIM K12 K21 DOSE VOLUME TIV

$CP0 = DOSE/VOLUME$

$TSTAR = UNIT(TIME-TIV)*(TIME-TIV)$

$BB = -(K12+K21+KELIM)$

$ROOT1 = 0.5*(-BB+SQRT(BB*BB-4*K21*K12))$

$ROOT2 = 0.5*(-BB-SQRT(BB*BB-4*K21*K12))$

$ALPHA = UNIT(ROOT1-ROOT2)*ROOT1+UNIT(ROOT2-ROOT1)*ROOT2$

$BETA = UNIT(ROOT1-ROOT2)*ROOT2+UNIT(ROOT2-ROOT1)*ROOT1$

$A = CP0/TIV*(ALPHA-K21)/(ALPHA-BETA)/ALPHA$

$B = (-CP0)/TIV*(BETA-K21)/(ALPHA-BETA)/BETA$

$TERM1 = A*(EXP((-ALPHA)*TSTAR)-EXP((-ALPHA)*TIME))$

$TERM2 = B*(EXP((-BETA)*TSTAR)-EXP((-BETA)*TIME))$

$CONCENTRATION = TERM1+TERM2$

$AUC = DOSE/VOLUME/KELIM$

$KELIMHALF = 0.693/KELIM$

$ALPHAHALF = 0.693/ALPHA$

$BETAHALF = 0.693/BETA$

$CONCMAX = A*(1-EXP((-ALPHA)*TIV))+B*(1-EXP((-BETA)*TIV))$

APPENDIX 6

Non-steady-state model to describe the gastrointestinal absorption of drugs.

Independent Variables: T

Dependent Variables: F

Parameters: DE, Q, R, PE, X

$$A=DE$$

$$B=Q/(3.1416 \cdot R^2)$$

$$G=2 \cdot PE/R$$

$$E=B^2/(4 \cdot A)+G$$

$$W=X \cdot \text{SQRT}(E/A)$$

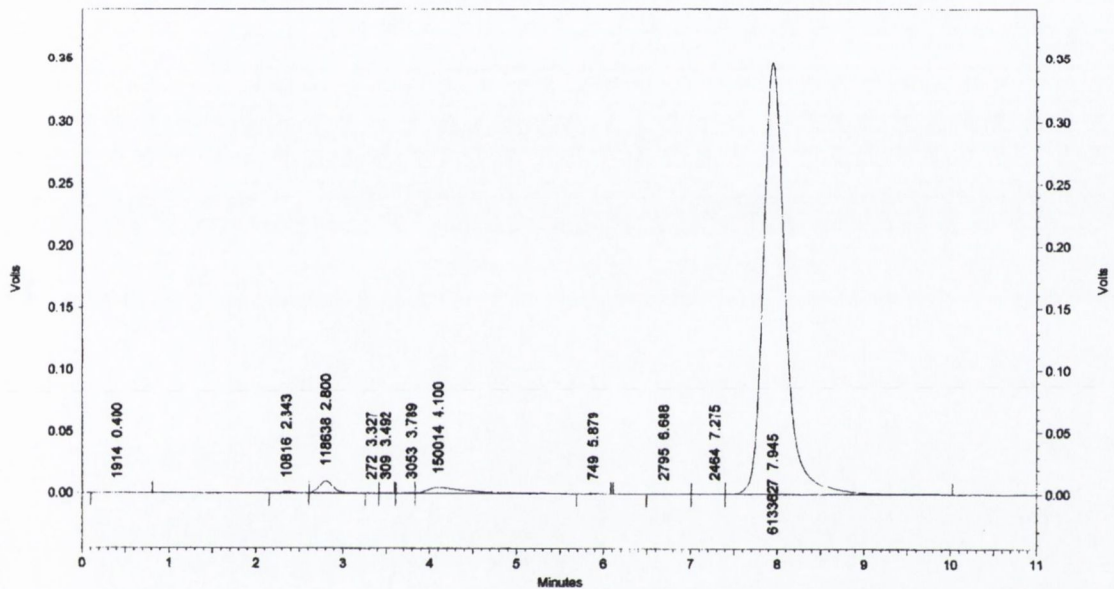
$$Y=X/(2 \cdot \text{SQRT}(A \cdot T))$$

$$Z=\text{SQRT}(E \cdot T)$$

$$F=100 \cdot \text{EXP}(B \cdot X/(2 \cdot A))/2 \cdot (\text{EXP}(-W) \cdot \text{ERFC}(Y-Z)+\text{EXP}(W) \cdot \text{ERFC}(Y+Z))$$

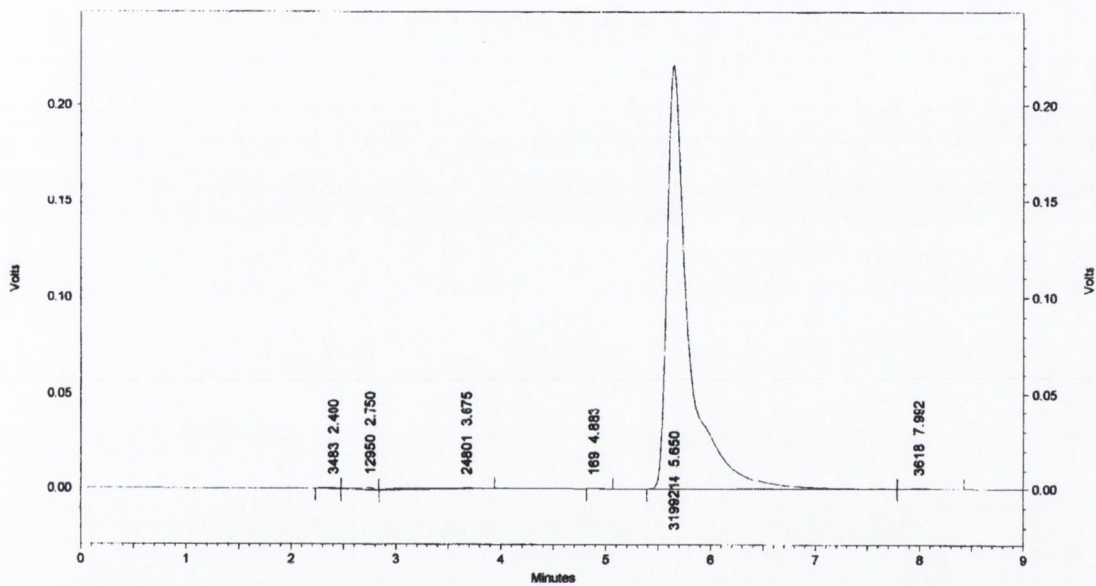
APPENDIX 7

HPLC trace of ibuprofen showing the principal ibuprofen peak with a retention time of 7.95 minutes.



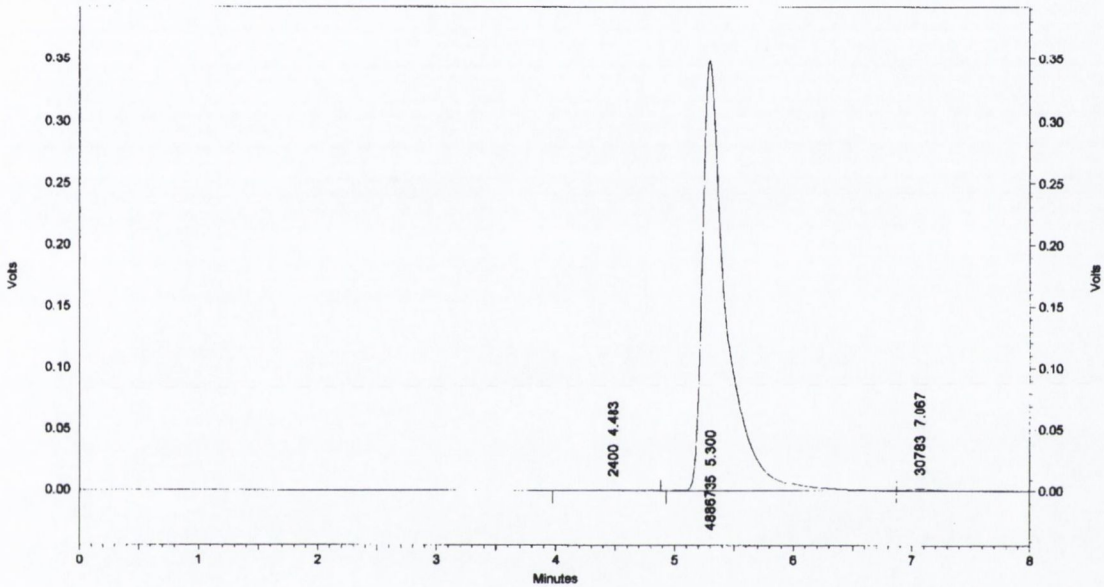
APPENDIX 8

HPLC trace of naproxen showing the principal naproxen peak with a retention time of 5.65 minutes.



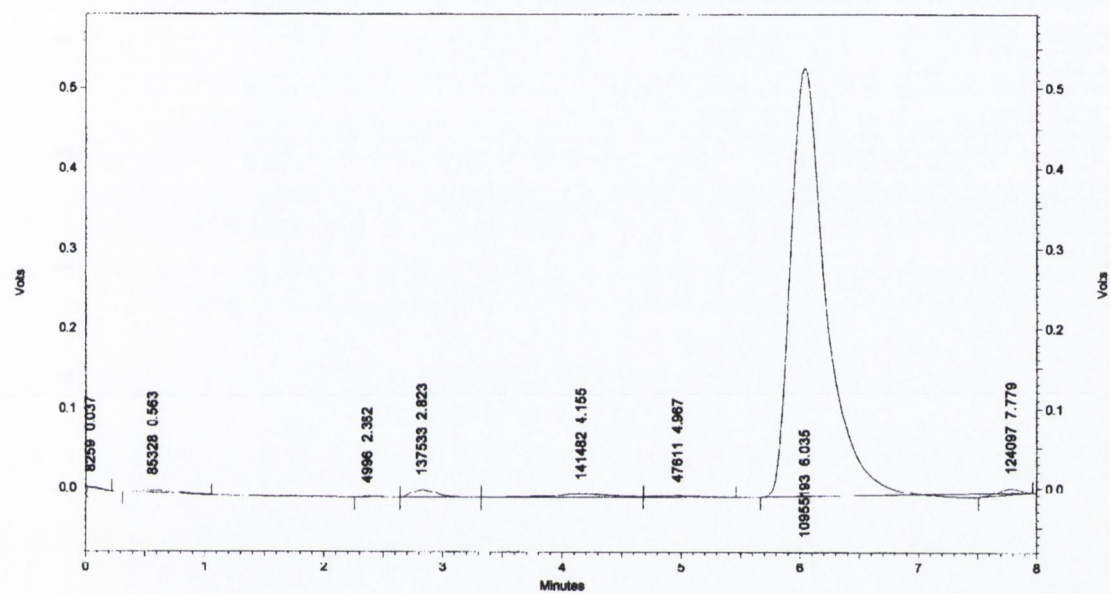
APPENDIX 9

HPLC trace of ketoprofen showing the principal ketoprofen peak with a retention time of 5.30 minutes.



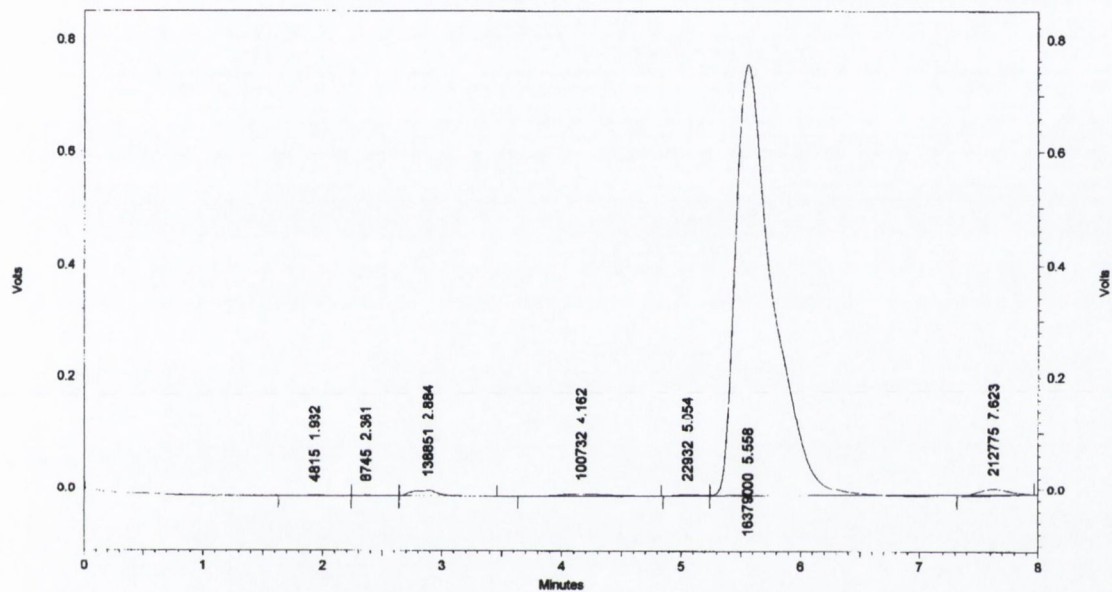
APPENDIX 10

HPLC trace of ibu-alanine showing the principal ibu-alanine peak with a retention time of 6.04 minutes.



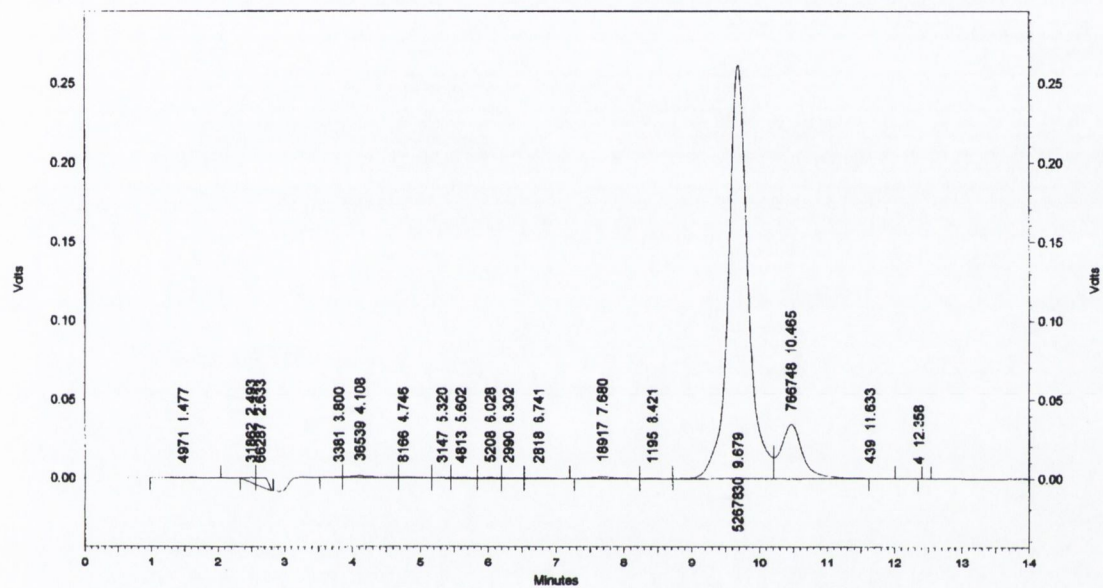
APPENDIX 11

HPLC trace of ibu-glycine showing the principal ibu-glycine peak with a retention time of 5.56 minutes.



APPENDIX 12

HPLC trace of ibu-phenylalanine showing the principal ibu-phenylalanine peak with a retention time of 9.68 minutes.



APPENDIX 14

Validation of the plasma assays for ibuprofen, ketoprofen and naproxen showing their extraction efficiency and precision.

Ibuprofen

Ibuprofen concentration ($\mu\text{g ml}^{-1}$)	Extraction Efficiency (%)	Precision (%)	
		Within-day	Between-day
47.07	86.89	1.08	4.08
94.14	87.38	0.98	7.16
188.28	89.71	1.23	9.34
376.56	92.17	0.77	6.01
753.12	91.38	0.75	10.03

Ketoprofen

Ketoprofen concentration ($\mu\text{g ml}^{-1}$)	Extraction Efficiency (%)	Precision (%)	
		Within-day	Between-day
66.34	90.35	1.37	10.09
126.68	93.74	2.48	5.87
253.36	88.71	0.68	6.17
506.72	95.06	0.74	8.21
1013.44	92.33	0.58	9.63

Naproxen			
Naproxen concentration (µg mL ⁻¹)	Extraction Efficiency (%)	Precision (%)	
		Within-day	Between-day
62.79	87.39	2.85	5.71
125.58	90.47	0.40	6.09
251.16	92.81	0.63	8.32
502.32	91.08	1.32	11.82
1004.64	90.34	2.05	7.62

Validation of the plasma assays for ibuprofen, ketoprofen, naproxen, ibu-alanine, ibu-glycine, ibu-phenylalanine and ibu-L-arginine, showing the linearity and limits of quantitation of the assays.

Compound	Linearity	Limit of Quantitation
	r ²	(µg mL ⁻¹)
Ibuprofen	0.997	2.35
Ketoprofen	0.998	1.87
Naproxen	0.999	2.73
Ibu-alanine	0.999	3.48
Ibu-glycine	0.999	4.20
Ibu-phenylalanine	0.999	3.08
Ibu-L-arginine	0.993	2.58