Transcutaneous Delivery of Anti-Arthritic Agents

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Summary

There is a substantial clinical need for improved therapeutic systems for the treatment of arthritis. This thesis concerns the development of a novel medication that is applied to the skin directly overlying the areas affected. The system comprises the ω-3 polyunsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), plus a non-steroidal anti-inflammatory (NSAID) drug. The source of EPA and DHA is fish oil which contains high proportions of these compounds and, although still largely considered a 'neutraceutical', its therapeutic value is supported by an ever increasing body of evidence, both scientific and anecdotal. The NSAID examined was ketoprofen, a widely used drug, found in a number of topical preparations. Synergistic action involving the two substances would be expected to provide a multi-faceted attack on the aetiology of arthritis. Ketoprofen and EPA/DHA were successfully delivered across full-thickness porcine ear skin *in-vitro*, although the presence of thickening agent retarded permeation of the latter. The successful delivery of these compounds into the joint capsule of a porcine forelimb was also demonstrated *in-vitro*. A novel transcutaneous delivery model was developed and used to provide preliminary data for the uptake of EPA into an *ex-vivo* cartilage ex-plant post transcutaneous permeation. The last three chapters can be considered collectively as an investigation into the unexpected phenomenon of enhancement of EPA/DHA by ketoprofen and two main hypotheses were tested; firstly, the formation of a π–π ketoprofen / EPA complex – the existence of which was strongly supported by the NMR/molecular modelling work of Chapter 8; secondly, the ketoprofen inhibition of epidermal enzymes active upon EPA, discussed in Chapters 7 and 9. In summary, the development of a novel dual-action, transcutaneous anti-arthritic formulation is possible and has been supported by this work. Furthermore, a hitherto unknown topical delivery mechanism has been elucidated.

List of publications

Heard CM, Kung D, Thomas CP, Skin penetration enhancement of mefenamic acid by ethanol and 1,8-cineole can be explained by the pull effect, International Journal of Pharmaceutics, in press.

Richards H, Thomas CP, Bowen JL, Heard CM, *In Vitro* Transcutaneous delivery of ketoprofen and polyunsaturated fatty acids from a pluronic lecithin organogel vehicle containing fish oil, Journal of Pharmacy and Pharmacology, 58 (2006) 903-980.

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List of abbreviations

15-HEPE	15-hydroxyeicosapentaenoic acid
15-HETE	15-hydroxyeicosatetraenoic acid
5-HPETE	5-hydroperoxyeicosatetraenoic acid
AA	Arachidonic acid
ALA	α-linolenic acid
APCI	Atmospheric pressure chemical ionisation
BHA	Butylated hydroxyanisole
$CDCl_3$	Deuterated chloroform
ClogP	Calculated logP
COX	Cyclo-oxygenase
CS	Chondroitin sulphate
DE	Dermo-epidermal layer
DGLA	Dihomo-γ-linolenic acid
DHA	Docosahexaenoic acid
DMARD	Disease modifying anti-rheumatic drug
DMMB	Dimethylene methyl blue
ECM	Extra-cellular matrix
EPA	Eicosapentaenoic acid
ER_{flux}	Flux enhancement ratio
FAME	Fatty acid methyl ester
FAPE	Fatty acid phenacyl ester
FLAP	5-lipoxygenase activating protein
GAG	Glycosaminoglycan
GAIT	Glucosamine/chondroitin arthritis intervention trial
GC	Gas Chromatography
GLA	γ-linolenic acid
GMS	Growth media sustained
HCl	Hydrochloric acid
HHBSS	Hepes buffered Hanks balanced salts solution
HPC	Hydroxypropyl cellulose
HPLC	High performance liquid chromatography
ICC	Immunocytochemistry
IL-1	Interleukin 1
J_{SS}	Steady state flux

K_p	Permeability coefficient
LA	Linoleic acid
LC	Liquid chromatography
LC-MS	Liquid chromatography-Mass spectrometry
LOD	Limit of detection
LOX	Lipoxygenase
LT	Leukotriene
LTA_4	Leukotriene A_4
LTB_4	Leukotriene B_4
LTB_5	Leukotriene B_5
LTC_4	Leukotriene C_4
LX	Lipoxin
M	Molar
MEME	Minimum essential medium Eagle
MeOH	Methanol
MMP	Matrix metalloproteinase
NGM	Non growth media
NMR	Nuclear magnetic resonance
NSAID	Non-steroidal anti-inflammatory drug
OA	Osteoarthritis
OAc	Oleic acid
PBS	Phosphate buffered saline
PEG 400	Polyethylene glycol 400N
PG	Prostaglandin
PGE_2	Prostaglandin E_2
PGE_3	Prostaglandin E_3
PGI_2	Prostacycline I_2
PGl	Propylene glycol
PUFA	Polyunsaturated fatty acid
RA	Rheumatoid arthritis
SEM	Standard error of the mean
SC	Stratum corneum
SD	Standard deviation
TNF-α	Tumour necrosis factor α
TXA_2	Thromboxane A_2
UV	Ultraviolet
WHO	World Health Organisation
WOMAC	Western Ontario and McMaster Universities Osteoarthritis

For Mum

Chapter 1

Introduction

1. Overview

There is a substantial clinical need for improved therapeutic systems for the treatment of arthritis. This thesis concerns the development of a novel medication that is applied to the skin directly overlying the areas affected. The system comprises the *ω-3* polyunsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), plus a non-steroidal anti-inflammatory (NSAID) drug. The source of EPA and DHA is fish oil which contains high proportions of these compounds and, although still largely considered as a 'neutraceutical', its therapeutic value is supported by an ever increasing body of evidence, both scientific and anecdotal (Calder, 2006, Curtis et al., 2004, Fortin et al., 1995). The NSAID examined was ketoprofen, a widely used drug, used in a number of topical preparations. Synergistic action involving the two substances would be expected to provide a multi-faceted attack on the aetiology of arthritis.

1.1 Arthritis

The term arthritis covers many different disease states all associated with inflammation of one or more joints. The most common forms of arthritis are rheumatoid arthritis (RA) and osteoarthritis (OA). The estimated cost of rheumatoid arthritis alone to the National Health Service is £240-600 million per year. The social cost of both diseases is also considerable, with significant numbers of patients being unable to work, requiring residential home care and having reduced life expectancy (Scott et al., 1998). The type of treatment an arthritic patient receives depends on several factors including the type of arthritis. Treatment programs can include combinations of rest, exercise, methods of protecting the joints and medication.

1.1.1 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is one of the commonest autoimmune diseases. It is a chronic, progressive, systemic inflammatory disorder affecting the synovial joints and

typically producing symmetrical arthritis. If left untreated it leads to joint destruction which is ultimately responsible for the deformity and disability seen in this disease.

The prevalence of rheumatoid arthritis is consistent worldwide, affecting about 0.5-1% of the population (Buch and Emery, 2002). Although the disease affects people all over the world, certain populations exhibit particularly low or high prevalence, for example the Inuit population and native Americans respectively. Studies of the Inuit population revealed a low incidence of a number of disease states common in Western society, including rheumatoid arthritis. This is believed to be linked to their diet and due to their high consumption of oily fish (Belch, 1990)

Pain and swelling are the main clinical symptoms seen in the early stages of rheumatoid arthritis and are caused by two factors. Firstly, inflammation of the synovium membrane that lines the joint capsule, referred to as synovitis, and, secondly, effusion of fluid into the cavity of the joint. Disease progression leads to cartilage degradation due to lysosomal enzymes and polymorphonuclear leukocytes present in the effusion fluid. Sustained inflammation leads to further erosion of cartilage and eventually bone. The management of rheumatoid arthritis aims to relieve pain, limit inflammation and improve mobility and function.

1.1.2 Osteoarthritis

Osteoarthritis (OA) is the most common disabling condition in the Western world. (Aigner et al., 2006) Osteoarthritis is the degeneration of, most commonly, the weight bearing joints of the body i.e. hips and knees and is most frequent and symptomatic in middle aged and older people. Once a patient has developed OA they will suffer with the disease for the rest of their lives with the severity and pain of the condition generally becoming progressively worse.

Primary or idiopathic OA occurs in the absence of a known cause of joint degeneration, age and general wear on the joints is the primary cause. Less frequently, infection or neurological disorders or damage to the joint for example, via mechanical stress, results

in secondary OA (Buckwalter and Martin, 2006). The World Health Organisation (WHO) estimates that 9.6% of the world's male population and 18% of women over the age of 60 suffer from OA. Due to the correlation between age and OA the prevalence of the disease is rising as the average age of the population increases (Buckwalter et al., 2003).

OA can affect any synovial joint although OA rarely occurs in the ankle, wrist, elbow and shoulder, however it is common in the hand, foot, knee, spine and hip joints. Of these the most common occurrence is in the knee joint. The main clinical symptoms of OA are similar to those of RA in that pain and stiffness are usually the symptoms experienced at the onset and throughout the disease. These symptoms are caused by pathological changes in and around the OA joint. Increased pressure within the joint capsule and surrounding bone, inflammation of the synovial lining and alteration in the function of the surrounding musculature are some of these changes. Current therapy aims to combat the pain caused by OA and in doing so increase the patient's ability to use the joint. There are no proven treatments currently available to slow or stop the progression of the disease.

The link between RA and OA lies with inflammation. RA is caused by the body over-producing inflammatory mediators and OA is potentiated by these same inflammatory mediators. If this inflammation could be inhibited then both disease states may be treated more effectively. Inflammatory processes must first be examined to allow identification of potential inhibitory mechanisms.

1.2 Inflammation

Inflammation is the body's response to tissue damage by a wide range of harmful stimuli, including mechanical trauma, tissue necrosis and infection. The purpose of inflammation is to eradicate the damaging agent, initiate repair processes and return the damaged tissue to normality. The body's initial reaction is to increase blood flow to the area and to increase permeability across blood capillaries. This allows larger molecules, antibodies and cytokines, to cross the endothelial wall and increases the movement of

leukocytes from the bloodstream into the surrounding tissues. The cells most prevalent at the onset of inflammation are granulocytes followed by monocytes / macrophages and lymphocytes. These cells are then triggered to produce inflammatory mediators which are involved in further processes including pathogen elimination, clearing cellular and tissue debris and tissue repair. The inflammation process is an essential function of the body and is usually well controlled via negative feedback loops and production of compounds which help to terminate the inflammatory process. In certain cases however termination does not occur and the inflammation process continues and propagates itself in an inflammatory cascade. High levels of cytokines, can lead to muscle wastage and bone and tissue loss. These cytokines are also responsible for the induction of other pro-inflammatory mediators as discussed below.

1.2.1 Cytokines

Cytokines are a group of proteinaceous signalling compounds that are used extensively for inter-cell communication, and are critical to the function of both innate and adaptive responses. Produced by a variety of cell types they can exert effects on both nearby cells or throughout an organism. The main inflammatory cytokines are detailed in section 1.2.1.1 and 1.2.1.2.

1.2.1.1 Interleukin 1 (IL-1)

The IL family contains two agonists (IL-1α and IL-1β) and one antagonist (IL-1ra). IL-1β is produced in response to injury, infection, or by induction from other cytokines and is mainly generated by monocytes and activated macrophages, but can also be generated by endothelial cells, fibroblasts and epidermal cells (Horai et al., 1998). IL-1β is important in systemic inflammatory responses and acts synergistically with tumour necrosis factor α (TNF-α). IL-1α, which is intracellular and not secreted from cells, and IL-1β have been implicated in the pathogenesis of several diseases including RA, inflammatory bowel syndrome, sepsis and several autoimmune diseases. Diseases associated with IL-1 may be caused by an imbalance between IL-1 agonists and the antagonist IL-1ra. IL-1 is responsible for the production of other cytokines, prostanoids

and nitric oxide, and also regulates extra-cellular matrices and causes cartilage and bone resorption (Stylianou and Saklatvala, 1998). IL-1 has also been shown to induce its own expression and that of cyclooxygenase-2 (COX-2), a pro-inflammatory mediator, in the brain (Horai et al., 1998).

1.2.1.2 Tumour necrosis factor (TNF)

Two types of TNF are known to exist namely TNF-α and -β. The two are structurally related but appear to have distinct three-dimensional structures as they differ in their sensitivity to various proteases and chemical agents, however, both are potent inducers of similar biological responses (Porter, 1990). TNF is responsible for the production of many systemic inflammatory responses including fever, mobilization / activation of polymorphonuclear leukocytes and induction of both COX-2 and lipoxygenase enzymes.

1.2.2 Cyclooxygenase (COX), lipoxygenase (LOX) and eicosanoids

The mechanism for the propagation of inflammation lies with cell membrane-bound fatty acids and the formation of eicosanoids: a group of biologically active lipids that act as chemical messengers within the immune system. They are synthesised from polyunsaturated fatty acids (PUFAs), in particular from arachidonic acid (AA), but also from dihomo-γlinolenic acid (DGLA) and eicosapentaenoic acid (EPA). The membranes of most immune cells contain large amounts of AA, compared to DGLA and EPA. AA therefore, is usually the principal precursor for eicosanoid synthesis. Three major classes of eicosanoids are formed from these compounds: prostaglandins (PG), formed via the cyclooxygenase (COX) pathway, leukotrienes (LT), produced by the lipoxygenase (LOX) pathway (in particular 5-lipoxygenase (5-LOX)) and lipoxins (LX), the enzymatic products of 5-, 12-, and 15-LOX. (Penrose, 2006)

There are two iso-forms of the enzyme COX: COX-1 and COX-2. COX-1 is the constitutive form and is responsible for regulating homeostatic processes such as platelet aggregation and gastric cytoprotection. COX-2 is thought of as the inducible iso-form and is involved principally in inflammatory conditions (Solomon, 2006).

COX-2 is induced by multiple inflammatory stimuli including cytokines such as IL-1 and TNF-α. Metabolism of AA by COX gives rise to the 2-series prostaglandin, e.g. PGE_2. (See Figure 1.1)

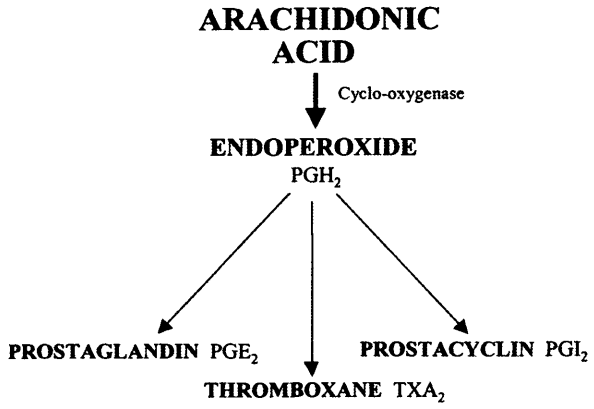


Figure 1.1 Prostanoid synthesis from AA precursor (Adapted from Calder, 2001))

PGE_2 acts synergistically with other inflammatory mediators such as leukotrienes (below) to cause hyperalgesia and increase venopermeability (Sperling, 1995). PGE_2 is produced in rheumatoid synovium where it stimulates osteoclastic (help to break down bone) activity and inhibits bone cell proliferation. (Paisz et al., 1993) It has also been demonstrated (Abramson and Yazici 2006) that the RA synovium can produce approximately ten times the concentration of PGE_2 compared to a normal healthy joint, indicating the importance of PGE_2 in the disease state. Table 1.1 indicates some of the roles of the prostanoids produced via the COX pathway.

Table 1.1 Prostanoids and the inflammatory process

The Role of Prostanoids in Inflammatory Processes	
Prostanoid	**Effects**
PGE_2	Dilates and increases permeability of microvasculature
	Hyperalgesia
	Pyrogenic
	Stimulates osteoclastic activity and bone resorption
	Inhibits bone cell proliferation
TXA_2	Bronchoconstricts
	Constricts miocrovasculature
PGI_2	Vasodilates

Release of AA from the cell membrane caused by phospholipase A_2 can also result in the production of LTs catalysed by the 5-LOX enzyme. 5-LOX has been found in granulocytes, monocytes, macrophages and mast cells. Metabolism of AA by 5-LOX first produces 5-hydroperoxyeicosatetraenoic acid (5-HPETE) which is dehydrated in the presence of 5-lipoxygenase activating protein (FLAP) to form leukotriene A_4 (LTA_4). This in turn is converted to leukotriene B_4 (LTB_4) by LTA_4 hydrolase (see Figure 1.2). LTB_4 is then exported from the cell to exert its inflammatory effects which include increased IL-1 and IL-6 production by monocytes and increased IL-1β in synovial cells. (Lewis et al., 1990) Table 1.2 lists some of the effects of LTs. LTB_4 not only initiates and amplifies an inflammatory response but may also initiate its own production (Devchand et al., 1996) as well as stimulating cytokine release and proliferation. LTB_4 concentrations have been shown to be in high in the synovial fluid and peripheral blood of patients with rheumatoid arthritis.

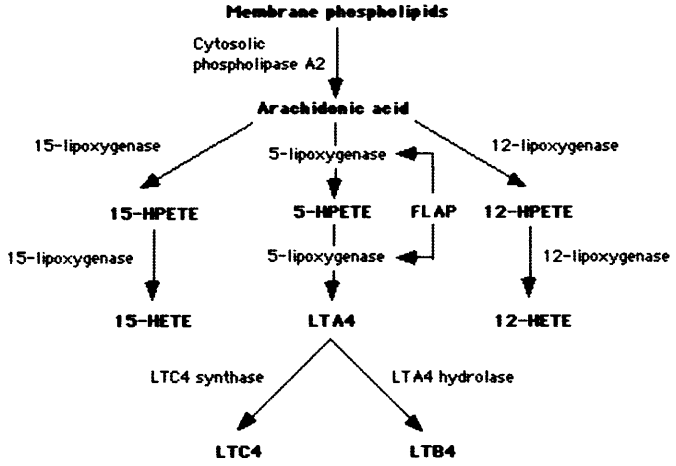


Figure 1.2 Leukotriene synthesis from AA precursor (adapted from Penrose, 2006)

Table 1.2 Effects of LTs on inflammation

Proinflammatory, Inflammatory and Immunomodulating Effects of Lipoxygenase Products	
Lipoxygenase Product	**Effect**
LTB_4	Hyperalgesia
	Stimulates monocyte and macrophage IL-1 and TNF-α production
LTC_4	Dilates and increases permeability of microvasculature
15-HETE	Hyperalgesia
	Inhibits PGH Synthase
15-HPETE	Suppresses T lymphocyte function

1.3 RA and OA therapies

Figure 1.3 illustrates the targets for inflammation therapy for the prostanoid pathway and the treatments used for those targets. The severity of inflammatory disease dictates the treatment, e.g. mild analgesics and complementary therapies such as acupuncture for mild cases or artificial joint replacement for more severe cases. Many drugs are currently used to treat the symptoms of both RA and OA and the choice depends on the stage and severity of the disease. These range from the commonly prescribed non-steroidal anti-inflammatory drugs (NSAID) such as ibuprofen, to disease modifying anti-rheumatic drugs (DMARDs), e.g. methotrexate. For the LT pathway, (1) in Figure 1.3 can be replaced with a LOX inhibitor such as Licofelone which would inhibit the production of the inflammatory mediator LTB_4. However, replacement of AA with a different fatty acid substrate allows (2) to be a potential target for treatment.

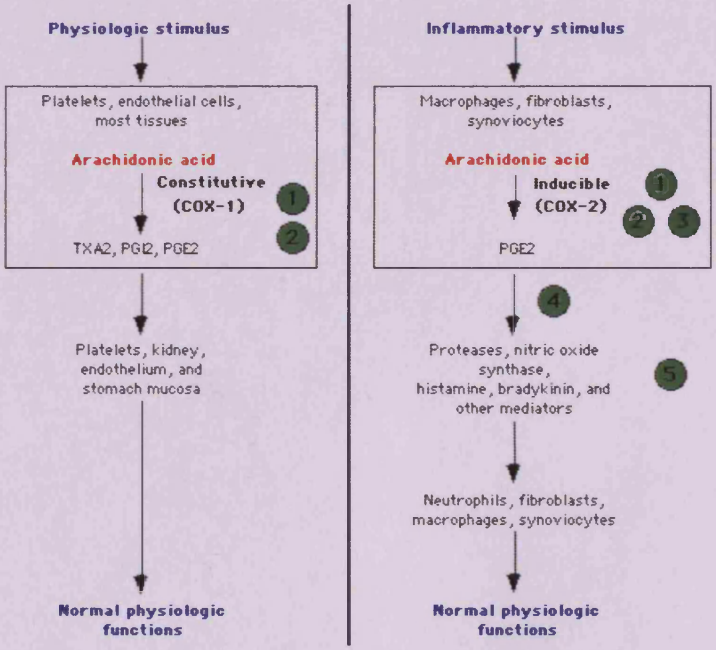


Figure 1.3 Representation of prostanoid synthesis and potential levels of inhibition, (1) Aspirin and other NSAIDs, (2) Alternative fatty acid substrates, (3) Selective COX-2 inhibitors, (4) Receptor antagonists, (5) Nitric oxide synthase inhibitors, antihistamines (edited from Solomon, 2006)

The following sections cover some of the current treatments used for the symptoms of RA and OA and include drug therapies, natural products and supplements.

1.3.1 NSAIDs

NSAIDs are the most commonly prescribed drugs for the treatment of rheumatic disease. Table 1.3 lists some of the commonly prescribed NSAIDs which are available in a range of formulations including oral, topical and injectable preparations. Their pharmacological action lies in their ability to inhibit the COX enzyme (Buchanan, 1997).

The main limitation to the use of NSAIDs is their side effect profile with gastrointestinal toxicity by far the most common adverse effect, although generally of a mild degree. Less frequent, but potentially serious, is renal toxicity. Renal complications fall into six categories; acute renal failure, interstitial nephritis and nephrotic syndrome, papillary necrosis, water retention and hyperkalemia. Acute renal failure is the most common renal complication described with NSAID therapy but is generally reversible within 24 to 72 hours.

Patients with interstitial nephritis / nephrotic syndrome did not necessarily have any former renal disease and by discontinuing NSAID use clinical manifestations are usually resolved. This syndrome is usually associated with propionic acid derivatives such as ibuprofen and ketoprofen. Sodium retention represents a universal side effect of NSAID therapy and essentially all NSAIDs have been implicated. Allergic reactions are also associated with the use of NSAIDs and aspirin. The clinicl symptoms can include anaphylactic shock, angiodema and types of skin eruptions (Stevenson, 1984). Also of significance is the effect of these drugs in asthma patients. A distinct clinical syndrome called aspirin–induced asthma can be caused by the use of aspirin and other NSAIDs (Szczeklik and Stevenson, 2003) In approximately 10% of asthma sufferers this condition can be induced after taking these medications (Szczeklik 1987). Topical treatment using NSAIDs can also cause reactions and this is discussed in detail by Ophaswongse and Maibach; (1993). Overall, NSAID therapy carries a low incidence of significant complications.

Table 1.3 Commonly prescribed NSAIDs

NSAIDs and usual doses		
NSAID	**Trade Name**	**Usual Oral Dose**
Aspirin (acetylsalicylic acid)	Multiple	2.4 – 6g / 24 hours (divided doses)
Ibuprofen	Brufen, Nurofen	3.2g /24 hours
Ketoprofen	Orudis, Oruvail	75mg three times daily
Diclofenac	Voltarol	50 – 75mg twice daily
Celecoxib	Celebrex®	100 – 200mg four times daily
Rofecoxib	Vioxx®	12.5 – 25mg four times daily

1.3.2 COX-2 inhibitors

The ideal aim for an NSAID would be to inhibit the trauma induced COX-2 enzyme and thus decrease inflammation, whilst having very little or no effect upon the constitutive COX-1 enzyme and normal physiological processes. Such a compound would maximize efficacy, without the associated toxicity, in particular, gastroduodenal erosions and renal effects. Two such selective COX-2 inhibitors, celecoxib (Celebrex®) and rofecoxib (Vioxx®) received approval from the Food and Drug Administration (FDA) in the United States. Both were approved for use in RA and OA. The selective COX-2 inhibitors demonstrate at least a 200 to 300-fold selectivity for inhibition of COX-2 over COX-1 at the defined therapeutic doses. Studies have shown that they are comparable to non specific NSAIDs in their analgesic effect for both RA and OA without the unwanted gastroduodenal toxicity (Emery et al., 1999; Silverstein et al., 2000). However, rofecoxib was recently withdrawn worldwide by the manufacturer due to an increased risk of adverse cardiovascular events. Celecoxib was also withdrawn for similar reasons.

1.3.3 Disease modifying anti-rheumatic drugs (DMARDs)

The class of drugs known as DMARDs, yield their action in their ability to affect the immune system and so stop disease progression. Generally these drugs are effective, but they take time to show positive results. For example, hydroxychloroquine

(*Plaquenil*^{TM}), used mainly in psoriatic arthritis, may take three or four months before a patient notices a significant effect. Other drugs, such as methotrexate, work more quickly, but often not quickly enough if the patient is suffering severe pain. For this reason, physicians often prescribe an additional drug such as an NSAID to help control pain and inflammation while the DMARD starts to work. DMARDs are most commonly used for RA, but some of the drugs are also used for juvenile RA, ankylosing spondylitis, psoriatic arthritis and lupus. The dosages of these drugs range from 200–600mg per day for hydroxychlorquine up to 7.5-20mg per week for methotrexate, usually given as one dose either orally or via injection. However depending on the specific condition and other factors including age, body weight and other medications, the dosages may vary.

1.3.4 Natural products

A number of natural products have received attention, all claiming to ease the pain of RA and OA and even in some cases stop the progression of the disease. Listed here are some of the more widely used natural products currently available.

1.3.4.1 Capsaicin

Capsaicin, Figure 1.4, is the active compound found in hot chilli pepper and is administered via a topical formulation. It exerts its biological effect by retarding the release of substance P, a polypeptide involved in the transmission of pain impulses from peripheral receptors to the central nervous system, from unmyelinated C nerve fibres. (Winter et al., 1995)

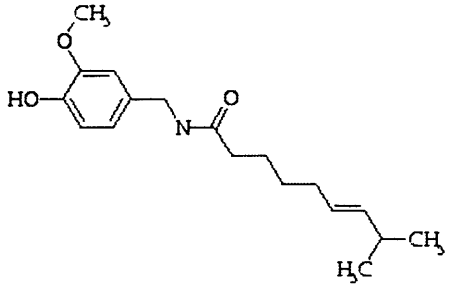


Figure 1.4 Capsaicin structure

The efficacy of capsaicin was assessed in a randomized, double-blind, placebo-controlled trial where both RA and OA patients demonstrated mean pain reduction of 57 and 33% respectively after four weeks treatment with a 0.0025% topical capsaicin formulation. Side effects reported were local irritation (burning, stinging, and erythema) which occurred in approximately 40% of patients. (Deal et al., 1991) The efficacy of this capsaicin formulation appeared good, however this study involved an application of the formulation four times daily. This would prove very expensive for patients for long term use and patient compliance would also be difficult to obtain.

1.3.4.2 Glucosamine and chondroitin

Glucosamine is an amino sugar which is a pre-cursor to proteoglycan which in turn is a major component of cartilage (Figure 1.5). Glucosamine is important for the repair and maintenance of cartilage and is usually delivered orally as the sulphate or chloride salt. It has been suggested from animal model studies that glucosamine sulphate may slow cartilage breakdown through the stimulation of cartilage to synthesize glycosaminoglycans (GAGs) and proteoglycans, and the inhibition of proteolytic enzymes (McAlindon et al., 2000).

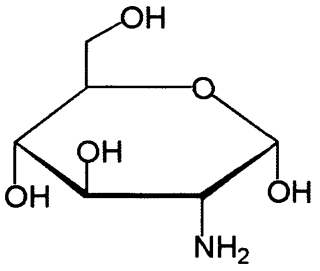


Figure 1.5 Glucosamine structure

Oral glucosamine is an increasingly popular supplement for the symptoms of OA. Some patients have reported glucosamine having a greater pain reducing effect in knee OA than NSAIDs (Dodge and Poole, 1989). Several studies have concluded that the addition of glucosamine into a cartilage culture system led to less matrix metalloproteinase (MMP) and aggrecanase activity within the cartilage extra-cellular

matrix (ECM). Both compounds cause breakdown of the ECM and further destruction of the cartilage (Poole, 1993). Cartilage degradation is discussed in detail in Chapter 6. However, promising as these results seem, the experiments were carried out *ex-vivo* and glucosamine is intrinsically difficult to deliver to joints due to its poor bioavailability from oral dosing. Persiani et al., (2005) found that an oral dose of 3000mg glucosamine sulphate only increased blood plasma levels by approximately 250ng ml^{-1} from baseline plasma levels. Studies have also revealed that the reported benefit in joint space narrowing is only limited and may have been due to overall pain relief and thus use of the affected joint (Reginster et al., 2001). A patch form of glucosamine is also available but with un-reported value.

Chondroitin sulphate (CS), (Figure 1.6), is a major structural component of the cartilage and is composed of repeating units of galactosamine sulfate and glucuronic acid. It is the predominant glycosaminoglycan found in articular cartilage. Chondroitin is believed to help stimulate the production of new healthy cartilage, block enzymes that break down cartilage, and normalize joint fluids to reduce friction. Oral availability of this compound is once again poor (Volpi, 2002) relying on intra-articular (into the joint) administration to ensure delivery to the site of action.

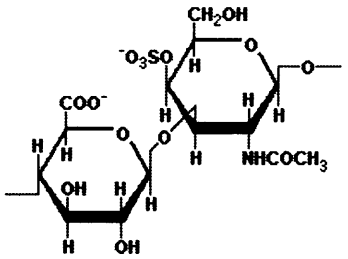


Figure 1.6 A Chondroitin Sulphate sub-unit

A 2000 meta-analysis involving 7 studies compared the pain relieving effects of CS with those of a placebo and found it to be significantly more effective. However patients were still allowed analgesic and anti-inflammatory medications and no dose effect of CS was observed (Leeb et al., 2000). It was also found that the efficacy of chondroitin sulphate was similar to that of glucosamine. Chondroitin sulphate used alone appears to provide little benefit among those with osteoarthritis. There is a

possibility that it is more effective when used in combination with glucosamine in those with more severe pain. However, the glucosamine/chondroitin arthritis intervention trial (GAIT) did not show glucosamine and CS to be significantly more efficacious than placebo for pain relief or functional improvement in patients with OA of the knee.

1.3.4.3 Hyaluronans / Hyaluronic Acid

Several studies have examined the effects of intra-articular hyaluronans in OA. Hyaluronan is a form of glycosaminoglycan (GAG) and one of the primary components of cartilage ECM. It is thought that intra-articular injections of hyaluronans can lead to decreased OA symptoms as these compounds are deficient in degenerating cartilage. Replacement of these missing hyaluronans leads to their beneficial effect. Commercial hyaluronan formulations are currently available in the US. The main limitation with this form of treatment comes from the patient as intra-articular injections can prove painful and lead to infection.

A 2003 meta-analysis examined the results of 22 trials of hyaluronan injections compared to intra-articular placebo injections. It concluded that hyaluronan injections were more beneficial than the placebo but had a relatively small effect (Lo et al., 2003). A similar study in 1998 compared the effects of hyaluronans with placebo and oral naproxen. They found the hyaluronans improved the symptoms of knee pain compared to placebo but found no significant difference against naproxen (Altman and Moskowitz, 1998).

In summary, the available evidence suggests that intra-articular hyaluronans have a small pain relieving advantage when compared to intra-articular placebo injections with little or no advantage over oral NSAID therapy.

1.3.4.4 Polyunsaturated fatty acids (PUFAs)

A polyunsaturated fatty acid is a carboxylic acid with a hydrocarbon chain of 4-36 carbons with multiple regions of unsaturation, i.e. double bonds. Three fatty acids

bonded as esters to a glycerol backbone form a triglyceride, and the hydrolysis of this triglyceride yields free fatty acids. The triglyceride can contain the same or a mix of fatty acids. Fatty acids are the main constituent of cell membranes and different types of fatty acids including saturated, mono or poly-unsaturated exhibit different characteristics when bound in cell membranes. Several fatty acids are classed as essential (see section 1.3.4.4.2) because the human body cannot produce them and they must therefore be obtained from the diet.

1.3.4.4.1 Nomenclature of polyunsaturated fatty acids (PUFAs)

Fatty acids have systematic names but most also have trivial names and are described by a standard shorthand nomenclature (British Nutrition Foundation, 1992 and 1999) This nomenclature indicates the number of carbon atoms in the chain, the number of double bonds in the chain and the position of the first double bond from the methyl terminus. There are rules regarding the position of double bonds in unsaturated fatty acids; these are determined by the specificity of the enzymes that insert those bonds into the hydrocarbon chain. It is the position of the first double bond in the hydrocarbon chain which is indicated by the *n–7*, *n–6*, part of the shorthand notation for a fatty acid. An *n–3* fatty acid, therefore has the first double bond on the third carbon from the methyl terminus. Another notation replaces the *n* with the Greek letter ω (omega) as this is the last letter of the Greek alphabet, it is indicative of the last carbon in the chain, thus *ω-3* notates a fatty acid with the first double bond three carbons from the methyl terminus. This is illustrated in Figure 1.7 below.

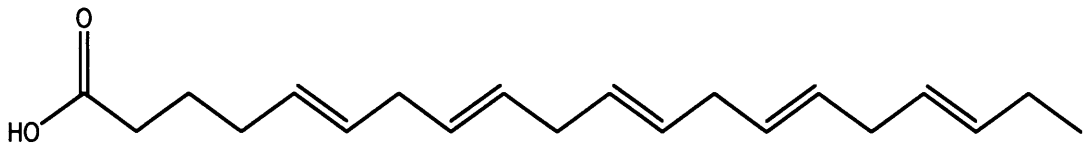


Figure 1.7 EPA – cis- 5, 8, 11, 14, 17-eicosapentaenoic acid (20:5*n-3*)

1.3.4.4.2 Biosynthesis of polyunsaturated fatty acids

Saturated fatty acids and most monounsaturated fatty acids can be biosynthesised in mammalian tissues from non-fat precursors like glucose or amino acids, but this does not usually occur in humans eating a Western diet since the consumption of fat in general, and of saturated and monounsaturated fatty acids in particular, is high. Mammals cannot convert oleic acid (OAc) (18:1*n*-9) into linoleic acid (LA) (18:2*n*-6) as mammals are unable to chemically introduce double bonds before the ninth carbon in OAc. The enzyme responsible for this, Δ^{12}–desaturase is found only in plants. Similarly, the conversion of LA to α-linolenic acid (ALNA) carried out by the Δ^{15}–desaturase enzyme, also responsible for the interconversion of *n*-6 and *n*-3 fatty acids, can only be achieved by plants, as shown in Figure 1.8. As these fatty acids cannot be made by mammals, they are deemed essential. Table 1.4 lists some of the most prevalent fatty acids.

Table 1.4 Common fatty acids

Carbon notation	Common Name
12	Lauric acid
16	Palmitic acid
18	Stearic acid
18:2	Linoleic acid
18:3	Linolenic acid
20:4	Arachidonic acid
20:5	Eicosapentaenoic acid
22:6	Docosahexaenoic acid

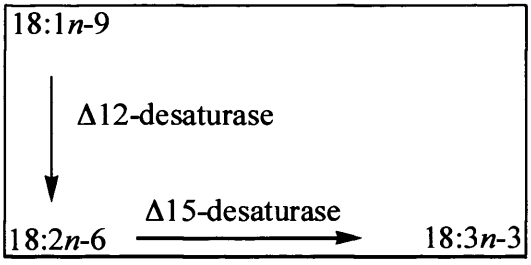


Figure 1.8 Plant metabolism of oleic acid

Once consumed in the diet LA can be converted via γ-linolenic (GLA 18:3*n*-6) and DGLA; 20:3*n*-6 acids to arachidonic acid AA; 20:4*n*-6. Using the same pathway dietary ALNA can be converted into EPA; 20:5*n*-3 and then to DHA; 22:6*n*-3, but this process is inefficient in the physiology of the Western society. Many marine plants also carry out fatty acid carbon chain elongation, and further desaturation of ALNA to produce EPA and DHA. It is the formation of these long chain *n*-3 PUFAs by marine algae and their transfer through the food chain that accounts for their abundance in the tissues of some marine mammals and fish (e.g. herring, tuna and mackerel; known as "oily fish"). EPA and DHA are found in relatively high proportions in the commercial products known as "fish oils" which are a preparation of the body oils of cold water fish e.g. salmon and tuna. EPA and DHA are also found in high proportions in the oils extracted from the livers of other species of fish which live in warmer waters e.g. cod (Calder, 2001).

1.4 Fish oil and inflammation

With increased bioavailability of EPA and DHA the production of the 2-series PGs and the 4-series LTs from AA is diminished as cell membranes incorporate a larger proportion of these fatty acids at the expense of AA. As a result the severity of the inflammatory response is modulated as EPA and DHA also act as substrates for COX and LOX enzymes and produce a different class of PGs and LTs (Figure 1.9). The 3-series PGs and the 5-series LTs are comparably less potent than their AA relatives. It has been found by Goldman et al., (1983) that LTB_5 is 10 to 100 fold less potent as a neutrophil chemotactic agent than the equivalent LTB_4. Similarly, prostaglandin E_3

(PGE_3) has been shown to be a less potent inhibitor of COX-2 gene expression in fibroblasts and of IL-6 production by macrophages compared to PGE_2 (Calder, 2006).

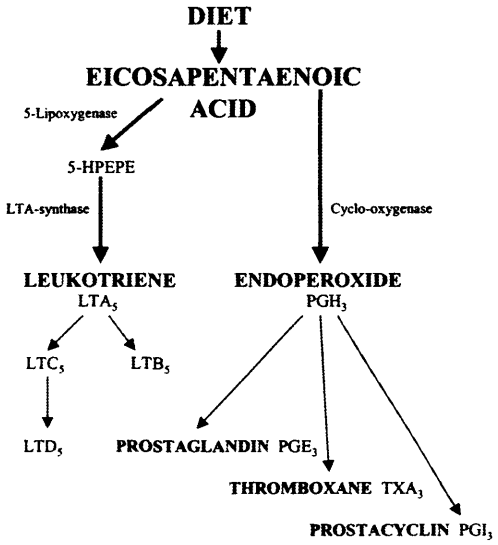


Figure 1.9 Prostanoid and leukotriene synthesis from EPA

Long chain *n-3* PUFAs have also been shown to effect the synthesis of inflammatory cytokines. The mechanism of this is thought to lie with the reduction of the cytokine regulating PGE_2 and 4-series LTs. Cell studies have demonstrated that both EPA and DHA can inhibit the production of TNF-α and IL-1β by monocytes (Calder, 2006). Several studies have also shown EPA and DHA to inhibit the expression of COX-2, IL-1α, IL-1β, TNF-α, 5-LOX, 5-LOX activating protein and MMP genes in OA cartilage cultures and bovine chondrocytes (Curtis et al., 2000). Figure 1.10 is a summary of the differences between the products of AA and EPA via the COX and LOX inflammatory pathways.

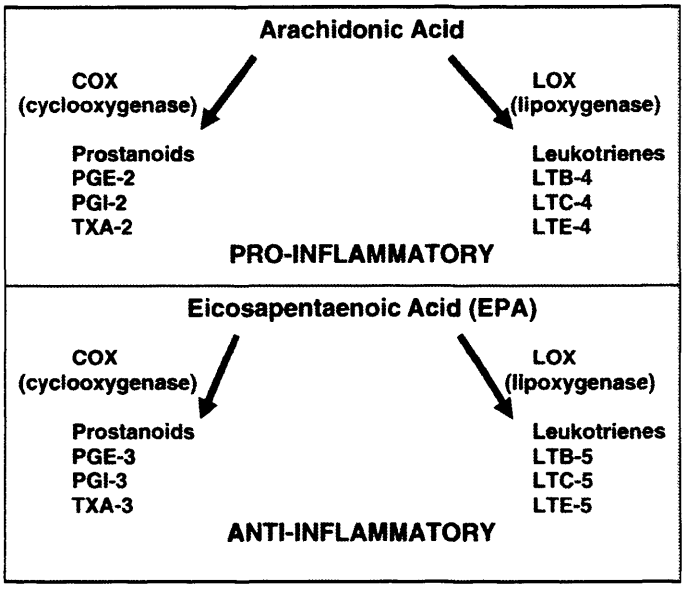


Figure 1.10 Summary of COX and LOX mediated metabolism of AA and EPA (Adapted form Shapiro, 2003)

1.5 Delivery of fish oil into the body

Several studies have been carried out concerning the effect of an EPA enriched diet on the symptoms and progression of RA and OA. James et al. (2000), showed that after 4 weeks of 9 g day^{-1} fish oil supplementation both the TNF-α and IL-1β concentrations within human peripheral blood mononuclear cells were significantly reduced. Marsen et al. (1992), found a concentration of 19.9 ± 3.3 mg L^{-1} in plasma after 28 days after receiving a 12g day^{-1} fish oil formulation. A different study by Fortin et al. (1995) looked at the efficacy of fish oil in relation to joint tenderness and morning stiffness associated with rheumatoid arthritis. This study concluded "a statistically significant improvement in tender joint count and morning stiffness" in those treated for three months with fish oil compared to a placebo. All studies carried out involve the patient taking approximately 3-12g day^{-1}, a large dose to achieve a relatively low plasma concentration. If EPA could be delivered straight to the site of action i.e. the joint, it would eliminate the need for such high oral doses.

Drug delivery into the body is a fundamental research area in the pharmaceutical industry. Most drugs are administered orally or intravenously and enter the systemic circulation to exert their activity after transport to their site of action. Some drugs

however are not suited to this form of delivery, either due to the high metabolic activity and consequential breakdown within the stomach or liver (first pass effect) or due to their incompatibility with the oral route of administration. The concentration of a drug within the body is also of importance as when an oral dose is given there is an initial increase in the plasma concentration and these peaks can cause undesirable effects. Troughs associated with this dosing, post administration, may be sub therapeutic. Pulse dosing (see Figure 1.11) is common with oral drug delivery but can be avoided by the delivery of drugs via the skin.

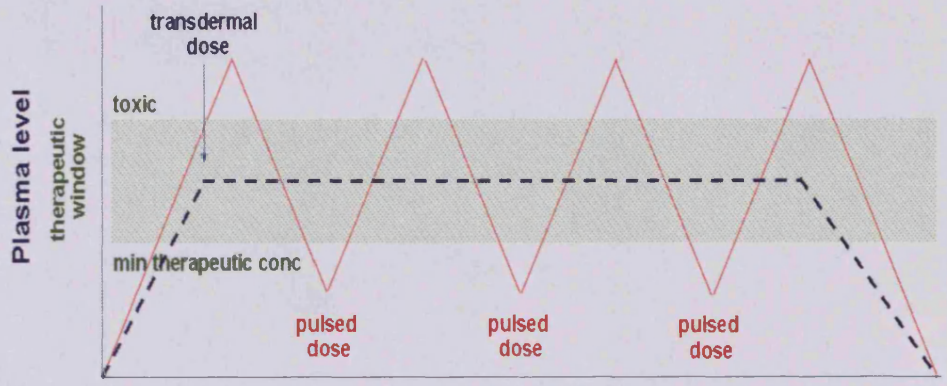


Figure 1.11 Pulse effect of oral dosing

Localised topical delivery has several advantages over other routes. It allows specific targeting of drugs to their site of action and minimises the side effects that can be seen after oral administration and reduces the frequency of dosing. This is particularly beneficial in diseases such as RA and OA as the pain and inflammation associated with the diseases are localised at individual joints. Topical drug delivery ensures that the drug enters the systemic circulation directly, eliminating the first pass metabolism of the liver. Direct delivery of both an anti-inflammatory drug and an alternate fatty acid substrate to compete with AA, to the site of the inflammation would provide a two-pronged attack on the inflammatory mediators and thus progression of RA and OA. With the combination of an NSAID and alternate fatty acid substrate both the main inflammatory mechanisms will be modulated and lead to a diminished inflammatory response. While the NSAID acts on the COX pathway the fatty acid can also act on the

COX and LOX pathways as well as inhibiting further inflammatory cytokines and mediators from being expressed by relevant cells.

The action of applying a topical formulation also has its benefits, primarily the psychological effect of applying a treatment to the site where pain is felt and the rubbing action when applying the formulation may help absorption of the drug into the skin. Particularly in arthritic conditions the massaging of the affected area has the effect of increasing blood flow, thus providing warmth and a soothing feeling during application. Many elderly patients are now subject to taking large quantities of oral medications and are averse to the constant swallowing of tablets / capsules. Combining this with the soothing effect of massaging, many patients would prefer to apply a medication to an arthritic joint as opposed to taking further oral medication.

Drug delivery through skin is not simplistic and many considerations must first be taken into account concerning the barrier function of skin and possible delivery mode of the chosen compounds.

1.6 The structure and function of the skin

Skin is a complex organ that has many functions including protection; a barrier to physical and biological threats from the external environment as well as ultra violet (UV) light and the regulation of water loss, body temperature and synthesis of vitamin D with UV absorption. The heaviest organ of the body, the skin comprises approximately 16% of total body weight, is in constant regeneration and is metabolically active in its immunological and histological response to trauma (Williams, 2003). The skin is composed of three histologically distinct layers known as the epidermis, dermis and hypodermis or subcutaneous tissue layer (see Figure 1.12).

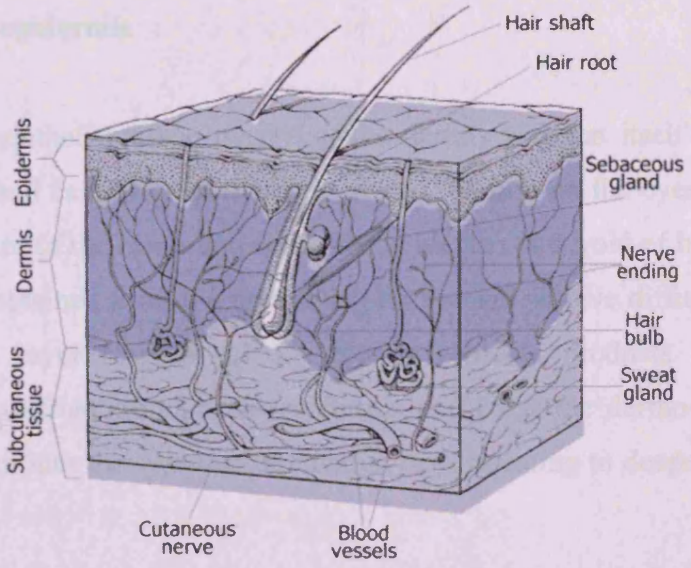


Figure 1.12 Diagrammatic representation of the skin structure (American Medical Association, 1998)

1.6.1 The hypodermis (subcutaneous layer)

The hypodermis is the bridge between the dermis and underlying body constituents (its principal function is body insulation and resistance to mechanical shock). This layer of adipose tissue provides the body with readily available energy-providing molecules and carries the principal blood vessels and nerves to the skin. (Williams, 2003)

1.6.2 The dermis

The dermis is the major component of skin at 3-5mm thick. The hydrophilic nature of the dermis, composed of a network of collagen fibrils and elastic tissue embedded in a mucopolysaccharide gel (Williams, 2003), poses the minimal barrier to hydrophilic compounds but may limit the permeation of lipophilic molecules such as fatty acids / triglycerides to a higher degree. The dermis lies below the epidermis and contains sweat glands, hair follicles, lymphatic vessels, nerve endings, and blood capillaries.

1.6.3 The viable epidermis

This is the live epithelial tissue overlying the dermis and can itself be divided into distinct sections and can differ in thickness from 0.06 mm on the eyes to 0.08 mm on the palms and soles of the hands and feet. The epidermis is devoid of blood vessels and all nutrients are obtained from the underlying dermis via passive diffusion through the dermo-epidermal layer (Figure 1.13). Similarly waste products and permeating compounds must diffuse through the epidermis and cross the dermo-epidermal layer before being taken into the systemic circulation or permeating to deeper regions (Elias, 1989).

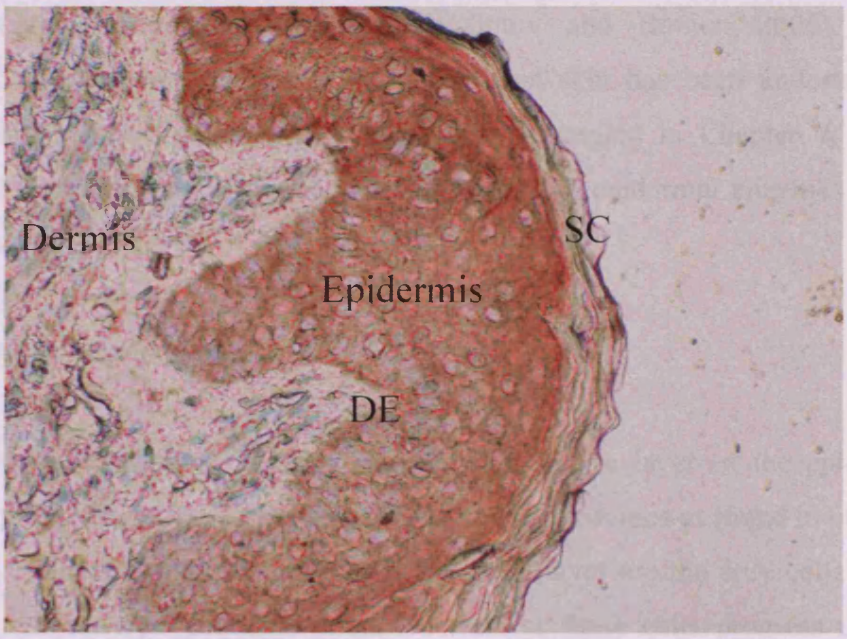


Figure 1.13 Skin section highlighting dermis, epidermis, dermo-epidermal layer (DE) and stratum corneum (SC)

The epidermis is made up of four histologically distinct layers: the stratum basale (the layer closest to the dermis), stratum spinosum, stratum granulosum, with the uppermost layer comprising of the stratum lucidum the stratum corneum (outermost layer). These last layers can generally be thought of as one layer as the stratum lucidum is thought of as the first layer of the stratum corneum. The main cells present in the epidermis are keratinocytes and these cells differentiate as they progress from the basal layer to the SC where they terminally differentiate to dead corneocytes.

1.6.3.1 Enzyme activity of the viable epidermis

The enzyme activity within the viable epidermis has been estimated at approximately <10% of the specific enzymes found in the liver (Hotchkiss, 1998). Nevertheless metabolism of compounds permeating the skin does take place. For example Mavon et al. (2004) reported that 20% of the applied dose of δ-tocopherol glucoside was bioconverted to free tocopherol. Miller and Ziboh, (1988) found that the human epidermis metabolised EPA to 15-hydroxyeicosapentaenoic acid via the 15-LOX pathway. It is therefore important to consider the possible metabolism when delivering drugs through the skin. In many permeation experiments the skin membrane used has been frozen / stored for some period. This may not have been found to effect the permeation rates of certain compounds (Sintov and Botner, 2006), but little investigation to the enzyme viability of the frozen skin has been undertaken. The viability of the skin during permeation process is studied in Chapter 7; Chapter 9 investigates the action of EPA and ketoprofen on two epidermal enzymes associated with inflammation, namely COX-2 and LOX.

1.6.3.2 Stratum basale (basal layer)

The basal layer represents the most metabolically active layer of the epidermis. It contains typical organelles such as mitochondria and ribosomes as found in other tissues in the body. The keratinocytes present in the basal layer are the only cells within the epidermis that undergo continual mitosis. Half of these cells progress upwards to differentiate while the other half remain behind to continue mitosis. The keratinocytes present in the basal layer also produce pro-inflammatory cytokines such as IL-1. The basal layer also contains melanocytes which produce the pigment melanin. This is transferred to keratinocytes and eventually reaches the SC where they are uniformly distributed to form a UV absorbing cover reducing the UV absorption deeper into the skin. Other cells present in the basal layer include, Langerhans cells, the major antigen-presenting cells of the skin, and the Merkel cell, associated with nerve endings beneath the dermis they are found in greater numbers in touch-sensitive areas of the body (Williams, 2003).

1.6.3.3 Stratum spinosum

In this layer of the skin the shape of the keratinocytes begins to change from columnar to polygonal and this represents the earliest stage of cornification. The synthesis of keratins, that aggregate and eventually form desmosomes which connect the cell membranes of adjacent keratinocytes, are formed by the evolving cells. The desmosomes are responsible for the maintenance of an approximately 20nm distance between the keratinocytes.

1.6.3.4 Stratum granulosum

Here the keratinocytes begin to mature further and membrane-coating granules are synthesised which form the precursors for the inter-cellular lipid lamellae seen in the SC.

1.6.3 The stratum corneum (SC)

The SC is the outermost layer of the skin and is a between 10 and 20 μm thick, consisting of terminally differentiated keratinocytes, now referred to as corneocytes, 20 to 40 μm long and approximately 0.5 μm thick (Schaefer and Redelmeier 1996). The corneocytes of the stratum corneum are considered largely impenetrable and are arranged in a tight interlocking fashion surrounded by complex lipoidal domains, composed of lipids, mainly cholesterol, free fatty acids and ceramides. The cells of the stratum corneum are continually shed and renewed from the underlying epidermal layers (Walters, 1990). The corneocytes embedded in the lipoidal domains have been compared to a brick wall with the corneocytes representing the bricks, and the intercellular lipids the mortar (Elias, 1983). The intercellular lipid matrix found between the corneocytes is arranged into bilayers and is formed from the exocytosis of lamellar bodies during terminal differentiation of keratinocytes. It is believed that these bilayers provide the SC its formidable barrier properties and may form the only continuous domain of the SC.

1.7 Routes of drug permeation across the skin

There are three main modes of delivery to be considered when applying a formulation to the skin. Diseases associated with the skin require the drug to be held within the skin and so exert their effect with minimal transdermal or transcutaneous permeation. Drugs with poor oral bioavailability are required to permeate through the epidermis and be carried from the dermis into systemic circulation. Tissues underlying the skin that require treatment need drugs to permeate through the dermis and hypodermis. These three modes of delivery are discussed in detail in the following sections.

1.7.1 Dermatological

Dermatological formulations produce a localised drug effect either on or in the skin. Besides the specific therapeutic action of incorporated active drugs, the formulations can also serve as lubricants or emollients. Treatments using dermatological formulations can help with minor skin infections, itching, burns, athlete's foot (e.g. Canesten® containing clotrimazole), acne (e.g. Oxy 10 containing benzoyl peroxide), psoriasis, and eczema are but a few examples. Such treatment requires the drug or compound of action to be retained within the skin and not pass through into the systemic circulation. In many cases the skin barrier is compromised and retaining the drug in the upper layers of the skin becomes difficult. Certain methodologies can be applied to maintain the concentration of drug within the skin, including the use of liposomes which are spherical vesicle with a membrane composed of a phospholipid bilayer used to encapsulate drugs or genes for delivery. The liposomes permeate the SC relatively easily but cannot permeate the more hydrophilic regions of the viable epidermis and so are retained in the SC where they can release the encapsulated drug. The formation of a reservoir within the SC by a rapidly permeating solvent alters the properties of the tissue into which the drug can partition and help to maintain a large drug concentration within the skin.

1.7.2 Transdermal

Transdermal delivery requires the drug of interest to permeate through the SC and the epidermis, reaching the blood vessels or lymphocytes in the dermis and consequential uptake into the systemic circulation and get transported to their site of action. Such treatments include nicotine replacement therapies and hormonal replacements.

1.7.3 Transcutaneous

Transcutaneous delivery involves the permeation of the drug across the complete skin membrane to underlying tissues. The drug must be able to permeate the lipophilic SC, then the hydrophilic viable epidermis before by-passing the blood vessels within the dermis and permeating through to the underlying tissue. This is particularly important in the case of arthritis as the inflammation process is occurring beneath the skin, within the joints e.g. Oruvail. This is an example of a formulation that that has been designed to deliver drug in this way. The main limitation to this mode of delivery through the skin is the clearance of the drugs into the systemic circulation. The rate of permeation of the compounds must out-weigh the rate of clearance to allow further permeation. Cutaneous dialysis is a method of determining the clearance rate of hydrophilic molecules but has had little success with molecules of a lipophilic nature (Morgan et al. 2003). This technique has also been used by Church (1997) to follow inflammatory reactions within the skin.

1.7.4 Crossing the stratum corneum

The three modes of topical delivery detailed above all share a common factor; the active agent must first cross the SC. Three main routes of entry into the skin from an applied formulation have been proposed (see Figure 1.14). The permeation of drugs via routes 1 and 3 also called the shunt route is an area of great scrutiny. Although the sweat glands and hair follicles only occupy approximately 0.1% of the total skin surface (Scheuplein, 1967) they have been shown to play a significant part in drug permeation. Illel et al. (1991) showed that appendage free rat skin produced four-fold lower

permeation values of several drugs. Barry (2002) provides many examples of formulations utilising this route including liposomes and naked DNA molecules and Williams. (2003) discusses how this route of permeation may be important for large polar molecules and ions that would have difficulty it permeating through the SC.

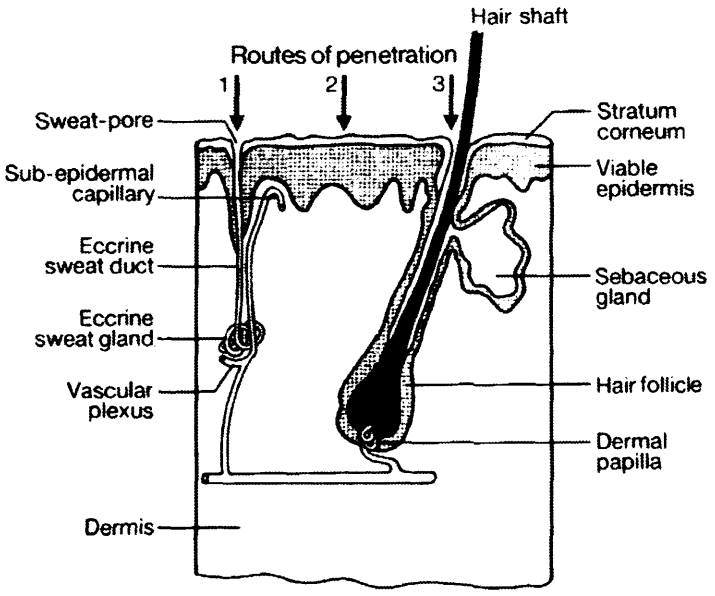


Figure 1.14 Diagram of skin structure and routes of drug penetration (Barry, 2002). Route 1, via the sweat ducts; route 2, across the continuous stratum corneum; route 3, through the hair follicles with their associated sebaceous glands

Route 2 can be sub-divided into two further permeation routes; transcellular, through the cell and intercellular, around the cells through the lipid bilayers. Figure 1.15 illustrates these two routes of permeation through the SC.

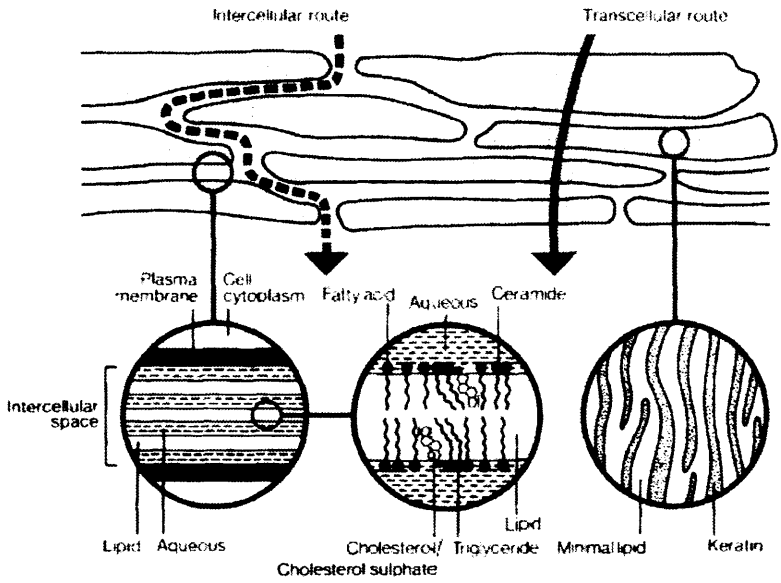


Figure 1.15 Diagrammatic representation of SC membrane (Barry, 2001)

1.7.4.1 Transcellular permeation

The transcellular route represents the most direct route through the SC. The molecule crossing the SC in this way is however presented with several challenges. The molecule must first partition into the top layer keratinocyte followed by diffusion through the hydrated keratin within the cell (Hadgraft et al., 1992). When delivering a compound such as a fatty acid the diffusion of such a compound into the cell may also lead to incorporation of the fatty acids within the cell membranes. This is more likely within the lower cell layers of the SC as the upper layers are terminally differentiated. It is generally assumed therefore that this route is likely to be avoided by most drugs. However, evidence supporting transcellular permeation has been found and is discussed in Williams (2003) and Perkins and Heard, (1999).

1.7.4.2 Intercellular permeation

This involves the diffusion of compounds through the lipid bilayers that surround the corneocytes. The lipid bilayers make up approximately 1 % of the SC diffusional area and represent the best permeation route for a lipophilic molecule although not a complete uninterrupted route. The diffusional pathlength that the molecule must travel

is significantly greater than the 15-20μm thickness of the SC and can be up to 350μm for methyl nicotinate (Albery and Hadgraft, 1979) and the pathlength for the diffusion of water was found by Potts and Francoeur (1991) to be 500μm. The actual pathlength of a molecule would heavily depend upon its physico-chemical characteristics and as such the diffusional pathlength differs from molecule to molecule. The composition of the lipid bilayers in the skin are unique, in that they lack phospholipids and contain a variety of ceramides.

The route of drugs through the skin is therefore arduous at best, and physicochemical characteristics of the drugs also play a large part in the permeation route. Ideal factors including molecular weight (not greater than 700Da), pKa (compatable with skin pH) and log P (the octanol / water partition, generally required to be between 2 and 5) all add to the complications of delivery through the skin. Ways of enhancing the permeation of compounds have been examined in great detail and Section 1.8 presents some of the enhancement techniques commonly studied.

1.8 Permeation enhancement strategies

To enhance the permeation of drugs through the skin, several possible strategies can be employed. Some techniques involve the disruption of the SC barrier, another involves the by pass of this barrier completely. The sections that follow summarise some of the common enhancement strategies.

1.8.1 Physical Modulation

1.8.1.1 Microneedles

A traditional solution to avoiding the SC is to use a hypodermic needle; however patient compliance due to the fear of needles, the pain caused and possible cross-contamination lead to the development of microneedles. When applied to the skin they puncture through the stratum corneum and into the viable epidermis. The heights of the needles are such that the pain receptors deeper in the skin are not triggered. The microneedle

device has recently been found to allow DNA delivery and subsequent gene expression within the viable epidermis (Coulman et al. 2005). The use of microneedles does pose certain problems. Breakage of the silicon needles can occur and these fragments may remain in and irritate the skin. Needle-less injection employs a gas powered gun to fire small particles into the skin in an attempt to avoid pain receptors. This strategy does have its benefits such as localised delivery but also its disadvantages as SC variation between subjects would vary the depth to which the particles could penetrate. Damage to the SC is also of concern as leaving "holes" in the SC would lead to reduced barrier function and possibly infection (Williams, 2003).

1.8.1.2 Iontophoresis

Other physical techniques include iontophoresis, employing an electrical potential gradient to aid drug delivery. The action of iontophoresis lies in the ability to repel charged molecules from two electrodes, with positively charged molecules being repelled by the anode and negative molecules by the cathode. The majority of molecules permeate via appendageal routes although some via paracellular pathways. Factors including skin pH, molecular weight and ionic competition affect the usefulness of this technique but it has found uses for the delivery of anti-inflammatory agents. Mathy et al., (2005) found that iontophoresis was efficient in delivering high concentrations of flurbiprofen to the dermis and underlying tissues of hairless rats. Similarly Curdy et al., (2001) found that iontophoresis improved the delivery of piroxicam into the SC of human volunteers compared to passive diffusion alone. Both results could be due to the increased permeation via appendageal routes when using inotophoresis. The use of iontophoresis in delivering drugs for the treatment of arthritic conditions is discussed in detail by Rosenstein, 1999.

A similar method is ultrasound or sonophoresis, using ultrasound with a frequency beyond 20kHz to facilitate the drug through the skin with an ultrasound probe.

1.8.2 Chemical enhancement

This thesis is focused on delivering a fatty acid / triglyceride with ketoprofen through porcine skin *in-vitro* and so these physical methods would be impractical and so were not considered. The second strategy of enhancement uses chemical enhancement, studied in Chapter 4. It is important to evaluate the possible chemical enhancers to demonstrate that the correct method was employed for the proposed application.

1.8.2.1 Dimethylsulfoxide (DMSO)

DMSO is a powerful aprotic solvent and is one of the earliest and most widely studied enhancers. Barry and Williams (2003) discuss the use of DMSO as an enhancer and conclude that it enhances both hydrophilic and lipophilic drugs. They also state that the levels required to attain the greatest enhancing effects are >60% DMSO which at that concentration causes erythema and damage to the skin.

1.8.2.2 Fatty acids

Fatty acid permeation enhancement using oleic acid has been well studied. (Barry and Williams, 2003) but like several other mechanisms of enhancement, the compound acting as the enhancer is assumed to do no more than temporarily effect the skin before being washed out of the stratum corneum. Fatty acids as enhancers are discussed in detail in Chapters 3, 4 and 7.

1.8.2.3 Alcohols

Ethanol is a common solvent for many formulations as not only does it act as a solvent but enhances the permeability of the drug within the formulation. Recently Kung et al. (2006) have shown the flux of mefenamic acid to be proportional to the flux of ethanol. As the concentration of ethanol increased in the formulation a corresponding increase was seen in the concentrations of permeated mefenamic acid and ethanol. This novel work has indicated the possibility of co-permeation related to the enhancers ability to solvate the permeating drug.

1.8.2.4 Pro-drugs

A form of chemical enhancement is the use of pro-drugs. These are compounds which typically aren't suitable for transdermal / transcutaneous delivery and are chemically modified to allow permeation through the skin. The most common form of modification is the formation of ester linkages. This allows greater permeation of drugs through the lipophilic SC. Once the pro-drugs reach the viable epidermis the esterases present within these skin layers can break this ester linkage and thus return the drug to its original, active form.

1.8.2.5 Terpenes

Terpenes are a group of non aromatic compounds comprising of carbon, hydrogen and oxygen and found in essential oils including menthol and limonene. 1,8-cineole is the major terpenoid of eucalyptus oil and has found application in a number of enhancement studies (Thomas and Heard, 2005; Feminia-Font et al. 2005; Yamane et al. 1995). 1,8-cineole was also found to have a concentration dependent effect on mefenamic acid and again implies a co-permeation or drag effect upon the drug by the enhancer. The action of 1,8-cineole is discussed in Chapter 4.

1.9 Objectives and Aims

The objectives of this project was to test the hypothesis that a dual action formulation containing ketoprofen and fish oil could be efficacious in the treatment of symptoms associated with arthritic conditions, in particular RA and OA.

The associated aims were:

i. To develop a formulation capable of transcutaneously delivering ketoprofen and EPA from fish oil
ii. To investigate the delivery of the active compounds into a joint capsule and diffusion into synovial fluid
iii. To determine the efficacy of the formulation against the biological symptoms of arthritis within cartilage
iv. To investigate the fate of the compounds during skin permeation
v. To probe complexation issues of formulation components

Chapter 2

Analytical Methods

2.1 *In-vitro* permeation experiments

2.1.1 Skin preparation

Porcine ears were obtained from a local abattoir, cleaned under running water and the hair shaved using electric clippers. Full thickness dorsal skin was removed from the underlying cartilage using a scalpel, cut into approximately 2cm x 2cm and stored at –20°C until required. Porcine skin was used as it has been shown to be one the best *in-vitro* models for human skin available (Simon and Maibach, 2000). As this thesis is studying transcutaneous delivery full thickness skin was used.

2.1.2 Franz cell diffusion

The standard tool for determining *in-vitro* skin penetration and permeation is the Franz diffusion cell or variants thereof (Williams, 2003). All *in-vitro* permeation experiments throughout this thesis were carried out using all glass Franz diffusion cells, of the type illustrated in Figure 2.1. The complete cell consists of two chambers, the lower (receptor) and the upper (donor). The nominal receptor volume and diffusional area was 3.5 ml and 1.5cm^2 respectively – these are important parameters for the accurate quantitative determination of permeation parameters. To limit leakage the mating flanges of both halves were pre-cleaned and greased with high vacuum grease (Dow Corning, Barry, UK). The square skin sections were placed between the two chambers with the SC uppermost and the chambers clamped together. The appropriate receptor phase (de-gassed beforehand) was then added to the receptor chamber ensuring no bubbles were present beneath the underside of the skin. To assist mixing (regarding stagnant diffusion layers) a micro-stirrer bar was added and the complete cell placed on a magnetic stirrer plate (Variomag, Daytona Beach, USA) in a water bath (Clifton, Fisher, UK) set at 37°C (to provide a skin surface temperature of 32°C via heat dissipation) (Figure 2.2). The sampling arm of the receptor compartment was occluded using a cap to ensure no loss of receptor phase. After a pre-determined equilibration time (typically 10 minutes) the cell was dosed via the donor chamber which was

occluded using laboratory film. Each bath can accommodate up to 15 cells. All other details for specific experiments are detailed in individual chapters.

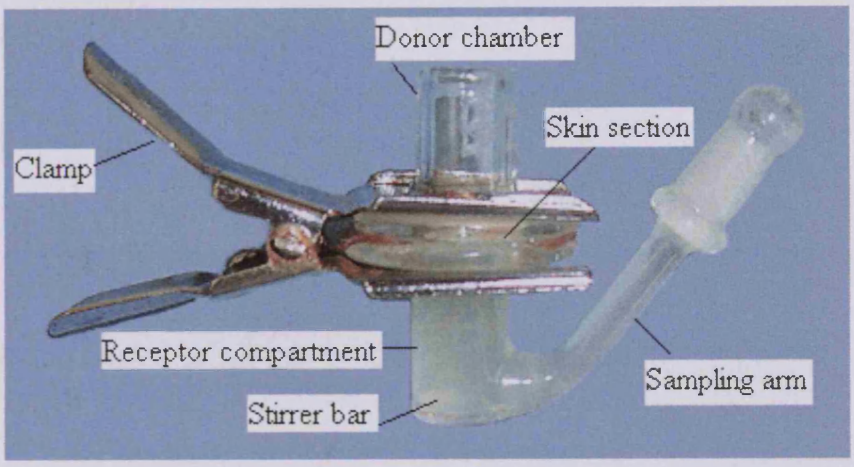


Figure 2.1 Glass Franz-type diffusion cell

Figure 2.2 Set of 12 diffusion cells in water bath

2.1.3 Receptor phase

The choice of receptor phase in any membrane permeation/penetration experiment is of major importance. The primary function of a receptor phase is to provide a sink for the permeated compounds and thus an optimum receptor phase should be one in which the amount of permeated drug contained should never exceed 20% of its solubility in that liquid (Williams, 2003). This is particularly an issue for lipophilic permeants. A further requirement of a receptor phase is that it does not adversely affect the membrane

by reverse diffusion, thus those with high alcohol content should be avoided as, e.g. ethanol is capable of leaching lipids of the stratum corneum (Barry, 1987).

In this work the receptor phase used for EPA / DHA and ketoprofen was cetrimide. Cetrimide solution (30 mg ml^{-1}) containing 0.05% butylated hydroxyanisole (BHA) as a standard reagent to inhibit decomposition of the polyunsaturated fatty acids was found to provide an effective sink for ketoprofen and fish oil. In this solution, ketoprofen was freely soluble and fish oil solubility was estimated at 5.1 ± 0.3µl ml^{-1} (5.2 ± 0.3mg ml^{-1}), approximating to 1.73 ± 0.1 mg (5.72 ± 0.33µmol) EPA ml^{-1}, by adding sequential aliquots of the oil to receptor phase at 37°C until a clear solution was no longer attainable. Furthermore, a preliminary study indicated that this receptor phase performed in a similar manner to the more ubiquitous alcohol-in-water system, giving rise to no perceptible deleterious effects on the skin (Heard et al, 2002). The optimum receptor phase would be to use the phase that the compounds would eventually permeate i.e. blood or specifically in this case synovial fluid. For the purposes of the initial permeation experiments it was thought that the use of cetrimide would be an acceptable receptor phase, offering a suitable sink, as the acquisition of large quantities of blood and synovial fluid is difficult due to regulatory implications. Chapter 5 uses the whole joint of a porcine forelimb and therefore provides the best receptor phase available, synovial fluid.

2.2 Quantitative determination of EPA and DHA

Several methods were investigated to determine the concentration of fish oil (EPA/DHA) from the receptor phase samples during a permeation experiment. The usual analysis method for fatty acids is gas chromatography (GC), due to the unavailability of this instrument HPLC analysis was used. As the concentrations of individual fatty acids present in the fish oil was to be determined by HPLC, it was thought that they must first be liberated from the triacylglycerols as which, they are bound. To accomplish this, the fatty acids were derivatised. The derivatised sample must be highly UV active in order to analyse the samples using an HPLC with UV detection and the conditions harsh enough to allow the derivatisation of each individual

fatty acid present as triacylglycerols. The first derivitisation method used was the formation of fatty acid phenacyl esters.

2.2.1 Preparation of fatty acid phenacyl esters

2-bromoacetophenone in acetone (10mg/ml) (10ml) and triethylamine in acetone (10mg/ml) (10ml) were added to a sample of fish oil (50mg) prior to heating under reflux at 70°C for 30 minutes. The reaction was kept under nitrogen throughout. Acetic acid (7ml) was then added and the reaction mixture heated to reflux for a further 5 minutes (Nichols and Davies, 2002). The solvent was then evaporated under vacuum and a pale yellow liquid remained. The sample was then dissolved in acetonitrile / water, 95/5 for HPLC analysis.

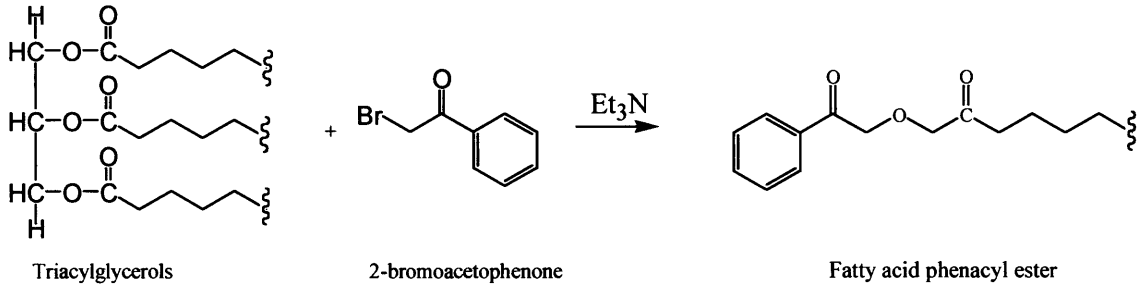


Figure 2.3 Reaction scheme for polynunsaturated fatty acid phenacyl ester preparation

Note: As this reaction must be free of water to go to completion, the receptor phase samples were freeze dried to remove any traces of water which would compete with the 2-bromoacetophenone.

2.2.1.1 HPLC analysis of fatty acid phenacyl esters (FAPE)

FAPE samples were analysed on a Hewlett Packard 1100 HPLC system with a variable wavelength detector. LC separations were carried out on a Phenomenex Kingsorb C18 column (150 x 4.6mm, 5μm) fitted with a Phenomenex Securiguard guard column. The mobile phase conditions were acetonitrile / water (95:5; v/v) run, isocratically, for 20

minutes at a flow rate of 1.5ml/min. UV detection was set at 240nm. Retention times of EPA and DHA were 6.0 minutes and 6.7 minutes respectively.

2.2.1.1.1 Liquid Chromatography – Mass Spectrometry (LC-MS) analysis of FAPE samples

FAPE samples were analysed on a Finnigan SN 4000 series with a diode array detector in series with a Finnigan LCQ mass spectrometer. HPLC separations were as above. The mobile phase conditions were acetonitrile / water (95:5; v/v) run, isocratically, for 20 minutes at a flow rate of 1.5ml/min. Wavelengths from 190 to 300nm were monitored on the diode array detector. The LC-MS was run under the following conditions: APCI (atmospheric pressure chemical ionisation) source, vaporiser temperature 450°C, capillary temperature 150°C, discharge current 10.00μA, sheath gas 80 psi, auxillary gas 10 psi, capillary voltage –24.00V. The range from *m/z* 150-1000 was scanned. EPA and DHA eluted at 7.4 and 8.3 minutes respectively. Their corresponding *m/z* ions were 421 and 447 respectively.

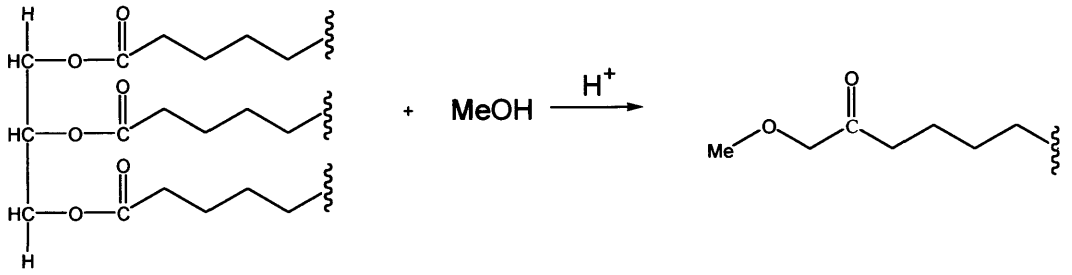
The mass spectrometry validated the FAPE HPLC analysis in that the peaks identified in the HPLC chromatogram were indeed the FAPEs of EPA and DHA. Quantification of EPA or DHA present in the fish oil was not carried out as the method provided suitable results but proved very time consuming. The preparation of fatty acid methyl esters (FAMEs) was therefore investigated.

2.2.2 Preparation of fatty acid methyl esters (FAMEs)

An improved method of derivatisation was found and validated involving the formation or fatty acid methyl esters (Heard et al., 2002)

PUFAs were transmethylated (see Figure 2.4) by heating under reflux in a sealed methylating tube with anhydrous methanol (10ml) in the presence of acid catalyst (2.5% v/v H_2SO_4) for 2 hours at 70°C. After this time the solution was allowed to cool to room temperature and the reaction quenched by the addition of aqueous NaCl (5% w/v)

(2.5mls). The FAMEs were then extracted with 3 x 30mls petroleum ether (60 – 80°C b.p.), the ether phases pooled and evaporated to dryness under vacuum. The remaining product was dissolved in methanol (2ml), bubbled with nitrogen, sealed and stored under light exclusion prior to HPLC analysis.



Triacylglycerols

Fatty acid methyl ester

Figure 2.4 Transmethylation of a triacylglycerol

2.2.2.1 HPLC analysis of FAMEs

FAMEs were again analysed on a Hewlett Packard 1100 HPLC. Separations were carried out on a Phenomenex Kingsorb C18 column (150 x 4.6mm, 5μm) fitted with a Phenomenex securiguard guard column. The mobile phase conditions were methanol / water (95:5; v/v) run, isocratically, for 20 minutes at a flow rate of 1.0 ml min^{-1}. UV detection was set at 210nm. EPA and DHA eluted at 10.8 minutes and 12.8 minutes respectively.

2.2.2.1.1 LC-MS analysis of FAMEs

FAMEs were analysed on a Finnigan LC SN 4000 series with a diode array detector in series with a Finnigan LCQ mass spectrometer. LC separations were carried out on a Phenomenex Kingsorb C18 column (150 x 4.6mm, 5μm) fitted with a Phenomenex securiguard guard column. The mobile phase conditions were methanol / water (95:5; v/v) run, isocratically, for 20 minutes at a flow rate of 1.0ml/min. Wavelengths from 190 to 300nm were monitored on the diode array detector. The LC-MS was run under the following conditions: APCI source, vaporiser temperature 450°C, capillary

temperature 150°C, discharge current 5.00μA, sheath gas 80 psi, auxillary gas 10 psi, capillary voltage 26.00V. The range from *m/z* 150-1000 was scanned.

The LC-MS analysis validated the FAME HPLC method. The values quoted by Boots PLC for the concentration of EPA and DHA were 330 mg per 1000 mg and 21 mg per 1000 mg respectively. The results from this method of analysis were found to correlate with the values quoted by Boots PLC. Therefore this method was used for the analysis of EPA and DHA in Chapters 3, 4 and 6.

2.2.3 HPLC analysis of free fatty acids

During the work carried out in Chapters 5 and 7 it was discovered that the analysis of free fatty acid of EPA could be analysed in the same HPLC method as stated in Section 2.2.2.1. Under these conditions EPA free fatty acid eluted at 6.9 minutes. A standard calibration curve, prepared in methanol (extraction solvent), was constructed over the range 1-250 μg ml^{-1} and provided r^2 values of > 0.99. This was again validated by LC-MS, as Section 2.2.2.1.1, and the values of EPA present in the fish oil were found to correlate with values quoted by the supplier.

2.3 HPLC analysis of 15-HEPE

The HPLC conditions for the analysis of 15-HEPE are as Section 2.2.2.1. Under these conditions 15-HEPE eluted at approximately 4 minutes. A standard calibration curve, prepared in methanol (extraction solvent), was constructed over the range 1-250 μg ml^{-1} and provided r^2 values of > 0.99. The LOD of 15-HEPE was 0.7 μg ml^{-1}.

2.4 HPLC analysis of ketoprofen

Ketoprofen samples were analysed on a Hewlett Packard 1100 HPLC system with a variable wavelength detector. Separations were carried out on a Phenomenex Kingsorb C18 column (150 x 4.6mm, 5μm) fitted with a Phenomenex Securiguard guard column. The mobile phase was acetonitrile / potassium phosphate buffer (pH 1.5) (55:45; v/v)

run isocratically for 10 minutes at a flow rate of 1.0 ml/min. UV detection was set at 258nm. The retention time of ketoprofen was 6.8 minutes and the limit of detection (LOD) was 0.03 $\mu g\ ml^{-1}$. A standard calibration curve was constructed over the range 1-120 $\mu g\ ml^{-1}$, prepared in receptor phase, which provided an r^2 of 0.99

2.5 HPLC data analysis and construction of permeation profile

The standard calibration curve along with the sample peak area was used to calculate the concentration of compound in the receptor phase. To avoid solvatochromatic effects, calibration curves were always prepared using the same medium as the test samples. Cumulative ketoprofen, EPA and DHA concentrations ($\mu g\ cm^{-2}$), corrected for sampling were plotted against time, ± the standard error of the mean (s.e.m). S.E.M was used instead of SD as replicates were performed over 2 or more occasions. Steady state flux, J_{ss}, was determined from the gradient of the linear portion of the plots. Lag time, where applicable, was determined by extrapolation to the x axis from the linear portion of the graphs. The partition coefficient k_p was determined by the division of the steady state flux J_{ss} by the concentration of drug in the formulation applied to the skin.

2.6 Depth profiles by tape stripping

Whereas permeation parameters can provide information concerning the transport of molecules across skin membranes it tells us little about the deposition or localisation of drug within the skin. Tape stripping is a technique which allows the concentration of a drug to be determined as a function of depth through the layers of the skin.

2.6.1 Depth profile methodology

A D-Squame® adhesive disc (1cm in diameter) was placed firmly over the diffused area of the skin sample. The strip was then removed using tweezers and placed in a clean glass vial. This was repeated using 30 individual strips which were grouped three per vial before methanol (2ml) was added and the vial left for approximately 24 hours under light exclusion on a laboratory shaker. The remaining epidermis was heat separated

from the dermis by placing the skin section epidermis side down onto a glass plate maintained at 55°C for approximately 30 seconds. The remainder of the epidermis was removed and both the dermal and epidermal tissue placed in separate glass vials with methanol (4ml) and extracted as above.

2.7 Immunocytochemistry (ICC) methodology

Immunocytochemistry is a method used extensively to probe the activity of enzymes within tissues and cells. It has previously found use for the analysis of COX, in UVB induced skin inflammation, (Athar et al. 2001) and for LOX in lung tumours in a study carried out by Gonzalez et al. (2004).

Stage 1 of any staining process is the fixing and dehydration of the skin samples. After the appointed time within the Franz cell apparatus, the skin is removed and the donor phase / formulation is removed from the surface of the skin with distilled water and wiped with a fresh tissue. The skin is then cut into approximately 2mm thick strips using a clean surgical blade, ensuring that the strips are taken from the area on which the formulation was applied. Following this, the skin is placed into 7 x 5 cm cassettes and the cassettes are snapped shut. The skin within the cassettes then undergoes the procedure as detailed in Table 2.1. The formaldehyde treatment fixes the skin while the ethanol treatment removes water. The xylene steps then displace the ethanol for further treatment.

Table 2.1 Procedure of fixation and dehydration of skin samples for ICC analysis

Solvent	Time of Treatment
4% Formaldehyde	24 hours
70% Ethanol	~16 hours
70% Ethanol	30 minutes
90% Ethanol	2 x 30 minutes*
100% Ethanol	2 x 30 minutes*
100% Xylene	60 minutes
100% Xylene	3 x 30 minutes*

*Each treatment carried out using fresh solvent

Once the fixation and dehydration procedure has been carried out the skin is ready to be embedded in paraffin to enable accurate sectioning. Before the embedding can take place the removal of xylene must first occur. This is achieved by placing the cassettes into 3 equivalent molten wax baths and applying a vacuum for a specified interval (see Table 2.2).

Table 2.2 Removal of xylene

Wax bath no.	Vacuum off (min)	Vacuum on (min)	Vacuum off (min)
1	15	10	5
2	10	15	5
3	10	15	25

The lid of the storage cassette is then removed and molten paraffin wax is poured over the skin and allowed to cool. The embedded skin is now in such an orientation as to allow cross sections from SC to dermis to be taken. The sections are cut using a Shandon Finesse microtome to a thickness of 5μm and are placed onto 2.5 cm x 7.5 cm x 1mm Surgipath pre-cleaned microslides.

The skin is then heated at 37°C for one hour and undergoes the following set of procedures to de-wax and re-hydrate the skin (Table 2.3).

Table 2.3 De-waxing and re-hydrating

Solvent	Treatment Time (mins)
Xylene	2 x 7
100% Ethanol	2 x 3
90% Ethanol	2 x 3
70% Ethanol	2 x 3
Distilled H_2O	1 x 5
PBS*	1 x 5

*Phosphate buffered saline

Xylene is used to remove any wax within the skin and present on the slide. Ethanol displaces the xylene from the cells and the skin is gradually re-hydrated.

2.7.1 Staining Procedure

To block endogenous peroxidases that would interfere with the staining process, an aqueous solution of hydrogen peroxide (3%) was applied to each section and left for 5 minutes. Excess solution was then removed by 2 x 3 minute washes in PBS.

2.7.2 Antigen unmasking

The sections were then micro-waved in 1L sodium citrate buffer (0.01M), adjusted to pH 6 with HCl for 30 minutes at 560 Watts. The slides were then cooled for ten minutes under running water and then washed twice for three minutes using PBS.

2.7.3 Application of primary antibody

Unspecified proteins were blocked by applying a PBS plus 0.2% Tween solution to the sections for 15 minutes. The excess blocking reagent was then removed and the primary antibody for the specific enzyme was applied to the sections and left at 23°C overnight. See Chapter 9 for details of primary antibody.

2.7.4 Application of secondary antibody and label

Slides were first pre-washed using a 1 x 3 minute PBS wash and then 2 x 5 minute PBS/Tween washes. A Dako Corporation horse radish peroxidase (HRP) labelled rabbit EnVision detection system was applied and the slides incubated for 2 hours at 23°C. The detection system contains both secondary antibody and label. The slides were then placed into a 1 x 3 minute PBS wash and then 2 x 5 minute PBS/Tween washes. Dako DAB was then applied to the sections and left for 10 minutes. The slides were then rinsed in distilled water for 3 x 2 minutes.

2.7.5 Counterstaining

An aqueous solution of 0.5% methyl green was applied to the sections for 5 minutes and was then rinsed off with distilled water for 2 x 3 minutes. The sections were then dehydrated at 37°C and Menzel-Glaser 22 x 40 mm cover-slips fixed over the sections using a distyrene, plasticizer, xylene (DPX) mix. The sections were then oberserved and photographed using an Olympus BH-2 microscope with Olympus digital camera.

2.8 Nuclear Magnetic Resonance (NMR) Spectral Modulation

^{1}H NMR spectra were obtained using a Bruker Avance DPX400 spectrometer operating at 400MHz and 27°C. Sub-saturated solutions of ketoprofen (2.5% w/w) in fish oil, mixtures of fish oil and Miglyol 812N, 1:10, 1:20, 1:1, 10:1 and 20: and pure Miglyol 812N were prepared and used as a control vehicle. A fixed volume of formulation (25µl) was added to separate NMR tubes and $CDCl_3$ (475µl) was added as solvent. These volumes were found to produce suitability strong NMR signals. The differences in shifts of the aromatic protons compared to a ketoprofen control were calculated and the individual proton shifts were plotted. These experiments were repeated using EPA and DHA free fatty acid standards. Due to the small amount of material available, serial dilutions of a 25mg ml^{-1} solution in Miglyol 812N were prepared and saturated with ketoprofen. Control experiments were carried out using ketoprofen in $CDCl_3$.

2.9 Molecular modelling and binding energy calculation

In collaboration with Dr. Jamie Platts, Chemistry Department, Cardiff University, individual molecules of ketoprofen, EPA and the triglyceride of this fatty acid, were built using the Molecular Builder function of the MOE package (http://www.chemcomp.com/), and minimised using the MMFF-94 force-field (Halgren, 1996) until no force on any nucleus exceeded 0.05 kcal / (Ång mol).

Complexes between ketoprofen and EPA constructed by bringing together optimised structures and re-optimising using the same force-field and force cut-off. This resulted

in little or no change to the conformation of either drug or fatty acid. The conformational energy landscape of these complexes was explored by randomly altering the mutual orientation of drug and fatty acid as well as the dihedral angles of any rotatable bonds. This was followed by full energy minimisation. Throughout this procedure, the cis orientation of the C=C bonds in fatty acids was maintained using harmonic restraints, which were subsequently removed in the final minimisation. These stochastic searches proceeded until either 1000 conformations were found, or no new low-energy conformations were found after 1000 searches.

Binding energies were calculated by subtracting the energies of the individual molecules from the total energy of the complex, all calculated using the MMFF-94 force-field. In all cases the contribution to binding energy from bond length, angle and dihedral terms was effectively zero, such that binding was a combination solely of electrostatic and Van der Waals forces.

Chapter 3

In-vitro transcutaneous delivery of ketoprofen, EPA and DHA from simple gel formulations

3.1 Introduction

It has previously been demonstrated that the simultaneous transcutaneous permeation of NSAID and essential fatty acids from fish oil across excised porcine skin using a saturated fish oil vehicle is possible (Heard et al., 2003). Solanki et al, (2005) have also shown that topically applied safflower oil (rich in linolenic and arachidonic fatty acids) can be absorbed in neonates and may be available for nutritional purposes. They also concluded that the fatty acid constituents of topically applied oil can "influence the changes in the fatty acid profile of the massaged babies." A transcutaneous system containing fish oil as a source of EPA and DHA therefore also represents a potentially useful vehicle for the delivery of these essential fatty acids and thereby treatment of inflammatory conditions.

This chapter studies three basic formulations containing fish oil and ketoprofen. Ketoprofen was selected as the NSAID of choice as Hadgraft et al., (2000) showed ketoprofen to provide the best predicted bio-effectiveness when considering maximum flux and IC_{50} values (Cordero et al., 2001). The formulations prepared in this chapter are the first stage in the development of a commercial formulation as previous work (Heard et al., 2003) employed saturated solutions of fish oil and NSAID but this does not represent commercially available NSAID products. In most available products this is 5% w/w for ibuprofen formulations (e.g. Ibuleve™) and 2.5% w/w for ketoprofen (e.g. Oruvail™). For this reason 2.5% w/w ketoprofen is used throughout the work. Furthermore, as it has already been demonstrated that significant amounts of ketoprofen and fish oil permeate skin (Heard et al., 2003a), and thermodynamic activity considerations were considered to be of less relevance. In this case the pull / drag effect is thought to be of more importance due to the solvation effects that may be seen within a formulation of this kind (Heard et al., 2006). BHA antioxidant was added to limit PUFA oxidation.

3.2 Materials and methods

3.2.1 Materials

Boots Super Strength concentrated fish oil capsules 1000mg (Batch number: BE09) were used as a source of EPA and DHA. Ketoprofen, polyethylene glycol 400N (PEG 400), hydroxypropyl cellulose (HPC), cetrimide and butylated hydroxyanisole (BHA) were obtained from Sigma – Aldrich Company Ltd., Poole, UK.

3.2.2 Gel preparation

Four gels were prepared for the initial skin permeation experiments with the compositions shown in Table 3.1. PEG 400 was heated to approximately 40°C on a temperature controlled heating mantle and the required mass of BHA was then added and allowed to dissolve at 40°C. The required mass of ketoprofen was added and allowed to dissolve followed by the fish oil. HPC was then added and the gels were allowed to mix overnight using a blood serum rotator. This produced a clear, bubble-free gel of appropriate (although arbitrary) viscosity.

Table 3.1 Gel formulations

Formulation	Fish Oil (%)	Ketoprofen (%)	PEG (%)	HPC (%)	BHA (%)
I	0	2.50	89.50	8.00	0.05
II	5.00	2.50	84.50	8.00	0.05
III	10.00	2.50	79.50	8.00	0.05
IV	20.00	2.50	69.50	8.00	0.05

3.2.3 Skin preparation

Skin was prepared as in Chapter 2, section 2.1.1.

3.2.4 Skin permeation experiments

Skin permeation experiments were carried out as in Chapter 2, Section 2.1.2. Porcine ear specimens were equally distributed between the gels to eliminate donor variability. The receptor phase chamber was filled with a cetrimide solution (30 mg/ml) and BHA (0.05% w/v) receptor phase and a micro stirrer bar added. After 10 minutes temperature equilibration time an infinite dose of 0.7g (approximately 1ml) gel and was added to the donor chamber. A total of three replicates were carried out on four occasions. At 3, 6, 12, 24, 36 and 48 hours the entire receptor phase was removed with 500µl transferred to a HPLC auto-sampler vial for the analysis of ketoprofen and the remainder of the receptor phase transferred to a larger screw-capped vial for the analysis of the fish oil. The Franz cell was replenished with fresh receptor phase after each point.

3.2.5 HPLC Analysis

Fish oil and ketoprofen were assayed as outlined in Chapter 2 Section 2.2.2 and 2.4 respectively.

3.2.6 Data Analysis

The activity of fish oil as an enhancer was expressed as an enhancement ratio for the flux of ketoprofen (ER_{flux}), where ER_{flux} = Drug flux no fish oil/ Drug flux with fish oil.

Statistical analyses were carried out using Instat 3 for Macintosh (GraphPad Software, Inc), where non-parametric Kruskal-Wallis ANOVA and Dunn's multiple comparison tests were employed to determine differences between data sets and specific pairs respectively. These tests are used due to the no Gaussian population within and between data sets.

3.3 Results

3.3.1 Ketoprofen

Figure 3.1 shows the cumulative permeation profiles for ketoprofen across porcine skin from the four gels. Typical profiles were obtained, although steady state flux may not have been attained in all cases as the rate continued to increase by the end of the experiment. However a trend is clear in that there was a concentration-dependent relationship between ketoprofen permeation and fish oil content.

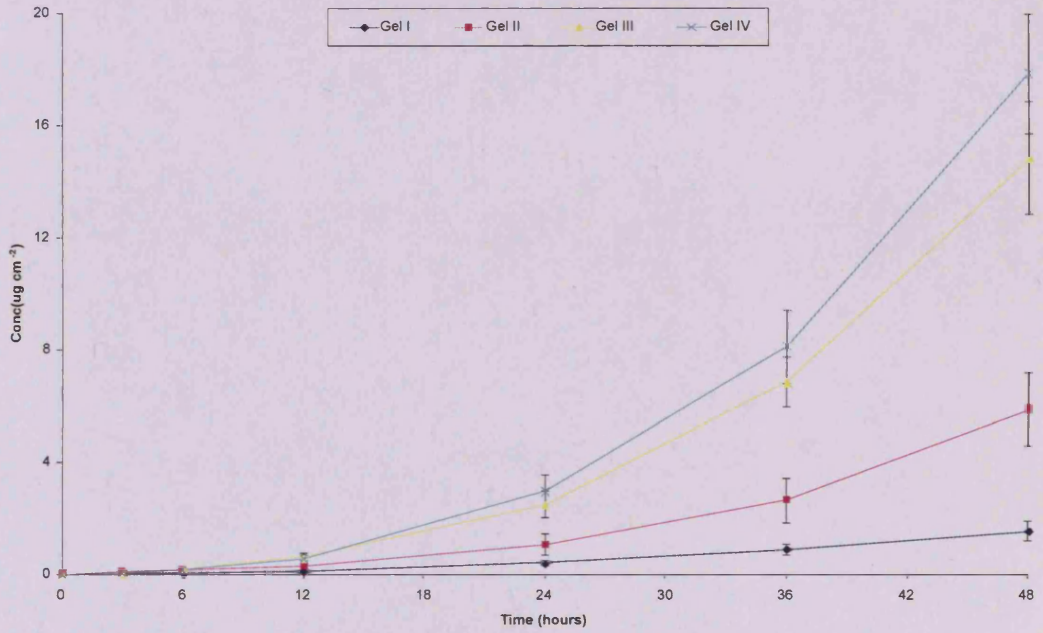


Figure 3.1 Cumulative permeation of ketoprofen across porcine skin from 4 gels (n=5 ± s.e.m)

The apparent steady state flux and permeability coefficient (K_P) for ketoprofen from each of the gels is shown in Table 3.2, where steady state flux was taken between 24-48 hours. All gels produced significantly different flux values from control, ($p = < 0.001$ for gels III and IV compared to control and $p = < 0.05$ for gel I compared to control). The steady state flux for ketoprofen from the control gel I was 4.72×10^{-2} μg cm^{-2} h^{-1}. Gels, II, III and IV showed a ketoprofen steady state flux of 20.07×10^{-2} μg cm^{-2} h^{-1}, 51.32×10^{-2} μg cm^{-2} h^{-1} and 62.03×10^{-2} μg cm^{-2} h^{-1} respectively. This equates to an

increase of steady state flux of ketoprofen in gel II, III and IV of 425%, 1087% and 1314% respectively compared to gel I.

Table 3.2 Mean apparant steady state flux, K_P and enhancement ratio of ketoprofen from fish oil gels (n = 12 ± s.e.m.)

Formulation	App. J_{ss} ($\mu g\ cm^{-2}h^{-1}$)	K_P (cmh^{-1}) ($x10^{-6}$)	ER_{flux}
I	0.0472 ± 0.0103	2.70	-
II	0.2007 ± 0.0444	11.47	4.25
III	0.5132 ± 0.0856	29.33	10.87
IV	0.6203 ± 0.0711	35.45	13.14

3.3.2 EPA and DHA

No EPA or DHA was detected in any of the receptor phase samples which is surprising given the results obtained by Heard et al. (2003).

3.4 Discussion

The results obtained from this short study clearly showed that in the presence of fish oil the permeation of ketoprofen is greatly increased. Numerous studies have previously shown that the presence of fatty acids significantly augments the permeation of certain drugs these are expertly summarised by Williams and Barry (2004) and detailed below. However, the anticipated (and desired) permeation of EPA and DHA from the fish oil appeared not to occur in these experiments. Had significant quantities of EPA and DHA been found to permeate the skin in proportion to fish oil content the ketoprofen data could have been attributed to the pull effect (Bowen and Heard, 2006) i.e. ketoprofen permeation was aided by the "pull" of EPA / DHA as they permeated the skin. Indeed, it has recently been proposed that the pull effect alone is sufficient to account for the enhanced permeation of mefenamic acid by ethanol and 1,8-cineole (Heard et al., 2006). It seems, therefore that the pull effect was not responsible for the ketoprofen permeation enhancement results in this study and a more traditional explanation seems appropriate.

Considering the fish oil as a penetration enhancer, a possible mechanism of enhancement concerns the reduction of skin resistance as a permeability barrier by disruption of tightly packed lipid regions of the stratum corneum. The disruption of these lipids allow molecules to flow through the intercellular space with greater ease than when these regions are present. This is well documented as leading to increased permeation across the intercellular lipid matrix (Barry, 1987). One study showed that the permeation of naphazoline was increased by the addition of fatty acids (Green et al., 1988). It was hypothesised that this could be further influenced by ion pair formation between drug and fatty acids, resulting in increased skin/vehicle partitioning of the drug. This mechanism could be in action in this investigation. The disruption of the lipid domains by the fish oil allows ketoprofen, already solubilised in the fish oil, to flow freely between cells and continue to permeate through into the receptor phase.

Electron microscope studies after treatment with oleic acid revealed discreet lipid domains within the stratum corneum. (Vavrova et al., 2005). The formation of such domains provides defects within the bilayer lipids and has been shown to aid the permeation of hydrophilic molecules (Williams and Barry, 2004). The "kink" in the chain of this cis isomer of oleic acid (Figure 3.2) was believed to be the main factor in this enhancement (Vavrova et al., 2005). EPA may act in a similar way as it is mainly present as the cis isomer, although the multiple double bond system may limit this effect. However, if EPA is present as the free fatty acid then the kink in the chain could provide a further explanation for the enhancement seen, if it is present as the triacylglycerol, then the formation of fish oil pools within the SC could allow increased ketoprofen permeation as discussed earlier. Both mechanisms acting simultaneously could then explain the magnitude of enhancement and the permeation pattern exhibited by the formulations studied may have been predicted.

The flux data from the permeation experiments also shows an interesting pattern. The flux enhancement ratio (ER_{flux}), Table 3.1, for gel II is 4.25 times that of gel I, and gel III at 10.87 is more than double that of gel II. Following this, gel IV would be expected to have approximately double the ER_{flux} of gel III but this is not the case. One study looking at the effects of fatty acids as penetration enhancers found that using 3% oleic

acid as an enhancer in propylene glycol (PGl) had very high enhancing effects on tenoxicam, yet when the concentration was increased to 5% OAc, the enhancing effect on tenoxicam permeation decreased (Gwak and Chun, 2002). A similar phenomenon could have been taking place here, in that the presence of 20% fish oil / fatty acids could be reaching a plateau of enhancement and any more could cause a decrease in the permeation of ketoprofen possibly via saturation of the SC and consequent reduction in the available routes for ketoprofen permeation.

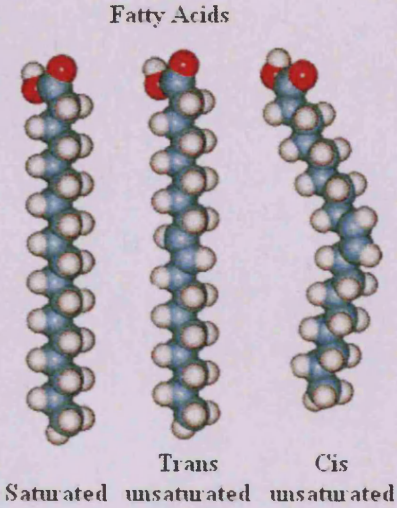


Figure 3.2 Depiction of a "kink" within a *cis* unsaturated fatty acid (oleic acid)

Given the permeation of EPA and DHA reported previously (Heard et al., 2003), the absence of EPA or DHA present in the receptor phases in the current work was surprising. It appears that there was some interaction between the fish oil in the gel and the stratum corneum as indicated by the pattern of ketoprofen enhancement. It has become a convention in skin permeation experiments to ensure test formulations are prepared at equal thermodynamic activities (Williams, 2003). This consideration was deemed less relevant to the current study as it was expected that the vehicle (i.e. fish oil) would permeate skin alongside the ketoprofen. As no EPA or DHA were found to permeate skin it may be contended that the results obtained were influenced by thermodynamic activity. However, at 2.5% w/w, ketoprofen is substantially below solubility (>320 mg ml^{-1}) therefore thermodynamic effects are less likely to be of relevance.

It is possible that the concentration of oil that penetrated was sufficient to form a reservoir within the stratum corneum, but insufficient to overcome the barrier presented by the hydrophilic areas of the epidermis and dermis. The resident oil could then be further subjected to metabolism, a topic explored in Chapter 7.

However, it is known that a simple solution of ketoprofen in fish oil does produce a steady state flux of EPA and DHA. The primary difference between the formulations used by Heard et al., (2003) and the current work was the use of a thickening agent, HPC, and it seems that its presence resulted in substantial retention of the fish oil within the vehicle, retarding its partition into the skin and subsequent permeation. However, ketoprofen appears not to be subject to similar retentive processes.

Furthermore, as release of fish oil from the gel seems to have been limited by the HPC, modulations to the skin barrier may still have been in progress at 48 hours. This could explain the lack of attainment of steady state (Figure 3.1). For future formulations different thickeners could be employed and their effect on permeation studied.

3.5 Conclusions

From this preliminary gel formulation study it would appear that the formulations prepared may be suitable for ketoprofen delivery (where fish oil content produced concentration dependent enhancement) but not for EPA and DHA. The principle cause for the lack of EPA and DHA permeation appears to be use of the thickening agent, HPC as previous work (Heard et al. 2002) used non-thickened formulations to obtain permeation.

Chapter 4

In-vitro transcutaneous delivery of ketoprofen, EPA and DHA from fish oil containing 1,8-cineole

4.1 Introduction

The results obtained in Chapter 3 suggest that the use of HPC as a thickening agent was detrimental in the development of a transcutaneous system for the delivery of EPA and DHA from fish oil. Thus a simple solution would appear to be a better option, despite its low viscosity. However, in order to achieve a useable transcutaneous system, the modest rates of permeation observed previously (Heard et al., 2003) would need to be optimised. The ClogP of EPA and DHA are 6.71 and 7.70 respectively, (ChemDraw) suggesting less-than-ideal permeation candidates and the potential benefit of employing penetration enhancement. As outlined in Chapter 1 penetration enhancement strategies generally fall in to one of two categories: physical and chemical, the latter the simplest and most patient friendly.

This chapter describes the effort to enhance the simultaneous transcutaneous delivery of EPA, DHA and ketoprofen via chemical enhancement using 1,8-cineole (eucalyptol), the major terpenoid of eucalyptus oil, which also fulfilled a secondary role of addressing the issue of odour arising from the use of this natural product, in particular oxidised PUFAs. 1,8-cineole, Figure 4.1, possesses a pleasant 'medicinal' odour, which was found to have a long acting odour-masking effect on the fish oil when applied to skin, has previously demonstrated to be a highly effective permeation enhancer (Yamane et al., 1995).

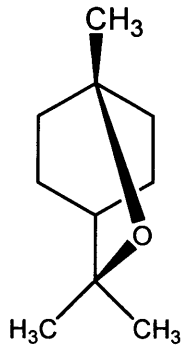


Figure 4.1 Structure of 1,8-cineole

This chapter aimed to address the issue of negligible EPA and DHA permeation as found in Chapter 3. The exclusion of the permeation inhibiting HPC should allow permeation of EPA and DHA. The effect of the proportion of 1,8-cineole on the delivery of ketoprofen, EPA and DHA was also investigated.

4.2 Materials and Methods

4.2.1. Materials

Boots Super Strength concentrated fish oil capsules 1000mg (Batch number: 100673) were purchased from a local store. Ketoprofen and BHA and cetrimide were obtained from Sigma – Aldrich Company Ltd., Poole, UK. Methanol (HPLC grade) and petroleum ether 60-80°C were obtained from Fisher Chemicals, UK. All other reagents were of analytical grade or equivalent.

4.2.2 Preparation of formulations

A fixed sub-saturated dose of ketoprofen (2.5% w/w) was used in each formulation as described in Chapter 3. The appropriate masses of ketoprofen and fish oil were combined and the formulation vortex mixed, followed by the drop-wise incorporation of 1,8-cineole in the quantities stated in Table 4.1. The formulations were thoroughly vortex mixed and stored at 2-4°C until required ($\leq$ 24 hours). In addition, three control formulations were employed: 2.5% ketoprofen in fish oil (enhancer-free) (I), 2.5% ketoprofen in de-ionised water (V) and fish oil without ketoprofen (VI).

Table 4.1 Percentage of components within formulations

Formulation	Fish Oil* (%)	Ketoprofen	1,8-cineole	H_2O
I	97.5	2.5	0.0	0.0
II	92.5	2.5	5.0	0.0
III	87.5	2.5	10.0	0.0
IV	77.5	2.5	20.0	0.0
V	0.0	2.5	0.0	97.5
VI	100.0	0.0	0.0	0.0

*Fish oil containing 0.05% BHA

4.2.3 In vitro transcutaneous delivery

Permeation experiments were carried out as described in Chapter 2, Section 2.1.1. A total of six replicates were carried out for each formulation.

4.2.4 HPLC analysis

HPLC analysis of EPA / DHA and ketoprofen was carried out as Chapter 2, section 2.2.2.1 and section 2.4 respectively.

4.2.5 Data analysis

Statistical analyses were carried out using Instat 3 for Macintosh (GraphPad Software, Inc), where non-parametric Mann-Whitney tests were employed to determine differences between specific pairs of formulations. This test was used as the data was of non-Gaussian distribution.

4.3 Results and Discussion

4.3.1 Transcutaneous delivery of ketoprofen

Figure 4.2 shows that steady state flux of ketoprofen was attained for each formulation between approximately 12 and 48 hours.

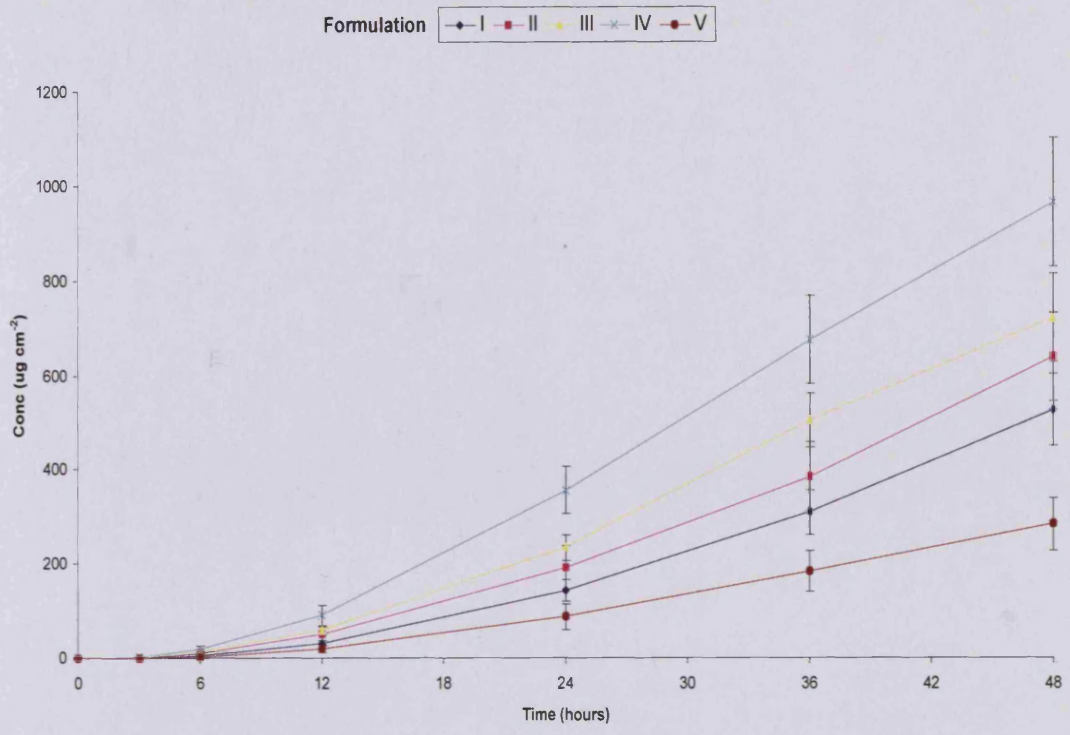


Figure 4.2 Permeation profiles of ketoprofen from formulations I - V

Permeation data is summarised in Table 4.2, where ketoprofen in water served as control and formulation VI is not shown as this was fish oil alone. The lowest flux was from the water vehicle control (V) and it is apparent once again that a fish oil vehicle alone (I) enhanced the permeation of ketoprofen relative to water, by a factor of 1.72; however this flux difference is deemed not significant ($p=0.1775$). The solubility of ketoprofen is 37.6 mg ml^{-1} which is significantly higher than in the water vehicle, however both solutions are prepared as 2.5% ketoprofen solutions and so once again thermodynamic activity is of less relevance.

Table 4.2 Summary of permeation data for transcutaneous delivery of ketoprofen (n=6, ± s.e.m.)

Formulation	Lag Time (h) ± s.e.m	J_{ss} (μg cm^{-2} h^{-1}) ± s.e.m	ER_{flux} Form./Keto H_2O	ER_{flux} Form./Keto FO
I	11.65 ± 0.51	11.54 ± 1.84	1.72	-
II	10.82 ± 1.05	13.87 ± 2.50	2.06	1.20
III	9.54 ± 0.87	18.51 ± 2.19	2.75	1.60
IV	8.55 ± 1.13	24.31 ± 3.18	3.62	2.11
V	11.0 ± 1.26	6.72 ± 1.78	-	0.58

This enhancement of ketoprofen is probably due to greater absorption of the oil vehicle, incorporating the ketoprofen solute, into the lipoidal domains of the stratum corneum, followed by diffusion across the viable epidermis and dermis. This was discussed in Chapter 3. The presence of the more hydrophilic layers of the epidermis appears not to have presented a major obstacle to the permeation of ketoprofen under these conditions.

The enhancement of ketoprofen by fish oil / fatty acids has been discussed in Chapter 3 and appears to be in action once again. The mechanism of enhancement by fatty acids is believed to be by reduction of skin resistance as a permeability barrier by disruption of tightly packed lipid regions of the stratum corneum (see Chapter 3). Perturbation has the effect of increasing the partitioning of a permeant molecule into the intercellular lipid matrix (Barry, 1987; Wang et al, 2003). However, within the fish oil the fatty acids are predominantly triacylglycerols, rather than free fatty acids. Therefore uptake of oil and ketoprofen would be more likely to be a consequence of absorption due to the miscibility between the skin lipids and the oil. In this case ketoprofen permeation in the oil would have been enhanced by the "drag" effect of the absorbing oil into the SC.

The enhancement potential of another PUFA, linolenic acid, has previously been studied in the transdermal delivery of melatonin across rat and porcine skin (Kandimalla et al, 1999) and for luteinizing hormone release hormone across human epidermis (Bhatia and Singh, 1999). As is typical in such reports the fate of the fatty acids and vehicle were not reported, although the effects of occlusion on the percutaneous penetration of linoleic acid was reported (Taylor et al., 2002). A major difference in

formulations is that this work involved triacylglycerols enhanced to incorporate greater proportions of EPA with a small amount of fatty acid ethyl esters, rather than free fatty acids – the latter being more amphiphilic and less liable to absorption by the stratum corneum lipids than the more lipophilic fish oil.

Figure 4.2 and Table 4.2 clearly show that there was a dose-dependent increase in the permeation of ketoprofen relative to the proportion of 1,8-cineole. The greatest flux of ketoprofen, 24.31 ± 3.18 μg cm^{-2} h^{-1}, was obtained with formulation IV which contained 20% w/w 1,8-cineole and was significantly different from both controls ($p=0.0043$ and 0.0152 compared to formulation V and I respectively). Terpenoids such as 1,8-cineole are believed to act by disruption of lipid bilayers and increasing diffusivity (Yamane et al, 1995). When EPA and DHA were present in the formulation a dual action mode of enhancement appeared to operate. The Q_{24} and Q_{48} data also express the same dose dependent relationship between ketoprofen and 1,8-cineole flux data with the highest Q_{24} and Q_{48} values, 355.78 ± 50.73 μg cm^{-2} and 963.29 ± 136.69 μg cm^{-2} respectively, being observed in formulation IV containing 20% 1,8-cineole.

Heard et al. (2003) showed the flux of ketoprofen through full thickness porcine skin from a saturated fish oil vehicle to be 6.12 ± 2.39 μg cm^{-2} h^{-1}. This study demonstrated a flux of 6.72 ± 1.78 μg cm^{-2} h^{-1} from a 2.5% w/w aqueous solution (formulation V). However the flux of ketoprofen from a 2.5% w/w fish oil vehicle (formulation I) was 11.54 ± 1.84 μg cm^{-2} h^{-1} demonstrating a significant improvement compared to either an aqueous or saturated fish oil vehicle. This difference may be explained by the dosing regime of both studies with the former, although using a saturated solution, dosed a finite 200μl indicating that the use of a saturated formulation appears to have a detrimental effect on ketoprofen permeation.

Loden et al. (2004) achieved a flux of 0.2μg cm^{-2} h^{-1} when using a 2.5% ketoprofen formulation with 30% ethanol. Ethanol is commonly used as an enhancer and has been shown to be very effective in this role. (Williams, 2003) When comparing this study with Loden et al. the presence of fish oil and 1,8-cineole appear to have a much greater effect than ethanol on the permeation of ketoprofen.

In a study by Rhee et al. (2001) the flux of a 3% ketoprofen / oleic acid micro-emulsion containing 5% cineole was shown to be 11.36 ± 2.17 μg cm^2 h^{-1}. This flux is very similar to the flux obtained in this study and indicates the presence of EPA and DHA had a similar if not slightly greater enhancing effect upon ketoprofen permeation.

4.3.2 Transcutaneous delivery of EPA and DHA

In contrast to the results using thickened (gelled) fish oil, transcutaneous delivery of fish oil was confirmed by the cumulative permeation EPA and DHA (Figures 4.3 and 4.4).

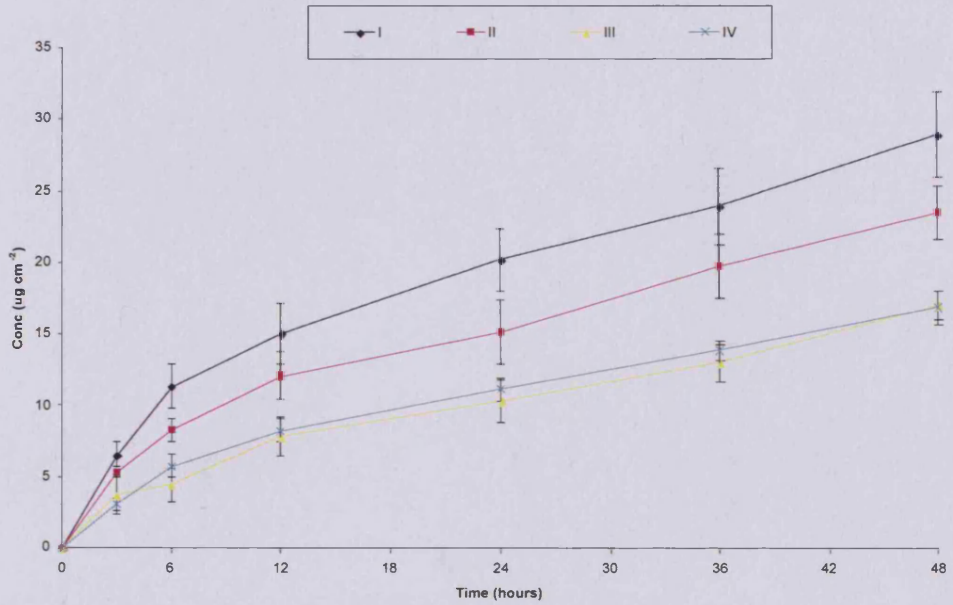


Figure 4.3 Permeation profile of EPA from formulations I - IV

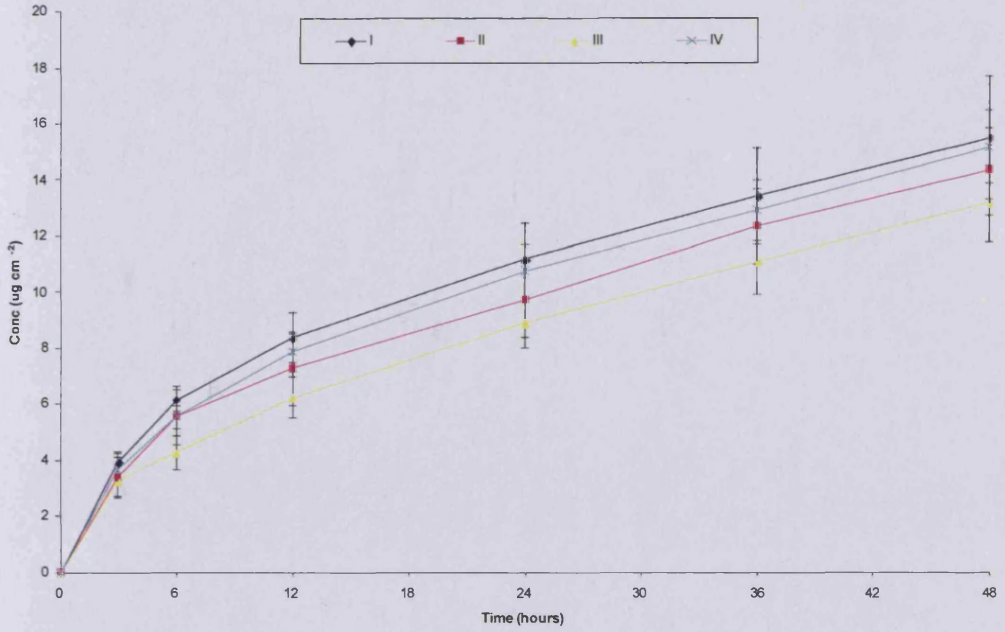


Figure 4.4 Permeation profile of DHA from formulations I - IV

The most surprising observation was that the general shape of the permeation profiles was dissimilar to those obtained for ketoprofen and, unlike ketoprofen, the presence of 1,8-cineole had no discernible enhancing effect on EPA and DHA which were both in much greater excess than ketoprofen and therefore have a greater possibility of interaction. This suggests that the enhancer was acting solely on ketoprofen, possibly as the 1,8-cineole may have solvated the ketoprofen in preference to the oil triacylglycerols. Furthermore, it is apparent that fluxes of EPA and DHA were not at steady state at any time throughout the 48 hour period, instead there was initially more rapid permeation followed by an approximately linear secondary permeation phase. Reasons for this behaviour are not clear although, breakdown of the barrier may be discounted, as ketoprofen provided classical permeation profiles. Saturation of the receptor phase can also be discounted as the entire receptor phase was sampled and the levels of EPA in the receptor samples were some three orders of magnitude below their maximum solubility level (see Chapter 2, Section 2.1.3). It is possible the morphology of the skin was altering in response to the permeating oil in a manner that restricted the permeation process. Seemingly, this response did not affect permeation of ketoprofen, the smaller molecule. The receptor phase itself could be the cause of the difference in permeation profiles. The receptor phase should act as a non invasive sink for the

permeating compounds and therefore produce a standard permeation profile as did ketoprofen. If the receptor phase was in some way encouraging the permeation of fish oil by provided a more than adequate sink, this may explain the initial rapid flux. However, if this was the case then the permeation profile would be expected to increase with time in a uniform manner following the pattern of the initial rapid flux, as the dose is infinite and at each time point the complete receptor phase is sampled. This does not happen and the permeation profile tails off as it would with a finite dose of formulation. Further explanations and possible experimentation for this must therefore be considered.

Table 4.3 summarises the permeation data. Formulation I, containing no 1,8-cineole, gave the highest first and second phase fluxes of EPA at 1.89 μg cm^{-2} h^{-1} (67.20 x 10^{-5} % of dose) and 0.36 μg cm^{-2} h^{-1} (8.44 x 10^{-5} % of dose) respectively. Surprisingly the inclusion of 10% 1,8-cineole (formulation III) produced the lowest first phase flux. Unexpectedly the lowest second state flux was seen with formulation IV containing the highest percentage of the enhancer, 20% w/w.

Table 4.3 Summary of the flux data for the in vitro transcutaneous delivery of EPA and DHA

Formulation	Dose	SSF 1st Phase (μg cm^{-2} h^{-1})		SSF 2nd Phase (μg cm^{-2} h^{-1})	
		EPA	DHA	EPA	DHA
I	Keto FO	1.89 ± 0.26	1.03 ± 0.06	0.36 ± 0.06	0.24 ± 0.03
II	5% 1,8-cineole	1.38 ± 0.14	0.93 ± 0.18	0.34 ± 0.04	0.21 ± 0.01
III	10% 1,8-cineole	0.74 ± 0.20	0.71 ± 0.11	0.25 ± 0.03	0.22 ± 0.02
IV	20% 1,8-cineole	0.96 ± 0.14	0.93 ± 0.08	0.24 ± 0.03	0.21 ± 0.03
V	Keto H_2O	-	-	-	-
VI	Fish oil	0	0	0	0

Statistical analysis showed there to be a significant difference in the first phase flux of EPA in formulations IV (p=0.0087) and III (0.0173) compared to formulation I. No significant difference was observed in the second phase fluxes of formulations II, III, IV relative to formulation I (p = >0.25 for each formulation). Lag times were too short to be measured.

No significant effect of 1,8-cineole was seen on the permeation of DHA. The greatest first phase flux, 1.03 μg cm^{-2} h^{-1} (57.50 x 10^{-5} % of dose), was once again seen in formulation I which contained no 1,8-cineole. The lowest flux, 0.21 μg cm^{-2} h^{-1} (12.74 x 10^{-5} % of dose), was seen in the second steady state in formulation IV containing 20% w/w 1,8-cineole. Statistical analysis showed no significant difference between either first or second phase fluxes of formulations II, III and IV relative to I ($p = >0.10$ in each case).

Neither EPA nor DHA were detected for formulations V and VI. As permeation of EPA and DHA was seen in formulations I, II, III and IV and the common component in these formulations was ketoprofen, it can be hypothesised that ketoprofen has a permeation enhancing effect on EPA and DHA. This is a subject studied in Chapters 7, 8 and 9.

The first phase flux data for both EPA and DHA show no clear pattern of the effect of 1,8-cineole. The second phase of steady state however shows an inverse dose dependent relationship between the concentration of 1,8-cineole and the flux of both EPA and DHA i.e. as the concentration of 1,8-cineole increases the second phase flux of both EPA and DHA decreased.

The very low permeation percentages (Table 4.4) are due in part to the high lipophilicity of the oil and its inefficiency in permeation (Taylor et al., 2002) and also reflect the fact that when infinite doses of EPA and DHA are applied, high absolute values may appear low when reported as a percentage of that infinite dose.

Table 4.4 Percentage of the applied dose of EPA and DHA

Formulation	Dose	Percentage of applied dose 1st Phase (10^{-5})		Percentage of applied dose 2nd Phase (10^{-5})	
		EPA	DHA	EPA	DHA
I	Keto FO	67.20	57.50	8.44	25.33
II	5% 1,8-cineole	51.55	54.60	7.87	20.17
III	10% 1,8-cineole	28.41	43.08	8.38	14.93
IV	20% 1,8-cineole	42.09	64.08	7.16	12.74
V	Keto H_2O	-	-	-	-
VI	Fish oil	-	-	-	-

The Q_{24} and Q_{48} data (Table 4.5) do not show a clear relationship between the permeation of EPA or DHA and concentration of 1,8-cineole, reflecting the pattern shown in Figures 4.3 and 4.4. There appeared to be an inverse dose dependent relationship until formulation IV where the permeation increased slightly.

Table 4.5 Amount permeated (Q_{24} and Q_{48}) values for EPA and DHA

Formulation	Dose	Q 24 Values ($\mu g\ cm^{-2}$) ± SEM		Q 48 Values ($\mu g\ cm^{-2}$) ± SEM	
		EPA	DHA	EPA	DHA
I	Keto FO	20.19 ± 2.18	11.14 ± 1.31	28.87 ± 2.97	15.61 ± 2.24
II	5% 1,8-cineole	15.17 ± 2.26	8.89 ± 1.56	23.42 ± 1.83	14.27 ± 1.55
III	10% 1,8-cineole	10.30 ± 1.44	8.88 ± 0.87	17.00 ± 0.95	13.11 ± 1.36
IV	20% 1,8-cineole	11.47 ± 0.92	10.75 ± 0.98	16.11 ± 0.79	15.13 ± 1.32
V	Keto H_2O	-	-	-	-
VI	Fish oil	-	-	-	-

The importance of vehicle formulation is well established. For example, it was recently demonstrated that it is possible to disturb SC lipids using fatty acids with short chains in a lipophilic mineral oil formulation, but not with formulations based on propylene

glycol (Wang et al., 2003). It is a basic physicochemical concept that solutes exist in discrete solvation cages, although a less well-probed phenomenon is that of differential solvation (Duffy and Jorgensen, 2000; Diaz and Berger, 2001) within a vehicle and its consequences in topical drug delivery (Heard et al., 2003: Karia et al., 2004). The nature of solvated permeant molecules depends on the mutual interactions between all species present within the formulation. It is feasible then, that the differences seen in enhancement between the PUFAs and ketoprofen were due to selective solvation by 1,8-cineole. Solvation of the smaller, more functionalised ketoprofen molecule is likely to have been preferential to the solvation of the large sterically hindered triacylglycerol units and therefore ketoprofen permeation may be expected to be relatively greater.

As EPA and DHA were both administered principally as triacylglycerols the main barrier to transcutaneous permeation would have been the aqueous domains of the viable epidermis and the dermis which are substantially more polar than the lipoidal domains of the stratum corneum. This may explain the very low levels of EPA and DHA in the receptor solutions. The lack of direct correlation between the concentration of 1,8-cineole and the permeation of EPA and DHA may be explained by the method of enhancement of 1,8-cineole. Terpenoids act as enhancers by the unpredictable disruption of lipid bilayers within the stratum corneum, although the dose dependency of such effects is less well documented.

Williams et al. (2006) have recently changed the common opinion of terpenoid enhancement by investigating the molecular interactions between 1,8-cineole and human skin using Raman spectroscopy. They concluded that 1,8-cineole causes both disruption of SC lipids and an increase of the order of these lipids. The difference between the areas of order and disorder allows the increased drug delivery seen after application of 1,8-cineole. At levels up to 10% 1,8-cineole the integrity of the skin may be intact and the balance between the ordered and disordered areas of the stratum corneum lipids may lie in the favour of ordered lipid domains, maybe incorporating a certain amount of the permeating lipids, i.e. EPA and DHA. However, at 20% 1,8-cineole far greater disruption to the integrity of the skin may have occurred and more disorder of the skin lipids takes place, allowing greater permeation of ketoprofen and

fish oil. This could explain the increase in the first phase flux of EPA and DHA in formulation IV. Formulation I has no interference from an increase or decrease in the order of SC lipids and so greater concentrations of EPA and DHA can permeate without uptake into cell bilayers.

Negligible amounts of EPA or DHA permeated from formulation VI which consisted of 100% fish oil. The main barrier to the transcutaneous permeation of a highly lipophilic substance would have been the dermis, which is substantially more polar than the lipoidal domains of the stratum corneum. With no other compound present it is possible that no oil could permeate as it may need to complex with another compound in order to permeate via the "drag" effect. The surprising inference from this chapter was that ketoprofen ***enhanced*** the permeation of EPA and DHA in the other formulations suggesting the need for a co-permeant. This phenomenon is studied in greater depth in Chapters 7, 8 and 9.

Differences in the permeation of EPA and DHA were expected due to the different concentrations of the two within the fish oil, although no distinct pattern in the permeation of the two fatty acids was observed. This could be explained by differential metabolism within the freshly excised skin.

4.5 Conclusions

Simultaneous permeation of ketoprofen and EPA/DHA has been confirmed. The permeation of ketoprofen from a fish oil vehicle increased by a factor of 1.72 compared to an aqueous solution of the drug. A concentration dependent relationship was found between the flux of ketoprofen and the concentration of 1,8-cineole, although no clear effects were seen on the permeation of EPA and DHA as fish oil. However, explanations of resulting patterns of permeation have presented themselves through recent findings concerning the action of 1,8-cineole (Williams et al., 2006). This may also have been due to differential solvation effects in the vehicle or in the permeation process, or modulation of the barrier which affected the permeation of oil but not

ketoprofen. 1,8-cineole has recently been shown to permeate full thickness porcine skin in a concentration dependent manner (Heard et al., 2006). The preferential solvation of ketoprofen by 1,8-cineole and the resulting drag effect could explain the concentration dependent enhancement seen throughout this chapter. The combination of both fish oil, aiding the permeation through the lipophilic SC, and 1,8-cineole, aiding permeation through the remainder of the epidermis and dermis via complexation, leads to the very large flux values of ketoprofen. The loss of this solvent from the formulation could cause an alteration to the thermodynamic activity of ketoprofen and act as another enhancement mechanism. If developing a formulation containing 1,8-cineole this must be studied further and toxicity and the fate of 1,8-cineole after leaving the formulation must also be determined.

The maximum flux of ketoprofen achieved in Chapter 3 was $0.6203 \pm 0.0711 \mu g\ cm^{-2}\ h^{-1}$ when using the 20% fish oil gel. This is ten fold less than formulation V and again indicates the severe retardation affect of the HPC thickening agent upon permeation.

Chapter 5

Delivery of EPA and ketoprofen to excised porcine joint capsule

5.1 Introduction

With the knowledge gained in the previous chapter concerning the delivery of EPA and ketoprofen through skin, this chapter investigated permeation deeper into the underlying joint capsule. All articular joint capsules are similar in nature comprising of two bones, with their ends covered in articular cartilage converging and held in place by tendons and ligaments. The joint is covered by a membrane called the synovium which produces synovial fluid. Synovial fluid is the main source of nutrients to the cartilage and contains hyaluronan to act as a shock absorber to the joint and to allow smooth movement between the limbs. The synovium outer layer is a fibrous joint capsule. See Figure 5.1.

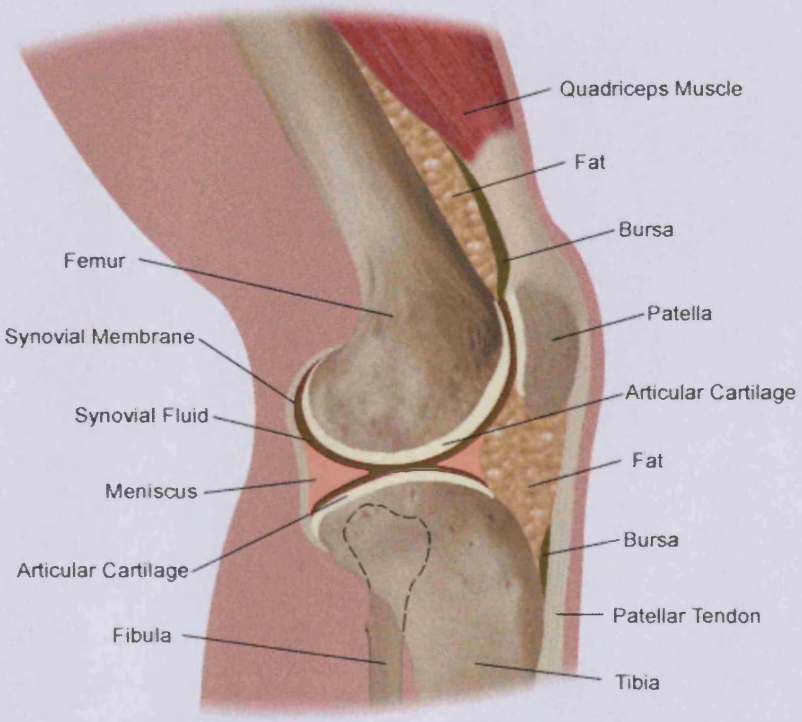


Figure 5.1 Anatomy of the knee
(http://www.healthsystem.virginia.edu/)

Intra-articular drug delivery is currently under great scrutiny. As RA is an inflammatory disorder, treatment of the external areas of the joint, the swollen joint

capsule and synovium is paramount. The link between this and OA is discussed in detail in Chapter 6, where the synovium and external areas of the joint contribute to the destruction of cartilage and overall progression of the disease. In OA, cartilage also releases pro-inflammatory mediators and cytokines and to treat this type of arthritis requires the delivery of drugs into the synovial fluid. When delivering EPA in either disease, RA or OA, the passage of EPA is the same. EPA must first permeate through the external joint, followed by the joint capsule, synovium, then into the synovial fluid and ECM of cartilage followed by incorporation into the chondrocytes. As EPA permeates these tissues its effect can be exerted on the inflammation process occurring in each tissue. The permeation process in itself has obvious benefits, not just the incorporation into cartilage, as by permeating each inflamed tissue it can help in the treatment of the symptoms and progression of the disease states. There are currently several DMARDs available for use in RA and these can slow the progression of this disease. OA has no such treatment available and current medical therapies are of limited effectiveness (Abramson, 2006). These treatments rely on the use of NSAIDs and nutraceuticals such as glucosamine given orally. Intra-articular therapies for OA and RA include injections of corticosteroids and the hyaluronans. The intra-articular route offers the possibility of delivering high concentrations at the site of action and also the delivery of drugs with low oral bioavailability. Intra-articular drug delivery does pose significant disadvantages. Multiple injections into the joint are very inconvenient and painful for patients and drugs injected into the joint space may also be cleared relatively quickly by the synovium. Transcutaneous delivery of a natural product which would be used in every part of the joint and if in excess likely to be incorporated into surrounding chondrocytes and synoviocytes, presents an ideal mechanism for the treatment and symptomatic relief and of both RA and OA. The sub-cutaneous approach has been used to deliver the anti IL-1β receptor antibody, AMG108, offering advantages by accessing all areas of the joint in both disease states (Abramson, 2006).

The delivery of ketoprofen into the joint space also has clear benefits as inhibition of COX has been well documented to assist with the inflammation and pain associated with both RA and OA. It has been reported, when using a multi-chamber distribution

dialysis system, that a non-stereoselective affinity of ketoprofen enantiomers for articular tissues is observed, with ketoprofen becoming more concentrated in solutions of synovial membrane, joint capsule and ligament compared to much lower concentrations achieved within those of articular cartilage (Lagrange et al., 2001). This may be due to the inability of ketoprofen to traverse the negatively charged ECM. However, when multiple topical applications of ketoprofen were employed, a 6 – 10 fold concentration increase in cartilage and menisci compared to synovial tissue was observed (Rolf et al., 1999). This again shows promise as the combined delivery of ketoprofen and EPA would help all areas of inflammation.

The concentration within the joint post application will be fundamental to the efficacy of an EPA / ketoprofen formulation.

The aim of this chapter is to determine the extent to which EPA and ketoprofen may permeate the joint capsule and become bioavailable within synovial fluid in a joint. This was achieved by using a porcine hock model. Clearly, the most appropriate model would have been a freshly excised joint complete with skin ***prior*** to steam cleaning. However, this was impossible to obtain due to food regulations. Furthermore, permeation data already exists for permeation of EPA and ketoprofen through skin, which would be expected to constitute the rate-limiting barrier.

All further work focused on EPA alone as Volker et al. (2000) found an EPA based diet to be more effective in the suppression of inflammation than a DHA diet in their rat model of streptococcal cell wall arthritis.

5.2 Materials and methods

5.2.1 Materials

Fish oil capsules 1000mg (Boots Super Strength, batch number: 30446M) were purchased from a local store. Ketoprofen, butylated hydroxyanisole (BHA) and cetrimide and HPC were obtained from Sigma – Aldrich Company Ltd., Poole, UK.

Methanol (HPLC grade) was obtained from Fisher Chemicals, Loughborough, UK. All other reagents were of analytical grade or equivalent. Porcine carpal joints were obtained from a local abattoir post steam cleaning and refrigeration for approximately 24 hours. The skin was then removed from the joint avoiding the removal of tendon and ligaments and used immediately.

5.2.2 Preparation of formulations

A fixed sub-saturated dose of ketoprofen (2.5% w/w) in fish oil was used as in previous chapters. The appropriate masses of ketoprofen and fish oil were combined, in addition to BHA (0.05% w/w) to inhibit PUFA degradation, and the formulation vortex mixed. Due to the difficulty of application of a liquid (fish oil) onto curvature of the porcine joint, it was necessary to thicken the formulation to stop the liquid running off the joint and was acheived by the addition of HPC, approximately 8%. In Chapter 3 it was found that this retarded the permeation of EPA. In this study however the vehicle itself was fish oil and not limited to a maximum of 20% and therefore the need for thickening of the formulation was thought to out-weigh the possible retardation affects. The formulations were stored at 2-4°C until required ($\leq$ 24 hours).

5.2.3 *In-vitro* joint capsule permeation experiments

Joint capsule permeation experiments were carried out using the carpal joint of the forelimb of the pig (Figure 5.2). A donor chamber was pre-greased and placed onto the skinless joint over the carpometacarpal compartment and secured in place using adhesive tape. The formulation (1ml) was then added into the donor chamber and occluded. After the required time the donor cell was removed, the joint cleaned thoroughly and washed several times with MeOH then wiped using paper tissues until no visible traces of formulation remained. The underlying synovial fluid was then extracted using a sterile 5 cm hypodermic needle and 5ml syringe and samples were stored at –20°C prior to analysis. A total of four replicates were carried out for each time point.

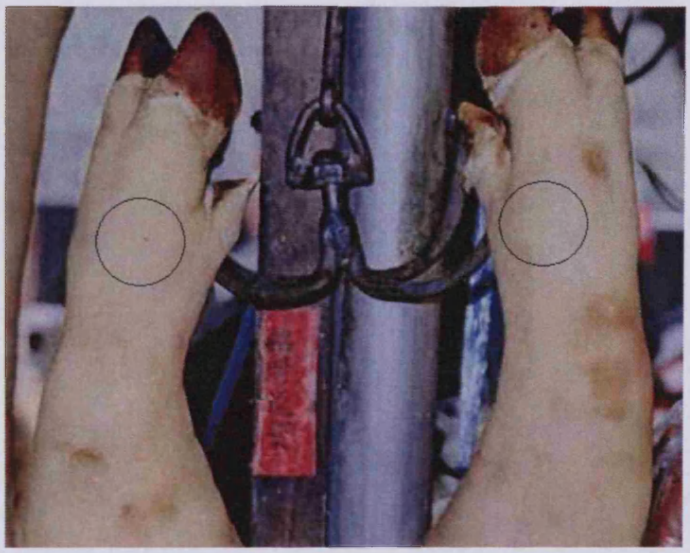


Figure 5.2 Carpo-metacarpal joint (highlighted) of a porcine forelimb

5.3 Analytical Methods

5.3.1 Extraction of ketoprofen and EPA from synovial fluid

The EPA within a fixed volume of synovial fluid (200µl) was extracted by the addition of methanol (4ml) to the synovial fluid and sonication for 10 minutes. The resulting solution was then centrifuged at 9000 rpm for 10 minutes. The supernatant was then sampled with EPA and ketoprofen concentrations determined by HPLC analysis as described in Chapter 2, Sections 2.2.3 and 2.2.4 respectively.

5.4 Results and Discussion

Figure 5.3 illustrates the cumulative permeation of both EPA and ketoprofen in the joint capsule synovial fluid. It must be noted at this stage that the concentrations shown are calculated from the sampling of 200µl of synovial fluid. This is due to the variations in the volume of synovial fluid removed from the joints, which is related to the size of the forelimb itself. To keep consistency between the different limbs a value of 200µl was chosen as this was the minimum volume of fluid that could be removed.

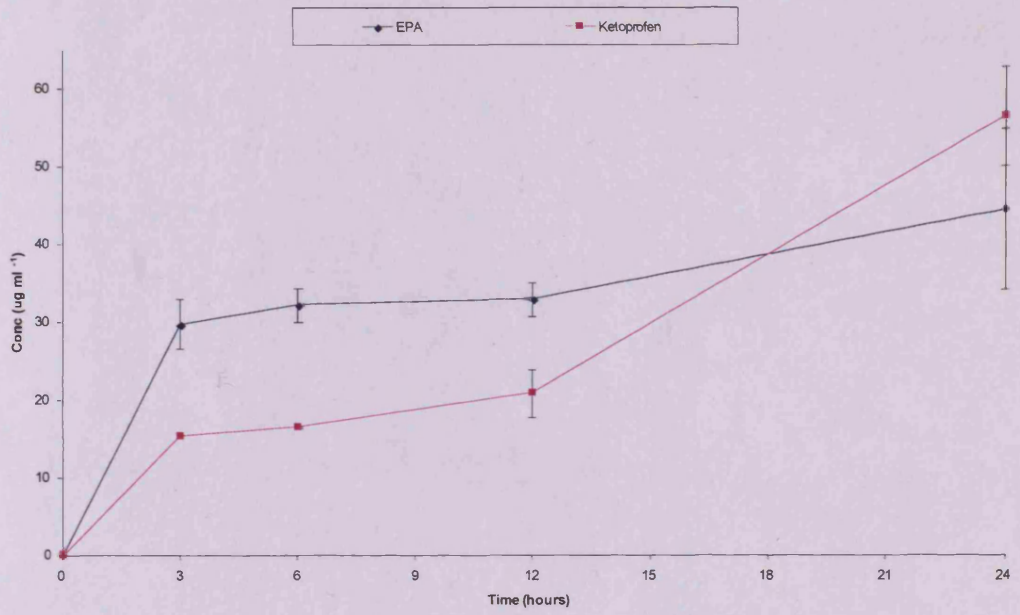


Figure 5.3 Delivery of EPA and ketoprofen into porcine joint capsule (n = 4, ± s.e.m.)

5.4.1 Permeation of EPA

Approximately 30–40µg of EPA permeated into the synovial fluid within the joint space after 24 hours. Interestingly there seems to be a plateau of delivery after only 3 hours. EPA is very lipophilic both as the triglyceride and free fatty acid and it is possible that the synovial fluid can become saturated with fats / fatty acids. If the majority of permeated EPA can become incorporated within the cartilage then this will dramatically reduce the inflammation cascade seen with RA and OA. The permeation through a swollen synovium will also be beneficial in both disease states as it is the synovium which contributes a large proportion of inflammatory cytokines and the presence of EPA will reduce the induction of these mediators. If this production can be reduced at the source then the benefits are obvious.

It is worthy of note that throughout the literature it is very difficult to find a therapeutic dose for EPA. Curtis et al., (2000) used an EPA dose of 70µg ml^{-1} when conducting their experiments with *ex-vivo* cartilage ex-plants, to mimic human plasma fatty acid

levels. In these experiments, detailed in Chapter 6, they found this to be a suitable dose to inhibit the inflammatory cascade.

The skin permeation seen in Chapter 4 indicated that only approximately 20µg EPA had permeated the full thickness porcine skin after 24 hours. This result suggests that the best use of an EPA topical formulation to treat arthritis would be repeated applications to accumulate EPA within the synovial fluid up to a concentration of approximately 70µg ml^{-1}. This repeated application would also serve to keep a constant concentration within the inflamed tissues surrounding the joint and help balance out the removal of these fatty acids into the systemic circulation. The percentage of EPA permeated from the applied dose is shown in Figure 5.4. The percentage values are small which is expected given the infinite dose regime. These values follow the permeation trend of a seemingly steady state and plateau of permeation.

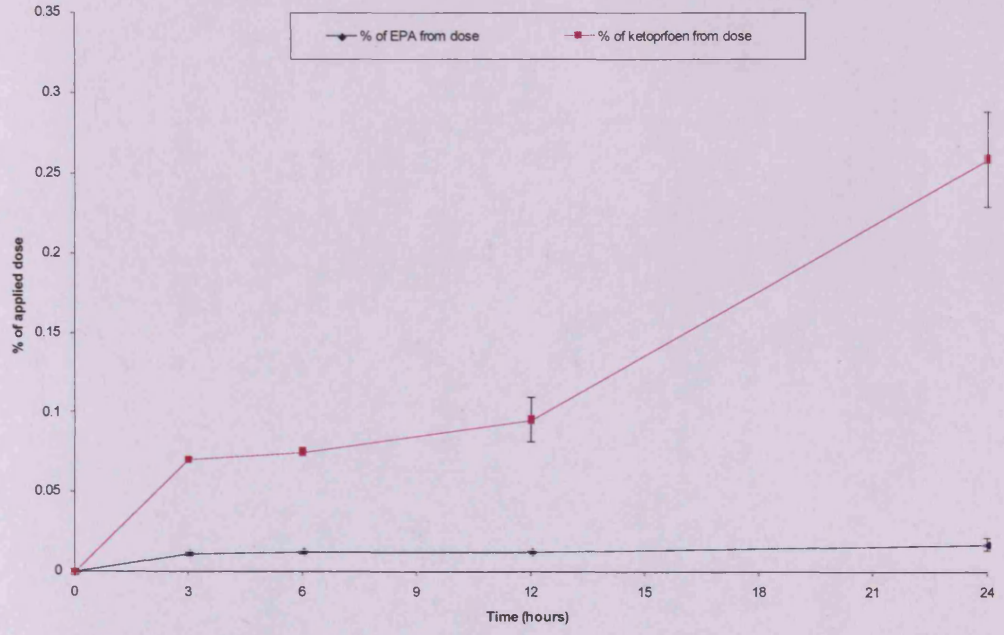


Figure 5.4 Percentage of EPA from applied formulation (± s.e.m.)

5.4.2 Permeation of ketoprofen

Figure 5.3 shows the permeation profile for ketoprofen. The synovial fluid solubility for ketoprofen appears to be higher than that of EPA which explains the greater

concentration of ketoprofen present after 24 hours. Comparing this to the skin permeation however shows that a lower concentration of ketoprofen permeated. The maximum ketoprofen concentration through skin after 24 hours was approximately 144μg as opposed to approximately 55μg through into the joint capsule. This difference could be explained by the difference in the type of membrane i.e. joint capsule versus full thickness skin. These results still show promise, as if 144μg permeates through the skin after 24 hours then this allows the 55μg (38% of the mass that can permeate full thickness porcine skin) to permeate into the synovial fluid in the same period. The bioavailability from an oral dose of 150mg can reach a plasma concentration of 15-25μg ml^{-1} which is much higher than the therapeutically active concentration (Upton et al., 1981). Roda et al. (2002) have studied a sustained release formulation of ketoprofen (100mg) and found plasma levels to reach 4μg ml^{-1} two hours after oral dosing and remain constant for up to twelve hours. In this chapter twelve hours after topical dosing onto the skinless joint allows a concentration of approximately 21μg ml^{-1} to be delivered into the synovial fluid. This would be less with the skin membrane present but should still deliver a therapeutic dose.

It is interesting to note that both EPA and ketoprofen show a similar delivery profile. In Chapter 4 it was noted that the two profiles were substantially different and showed that EPA appeared to have an initial rapid flux followed by a period of steady state. Ketoprofen showed a classic permeation profile. The similarity in profiles seen in this experiment might be due to the difference in the membrane that is being crossed. The joint capsule and synovium may be an easier barrier to traverse for EPA and combined with the decrease in ketoprofen permeation, would indicate that the nature of the barrier is more lipophilic. The skin barrier is designed to keep out exogenous molecules and is therefore expected to have a greater barrier function. The internal barrier of the joint capsule has no such function and permeation through to underlying tissue is important to allow adequate nutrition.

The difference may be due to the sampling method of the experiment. Samples from the same joint were not taken throughout the time period as this would have punctured the joint capsule. The size of the joints used in the experiment did differ slightly and as

a consequence the thickness of tissue between the surface and the synovial fluid within the joint capsule also differed. This made it very difficult to keep consistency within the experiment and could explain the permeation profiles becoming similar. However this slight inconsistency is representative of the variation between human joints and between individual patients that would be seen during *in-vivo* experiments.

Recently comparisons between routes of administration of nimesulide, a COX-2 inhibitor were made by studying the concentration of the drug within synovial fluid. It was found that nimesulide did indeed permeate into the synovial fluid from topical application and achieved a concentration of 19.7 ± 8.6 ng ml^{-1}. This was compared to 1958.8 ± 397.5 ng ml^{-1}following oral therapy. (Erdogan et al., 2006). They also found that a repeated dose, using one week of three times daily application, provided a significant improvement in all WOMAC Osteoarthritis Index parameters.

5.5 Conclusions

The delivery of EPA and ketoprofen through a typical joint capsule and into synovial fluid has been demonstrated after only 3 hours. The permeation through the joint capsule is itself a fundamental discovery for determining the efficacy of a topical fish oil / ketoprofen formulation. If both compounds can permeate this barrier then both compounds can act upon the barrier and exert their anti-inflammatory effects.

It has been shown that the solubility of these compounds within synovial fluid is an important factor in determining this delivery. This need not present a significant problem as the joints in this experiment were without a skin barrier. The rate of permeation combined with the uptake of EPA into the systemic circulation and the use of EPA by inflammatory mediators as it permeates the inflamed tissues would limit the concentration of these compounds reaching the joint and so saturation of the synovial fluid is unlikely. Also of note is the solubilisation of both compounds within synovial fluid. As in all mechanisms there must be a driving force for permeation and in most

cases this lies with the ability of the underlying tissues to accept / solubilise the permeating compounds. If this did not happen the compound would not permeate.

Despite the imperfections of the model, combining these findings with those of Chapter 4 demonstrates the potential of a formulation such as this. The ability of the formulation, even in such a basic form, to deliver both EPA and ketoprofen has been shown.

Chapter 6

Incorporation of EPA into *ex-vivo* bovine cartilage following *in-vitro* transcutaneous delivery

6.1 Introduction

Chapters 3 and 4 demonstrated that the simultaneous transcutaneous permeation of NSAID and essential fatty acids from fish oil across excised pig skin using a fish oil vehicle was possible. Chapter 5 examined the permeation through the joint capsule into synovial fluid. This was found to be possible, indicating the potential value of such a formulation in RA and OA. It was considered important to ascertain if the permeated EPA could be incorporated into cartilage using an *in-vitro* model. Following this, the possibility that incorporated EPA could inhibit the production of the potent inflammatory mediators and hence reduce or halt the degradation of cartilage was studied.

To establish the potential of EPA to exert an anti-inflammatory and anti-degenerative effect in both rheumatoid arthritis and osteoarthritis the pathology of the disease states must first be examined. As the two diseases are different in many ways, the plausibility of EPA being able to treat symptoms of both and to prevent further deterioration is a fundamental factor to consider. There are certain similarities between the two and these are discussed in the following sections and an insight to treating both diseases with EPA is established.

6.1.1 Cartilage metabolism

The subchondral bone is covered by articular cartilage that consists of chondrocytes embedded in a specialised ECM containing collagens and proteoglycans that are unique to cartilage. As there are no blood vessels, nerves, or lymphatics within such tissue, chondrocytes derive their nutrition in two ways. Firstly by passive diffusion from the blood supply in the subchondral bone and, secondly, from diffusion from the synovial fluid (Goldring and Goldring, 1999). Under normal conditions, chondrocyte production is limited and penetration of other cell types from the joint space or subchondral bone is restricted. Thus the chondrocytes actively maintain a stable equilibrium between synthesis and degradation of matrix components (Poole, 1993). During aging and in inflammatory and degenerative joint diseases, such as RA and OA, this stable equilibrium is disrupted and the rate of loss of collagens and proteoglycans from the

matrix exceeds the rate of deposition of newly synthesised molecules (Poole, 1993) This metabolic imbalance leads to a progressive loss of articular cartilage and disruption of normal joint function.

6.1.2 Cartilage loss in RA

Cartilage destruction in RA occurs primarily in areas connected with the proliferating synovial pannus (Kobayashi and Ziff, 1975; Woolley et al., 1977) and to some extent at the cartilage surface exposed to the synovial fluid (Kimura et al., 1977; Dodge and Poole, 1989) due to the release and activation of proteinases from the synovial cells and polymorphonuclear leukocytes, respectively. In early RA, loss of proteoglycan occurs throughout the cartilage matrix and is not limited to the synovial-pannus junction (Mitchell and Shepard, 1978).

Destruction and loss of cartilage is triggered by the release of IL–1, a primary regulator of inflammatory response, both within cartilage chondrocytes and cells in the surrounding synovial fluid. This in turn causes the stimulation of potent inflammatory mediators, namely prostaglandins and leukotrienes by increasing COX-2 levels as detailed in Chapter 1. These mediators inhibit proteoglycan synthesis and initiate the release of MMPs which are involved in the breakdown of collagen and other proteins within the cartilage ECM.

6.1.3 Cartilage loss in OA

Cartilage loss in osteoarthritis is a direct consequence of the disease and both primary (idiopathic) or secondary (caused by major trauma) OA result in a breakdown of articular cartilage. This is caused by an imbalance of applied mechanical stress and the inability of the cartilage to overcome this stress. (Aigner et al., 2006) The resulting osteoarthritis ensues from the destruction of the ECM and the inability of the chondrocytes present in the cartilage to respond to such stimuli. Kurz et al., (2005) have suggested that, when exposed to a large mechanical force, the chondrocytes appear to switch from a state of low metabolic activity to that of one that stimulates production

of the many inflammatory mediators, including IL-1. When coupled with the lipid based mediators, PG and LT, the chondrocytes serve to increase their own catabolic activity resulting in the release of proteolytic enzymes (such as aggrecanses and MMPs), culminating in the destruction of the ECM (Loeser, 2006).

6.1.4 Efficacy of EPA treatment

As discussed in detail in Chapter 1, the main mechanisms behind the anti-inflammatory action of EPA lies in its ability to actively compete against AA. This results in the production of less potent inflammatory mediators via the COX and LOX pathways and inhibition of the expression of inflammatory cytokines, namely IL-1, TNF-α and COX-2 itself. Both RA and OA disease states are facilitated by the production of these mediators and the further expression of cytokines in the form of an auto-catalytic production cycle. Abramson and Yazici (2006) state that at the molecular level joint tissue, including synovium, joint capsule and cartilage are the site of production of the aforementioned molecules which are associated with inflammation and perpetuate joint damage and inevitably destruction. Figure 6.1 illustrates the main characteristics of RA and OA. Studies have shown that the metabolism of AA by COX-2 and 5-lipoxygenase (5-LOX) yielding these inflammatory mediators can be inhibited by the long chain *n*-3 polyunsaturated fatty acids such as EPA. Substantial work that highlights the effectiveness of NSAIDs in inhibiting the COX enzyme has also been carried out. Coupled with the knowledge that the presence of an NSAID, in this case ketoprofen, also enhances permeation of EPA the rationale for a system containing both *n-3* fatty acids and an NSAID is justified.

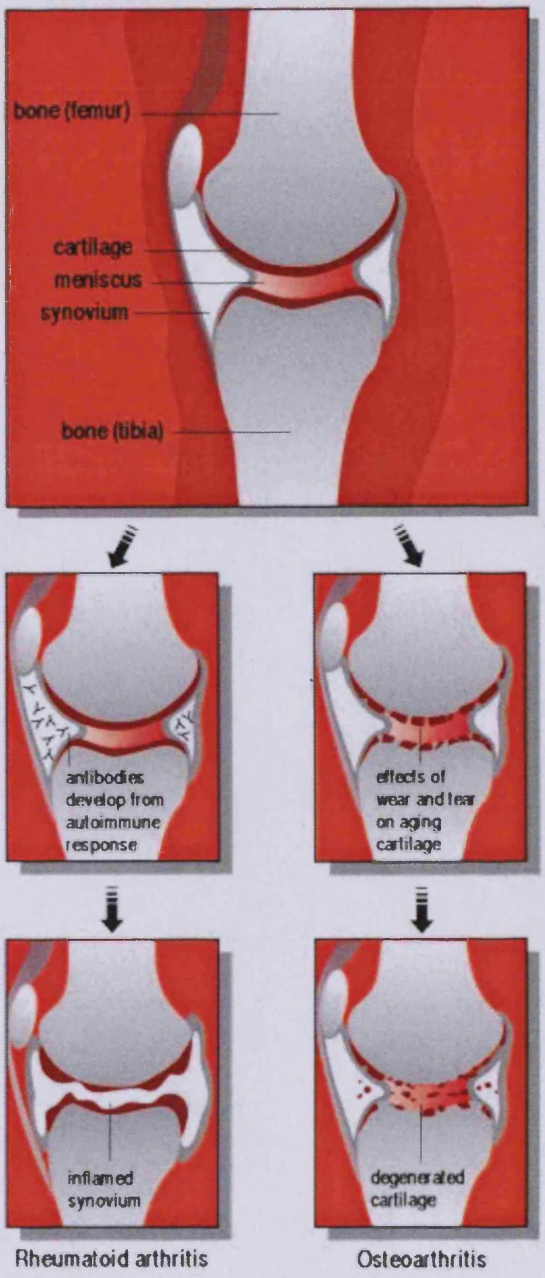


Figure 6.1 Disease progression of RA and OA

Curtis et al., (2002) have provided evidence supporting supplementation with *n-3* fatty acids. This study, carried out using well established culture models from normal bovine and human osteoarthritic cartilage, showed a dose-dependent reduction in the expression and activity of aggrecanases and the expression of inflammation-inducible cytokines, IL-1, TNF-α and COX-2.

In a study by Bousserouel et al., (2003) EPA and DHA were shown to abolish the production of the prostaglandin PGE_2 by inhibiting the IL-1β induced production of COX-2 mRNA. They concluded that EPA and DHA act in opposition to AA by altering different steps of the inflammatory process induced by IL-1β. In another study investigating the possible anti-inflammatory effects of EPA on the brain of aged rats via an IL-1β modulatory pathway, Lynch et al. (2003) showed EPA to exert an effect upon the IL-1β mRNA expression itself and thus prevented the age-related increase of the resulting IL-1β protein.

The aim of this chapter was to develop a novel *in-vitro* system for modelling the effect of fish oil on a sample of live cartilage following delivery across full thickness skin and further indicate the plausibility and efficacy of a transcutaneous fish oil formulation.

6.2 Development of 'model joint' system

To determine whether topically delivered EPA could be incorporated into a sample of viable cartilage, a 'model joint' system was developed. Ideally, this system would need to mimic a typical human joint (see Figure 6.2) undergoing topical treatment with an EPA formulation and must include the following:

1) A skin barrier overlying the model joint beneath.

2) Synovial fluid or an *in-vitro* substitute to determine the possibility of diffusion through the joint space and into the cartilage.

3) *Ex-vivo* viable cartilage ex-plant

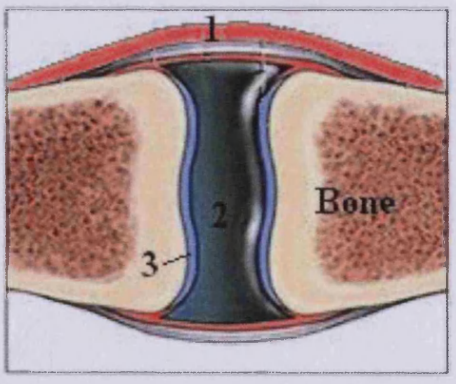


Figure 6.2 A normal joint indicating, (1) skin membrane, (2) synovial fluid and (3) cartilage

An appropriate model would allow determination of EPA permeation through skin, diffusion through a model synovial fluid and subsequent incorporation into ECM of cartilage.

The model joint pictured in Figure 6.3 is the most representative *in-vitro* model attainable in this work. Ligament and synovial membrane tissue is lacking from the model joint due to the inability to obtain viable tissue samples. Also absent is the process of dermal clearance. The absence of these tissues and process limits the model joint to a certain degree, however during rheumatoid and, to a lesser extent osteoarthritis, the synovial membrane is swollen and stretched which should allow greater permeation of both EPA and ketoprofen. Modelling the joint without these tissues should not detract from results gained from the system as the major components of the joint are still present. The presence of EPA and ketoprofen in the swollen synovial membrane will also allow the compounds to ease the pain and inflammation occurring within the tissue.

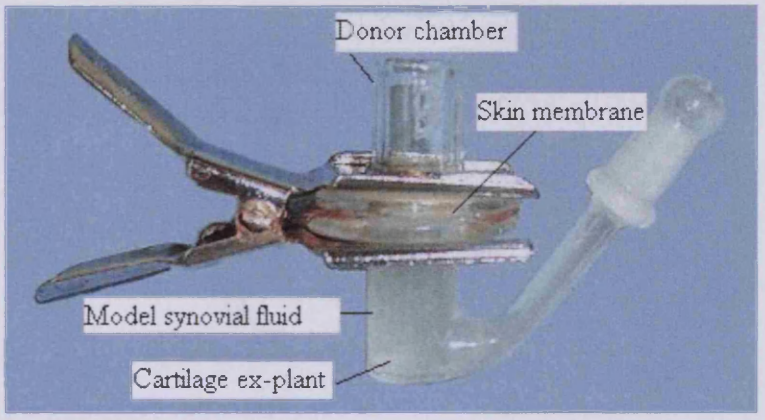


Figure 6.3 The model joint indicating, skin membrane, model synovial fluid and cartilage ex-plant

6.3 Materials and Methods

Viable bovine articular cartilage was obtained from the metacarpophalangeal joints of 1-2 week old calves. Ex-plant cultures of 1-2 mg wet weight of cartilage were established as described Little et al., (1999) and maintained for 4 days in approximately 10ml filtered bovine foetal calf serum. They were then washed with 4 x 10ml of filtered serum free medium containing 10% gentamicin. This section of work was carried out

under supervision by Dr. Clare Hughes and Prof. Bruce Caterson of the Cardiff School of Biosciences whose work on cartilage destruction has been well documented, Caterson et al., (2000); Curtis et al., (2000); Curtis et al., (2004).

6.3.1 Preparation of formulations

Each formulation was prepared as a 2.5% w/w formulation of ketoprofen in fish oil. The required mass of fish oil was weighed and BHA (0.05%w/w) added. The appropriate weight of ketoprofen was added and the formulation vortex mixed until full dissolution had occurred. An *n*-3 (EPA) formulation was also prepared by the addition of 240 µl (approximately 120mg) to 1ml of receptor phase solution (Dulbeccos serum free medium containing 10% gentamicin). The same receptor phase solution was used as a control formulation containing no *n*-3 fatty acids.

6.3.2 *In-vitro* skin permeation and cartilage incorporation study

Skin permeation experiments were carried out using glass Franz type diffusion cells as Chapter 2, Section 2.1. After sufficient culture time and rinsing protocol a sample of cartilage approximately 2mm x 2mm was added to each receptor phase compartment using sterile forceps within a laminar flow cabinet. The receptor phase chamber was then filled with receptor phase ensuring no air was present beneath the skin. The donor chambers were then filled with an infinite dose of formulation (1ml, approximately 0.8g) and the chamber occluded with perforated laboratory film. The complete cells were incubated at 37°C. A total of six replicates were carried out for the fish oil formulation and six replicates for the *n*-3 fatty acid formulation. Three cells of each formulation were removed after 48 hours incubation. The receptor phase was removed and frozen for analysis. The cartilage was stored separately, also at -20°C. The remaining cells were left to incubate until 72 hours when the receptor phase and cartilage were removed and stored as above. Three control cells were also analysed utilising the receptor phase solution as a control formulation. These cells were removed after 72 hours and the receptor and cartilage again stored as above.

6.3.3 Extraction of EPA from bovine cartilage ex-plant

To analyse any incorporated EPA the cartilage sample was first comminuted. This breaks down the cartilage to cellular level and allows extraction of the EPA. The cartilage was first frozen with liquid nitrogen and then added to the comminution chamber with a solid steel ball. After 5 minutes comminution at 9000 rpm, PBS (1ml) was added to the powdered cartilage. To this solution, chloroform / methanol (1:2) (3.75ml) was added and this mix vortexed and left at room temperature for 30 minutes. Chloroform (1.25ml) and Garbus solution (2M potassium chloride in 0.5M potassium phosphate buffer, pH 7.4) (1.25ml) were then added and the solution once again vortexed. The chloroform phase, containing the phospholipid, was then removed and evaporated to dryness under nitrogen. The remaining solid was then derivatised as detailed in Chapter 2, Section 2.2.2. The fatty acid methyl esters were then analysed by HPLC as described in Chapter 2, Section 2.2.2.1.

6.3.4 Initiation of IL-1 degradative response

After incubation for 72 hours the receptor phase was removed for analysis and the cartilage washed with 3 x 10ml Dulbeccos serum free medium. An appropriate aliquot of IL-1 was then added to 2ml of the chosen samples in a 24 well plate and incubated for a further 72 hours. The media was then analysed for lactate concentration (an indication of metabolism of the cartilage) and for GAG (indicative of cartilage degradation).

6.3.4.1 Lactate assay

A vial of lactate reagent was dissolved in water (10ml) with gentle inversion. The lactate reagent (250µl) was then added to a sample of media (5µl) from each well. The solutions were then placed in a spectrophotometer and analysed at 540nm. A standard curve of 25-400µg/ml was employed. Increased lactate is indicative of a higher metabolic activity and conversely decreased lactate shows a reduced metabolic activity.

6.3.4.2 Dimethylene methyl blue (DMMB) GAG assay

DMMB solution (200µl) was added to each well in a fresh well plate containing the sample media (40µl) and water (20µl). Analysis was carried out by spectrophotometry at 540 nm employing a standard curve of 0 - 40µg/ml GAG.

6.4 Results and Discussion

6.4.1 Transcutaneous delivery and incorporation of EPA into cartilage - 1

Table 6.1 shows the average mass of EPA detected in the receptor phases of all samples after 48 and 72 hours, indicating the average mass of EPA permeated after both time points.

Table 6.1 Average mass of EPA permeated into the receptor phase

Formulation	Time point	Average mass of EPA (µg) ± S.E.M.
Fish oil	48 hours	3.16 ± 0.004
Fish oil	72 hours	3.53 ± 1.5948
n-3 control dose	48 hours	2.64 ± 0.1154
n-3 control dose	72 hours	2.68 ± 0.9606

Table 6.2 illustrates the average mass of EPA present in the comminuted cartilage. This is indicative of the mass of EPA incorporated into the chondrocyte cells within the cartilage.

Table 6.2 Average mass of EPA incorporated

Formulation	Time point	Average mass of EPA (μg) ± S.E.M.
Fish oil	48 hours	0.71 ± 0.0451
Fish oil	72 hours	0.44 ± 0.1796
n-3 control dose	48 hours	0.30 ± 0.0306
n-3 control dose	72 hours	0.37 ± 0.0337

As previously demonstrated EPA has been transcutaneously delivered when using a fish oil vehicle. The permeated values are slightly less than those achieved in the previous study; however this could be due to a difference in receptor phase or a difference in incubation technique. It was shown that the fish oil formulation delivered a greater dose of EPA than the *n*-3 control dose. This can be expected, as the amount of EPA present in the fish oil formulation is greater than that of the *n*-3 control dose. As more EPA was seen to permeate it would be logical to assume a greater mass of EPA to be incorporated into the cartilage. This was found to be the case.

Interestingly, it was found that several of the receptor phases changed colour during the investigation and this was found to correspond with decreased metabolic activity and ultimately the death of the viable cells of the cartilage. For this reason a number of the cells were discarded and the receptor phase and cartilage samples were not used for further analysis. Due to this low number of repeats the investigation was repeated and the results are shown in Section 6.4.2.

6.4.2 Transcutaneous delivery and incorporation of EPA into cartilage – 2

Repetition of the same experiment produced a greater number of replicates but differing results. Only a limited number of cells appeared to be unusable and fewer problems were encountered. The average mass of EPA that permeated the skin membrane had increased by approximately ten fold as seen in Table 6.3.

Table 6.3 Average mass of EPA permeated into receptor phase

Formulation	Time point	Average mass of EPA (μg) ± S.E.M
Fish oil	48 hours	43.41 ± 5.6272
Fish oil	72 hours	66.12 ± 14.4272
n-3 control dose	48 hours	48.55 ± 4.3517
n-3 control dose	72 hours	48.11 ± 10.1966

Analysis of the receptor phase has shown that after 48 hours the permeation of EPA from fish oil and from the control formulation is comparable. However, after 72 hours, the permeation of EPA from the fish oil vehicle is approximately 1.4 times that of the control formulation. This is probably as a result of the higher dose present in the fish oil formulation.

The results of the cartilage analysis, Table 6.4, indicated an approximate level of saturation of EPA incorporation into the cartilage of 40 – 50μg. This is consistent between the fish oil formulations and the *n*-3 control dose. A plateau of EPA incorporation was anticipated as the masses of the cartilage are approximately the same.

Table 6.4 Average mass of EPA incorporated within bovine cartilage ex-plants

Formulation	Time point	Average mass of EPA (μg) ± S.E.M.
Fish oil	48 hours	40.81 ± 5.6620
Fish oil	72 hours	46.35 ± 4.5378
n-3 control dose	48 hours	39.58 ± 5.8198
n-3 control dose	72 hours	44.08 ± 9.9862

Table 6.5 shows the average percentage of EPA incorporated into the cartilage ex-plant from the applied dose. These results support the saturation of the cartilage as demonstrated above and it would appear that the cartilage can incorporate

approximately 2% of its mass within 72 hours. An encouraging factor is the dose that it is possible to incorporate within the cartilage. Curtis et al., (2002) when demonstrating the effects of EPA, used a concentration of EPA of between 50-70μg ml^{-1} which mimics human plasma levels of free fatty acids. The levels that were transcutaneously delivered in these experiments fall between these levels after 72 hours and fall just short after 48 hours. This would not cause a problem as re-application of a dose would occur approximately every 12 hours. Chapter 4 demonstrates a Q_{48} value through skin into a cetrimide receptor phase of approximately 28μg cm^{-2}, which again would increase after multiple dosing providing that saturation is not reached.

Table 6.5 Average percentage of EPA incorporated

Formulation	Time point	Average % of EPA from applied dose
Fish oil	48 hours	0.30
Fish oil	72 hours	0.38
n-3 control dose	48 hours	0.28
n-3 control dose	72 hours	0.42

These results differ from the previous analysis which again could be due to a difference in incubation from previous permeation experiments. However, this does not explain the discrepancy seen between the two studies and suggests that the life span of the cartilage could be an issue.

6.4.3 Stimulation of cartilage degradation by IL-1 and determination of the effect of permeated EPA

The results for both the lactate and DMMB analysis proved inconclusive. They were expected to confirm the initial degradation of the cartilage with an increase in metabolic activity and an increase in the GAG release. With EPA present these values would have been expected to be reduced.

Neither assay showed any difference between cartilage dosed with IL-1 and cartilage without IL-1 or between different formulations. In fact the results indicated a lower than normal metabolic activity within the cartilage indicating cartilage cell necrosis.

During the experiments it was noted that the colour of the receptor phase changed from red to cloudy yellow. It was concluded that this was due to a build up of waste material within the receptor phase and consequent change in pH which would have a debilitating effect on the cartilage and in this case caused cell death. This may explain the lack of metabolic activity seen after 72 hours. Another possible reason for this could be the incompatibility of the porcine skin with bovine cartilage. Enzymes leached from the porcine skin could contribute to the death of the bovine cartilage by being incompatible with the normal physiological function of the cartilage cells as these cells need optimum sterile conditions in which to stay alive.

6.4.4 Dialysis membrane

To determine if the porcine skin was having an effect upon the bovine cartilage a dialysis membrane was used in place of the porcine skin. This allowed permeation of EPA from the fish oil into the receptor medium and incorporation into cartilage without producing enzymes which may have been affecting the cartilage growth. Unfortunately no clear result could be gained from this experiment. The receptor medium once again appeared yellow after a short period of time. Loss of receptor fluid from the chamber was also apparent indicating a build up of pressure and the expulsion of fluid via the sampling arm of the chamber. As a result of continued negative results this area of work was postponed while other investigations were carried out.

As the receptor fluid changed colour when using the dialysis membrane, it would appear unlikely that incompatibility of the porcine skin with the bovine cartilage caused the necrosis. It is possible that unknown components present in the fish oil lead to the necrosis, but this hypothesis does not adequately explain the cartilage death seen when using a formulation of an EPA solution. It is thought that residual cleaning agents on the Franz cells (left after washing and rinsing) may be the cause, but further experiments are required to determine the true cause of cartilage death.

Since this work has been carried out a similar model joint has been developed by Kreiselmeier et al., (2005). They investigated a diffusion chamber for the analysis of the diffusion of a matrix metalloproteinase inhibitor through articular cartilage. The work employed a modified diffusion chamber with a sample of cartilage between the donor and receptor chambers but no skin membrane or model synovial fluid. For the investigation into permeation of compounds into the joint and incorporation within cartilage the work carried out in this chapter demonstrates a better model for joint delivery.

6.5 Conclusions

Incorporation of EPA into the cell membrane of the ex-plant appears to plateau after 48 hours but can be expected due to size limitations of the cartilage ex-plants and the unexplained necrosis of the cartilage during the experiment.

A novel system has been developed to determine uptake of free fatty acids into cartilage and has been used to prove EPA can be delivered transcutaneously via a fish oil vehicle. This EPA can then diffuse through a model synovial fluid and be incorporated into bovine cartilage ex-plants within approximately 48 - 72 hours and these concentrations were almost equivalent to test conditions where the anti-inflammatory effects of EPA have been investigated. Sample time points before 48 hours and after 72 hours could elucidate more information on the saturation of the ex-plant with EPA and also indicate a life span of the cartilage under these conditions. This "model joint" system could then be used to further study other formulations containing fish oil.

The results of this chapter in combination with Chapters 4 and 5 demonstrate the efficacy of a fish oil / ketoprofen topical formulation. It has been proven that both EPA and ketoprofen can permeate through the SC, epidermis and dermis and then permeate through the joint capsule (comprising of fibrous joint capsule and inner synovium lining) and into synovial fluid. Under the right conditions the EPA can then become incorporated into cartilage ex-plants. Under *in-vivo* conditions all of these actions would occur sequentially and the route of EPA and ketoprofen would be a lot more arduous. However repeated dosing of the compounds would allow a constant supply to the inflamed tissues and make a significant difference in joint pain and inflammation associated with RA and OA.

Chapter 7

Probing the phenomenon of permeation enhancement of fish oil / EPA by ketoprofen

i Metabolism of fish oil within full thickness porcine skin

7.1 Introduction

The transcutaneous delivery of EPA and ketoprofen from a fish oil vehicle was demonstrated in Chapter 3, where the data also seemed to indicate that an inter-relationship existed between the permeation of EPA and ketoprofen. Ketoprofen permeation was shown to be enhanced by EPA in Chapter 4, but it was difficult to explain the enhancement of EPA by ketoprofen using regular skin permeation theory. It was thought that epidermal metabolism or inhibition of such metabolism may go some way to rationalize the data.

The fate of polyunsaturated fatty acids following topical application to the skin has been discussed in several papers and is summarised in Williams and Barry, (2004). The general consensus is that the fatty acids become incorporated within keratinocytes and are subsequently converted to ceramide derivatives (Nugteren et al., 1995). Incorporation of EPA into cells plays an important role in inflammation as the replacement of AA results in a decrease in inflammatory mediators, as described in Chapter 1. One study showed that after 6 weeks of ingestion of 3.6g EPA daily, a decrease of AA composition of neutrophil membranes of 33% was induced (Simopoulos, 2002). This incorporation would also be expected to occur in keratinocytes.

However, the fact that EPA has been demonstrated to traverse skin intact (Thomas and Heard, 2004) indicates intercellular permeation occurs such that incorporation of the applied dose into keratinocyte membranes does not exclusively take place.

Fatty acids when used as penetration enhancers have been shown to pool in the stratum corneum and even form discrete lipid domains within bilayer lipids in the SC (Tanojo et al., 1997). From these pools within the SC it is plausible that the fatty acids could cause saturation of enzymatic pathways as opposed to a small constant supply which the metabolic processes can deal with efficiently. However, with high local bioavailability

of EPA it is probable that metabolism takes place to a certain degree possibly to an initial saturation limit before the production of further catabolic enzymes.

It has been proposed that, from oral doses, EPA is metabolised within the skin via epidermal 15-lipoxygenase to its monohydroxylated metabolite, namely 15-hydroxyeicosapentaenoic acid (15-HEPE) (Miller and Ziboh, 1988). It has also been reported that these metabolites accumulate in the epidermis of guinea pigs after ingestion of fish oil ethyl esters (Miller et al., 1991). 15-HEPE is also a non-potent inflammatory mediator with the ability to actively compete with AA and thus inhibit the production of the 2-series prostaglandins and the 4-series leukotrienes via the COX and LOX pathways. Interestingly, it was found that the 15-HEPE is more potent than EPA at inhibiting cellular growth and AA metabolism in human prostatic cancer cells in culture (Vang and Ziboh, 2005). It is for this reason that 15-HEPE has been studied, as converted esters or ceramide derivatives have little or no benefit in the eicosanoid cascade.

The co-permeation of molecules across skin is a recently probed phenomenon and the effect on metabolism exerted by a co-permeant has had little examination. With EPA and ketoprofen acting on the COX enzymes, whether the two actives will act synergistically or competitively with relation to COX in the skin and the extent to which one would enhance or retard the metabolism or function of the other was unknown. If an EPA-ketoprofen complex co-permeates then this also may play a part in the utilization of the two compounds.

To investigate the metabolism of compounds within the skin it is a vital pre-requisite that the skin be as fresh and as metabolically active as an *in-vivo* experiment would allow. The viability of the skin for the duration of normal skin permeation experiments has been investigated using a range of different receptor phases. Haberland et al., (2005) found that when using minimum essential medium Eagle (MEME) supplemented with PBS + 0.1% glucose as receptor fluids, the viability of the skin equivalents used, compared to keratinocyte medium, was reduced by 10-50%. The viability of the skin is paramount when investigating the metabolism or breakdown of

the drug / compound of interest. This issue is also of major importance when delivering co-drugs via the transdermal pathway as the co-drug requires metabolism to release the active compound locally or systemically. It is important, when designing such investigation, either into the metabolism of a drug or delivery of a co-drug, to ascertain whether the enzyme activity within freshly excised skin, using a non growth media receptor phase, is equivalent to the activity profile of growth media sustained skin. The simplest way of providing the growth media to the skin is utilising the growth media as the receptor phase solution in a normal Franz cell permeation experiment.

This chapter describes the investigation into the metabolism of EPA by 15-LOX as it permeates through the skin from a fish oil vehicle. The effect of the incorporation of ketoprofen into the formulation on the permeation and conversion of EPA to its 15-LOX metabolite is also examined, to observe if ketoprofen modifies 15-LOX metabolism in any way and to try and explain the increased permeation of EPA seen previously when incorporated within the same formulation. Additionally this metabolism will be examined when using both growth media sustained skin (GMS) and a non growth media (NGM) receptor phase solution, cetrimide. In this case the relative metabolite production will be an indication of the viability of the skin under the different conditions.

7.2 Materials

Fish oil capsules 1000mg (Boots Super Strength, batch number: 30446M) were purchased from a local store. Ketoprofen, butylated hydroxyanisole (BHA) and cetrimide BP were obtained from Sigma – Aldrich Company Ltd., Poole, UK. High vacuum grease was from Dow Corning, Barry, UK. Methanol (HPLC grade) was obtained from Fisher Chemicals, Loughborough, UK. All other reagents were of analytical grade or equivalent. D-Squame® Stripping Discs were obtained from CuDerm Corporation, Dallas, Texas. Freshly excised pig ears were obtained from a local abattoir prior to steam cleaning, cleaned under running water and the hair removed using electric clippers. The time interval between slaughter and commencement of experimentation was approximately 1 hour. Full thickness skin was then removed from

the dorsal side of the ear, cut into approximately 2cm x 2cm sections and used within two hours of excision.

7.3 Preliminary investigation into the metabolism of fish oil in full thickness porcine skin

A simple preliminary study was carried out to demonstrate that fish oil was metabolised in porcine skin following topical delivery. Fresh full thickness pig skin was prepared as described in Section 7.2, cut into approximately 3mm squares, placed in a screw top glass vial and ground for 30 seconds with a glass rod. After application of dose (see Table 7.1), the skin was again ground for a further 30 seconds and the glass vial sealed and placed in temperature-regulated oven at 37°C for 24 hours. The samples were then removed and stored at –20°C until needed. Each sample was prepared in duplicate. The samples containing cetrimide solution were freeze dried and then all samples were derivatised as per FAME method.

Analysis of the FAME's was carried out as Section 2.2.2.1.

Table 7.1 Metabolism sample preparations

Sample name	Skin	Fish oil[1]	Cetrimide[2]
Fish oil and skin	2cm x 2cm	10µl	X
Fish oil, cetrimide, skin	2cm x 2cm	10µl	3ml
Fish oil	X	10µl	X
Fish oil and cetrimide	X	10µl	3ml
Skin	2cm x 2cm	X	X
Skin and cetrimide	2cm x 2cm	X	3ml

[1] 10µl fish oil equates to approximately 10mg. This in turn equates to 3.3mg EPA and 2.1mg DHA using values quoted by Boots plc.

[2] 3ml was used to mimic the conditions during a skin permeation experiment i.e. the average volume of receptor phase is 3ml.

7.4 Depth profile analysis of EPA and ketoprofen

7.4.1 Preparation of formulations

A fixed sub-saturated dose of ketoprofen (2.5% w/w) was used in each formulation as described in previous chapters. The appropriate masses of ketoprofen and fish oil were combined, in addition to BHA (0.05% w/w) to inhibit PUFA degradation, and the formulation vortex mixed. The formulations were stored at 2-4°C until required ($\leq$ 24 hours).

7.4.2 Receptor phase

Two different receptor phases were used during these experiments to determine the viability of porcine skin with and without the presence of growth media. In this case Hepes modified Hanks buffer solution (HHBSS) was the growth medium of choice as its use is well established. HHBSS was used as its use has been well documented in prolonging the viability of skin post excision (Mukhtar, 1992). HHBSS was prepared by the addition of 25μM of HEPES and 50μg/ml of gentamicin sulphate to Hanks buffer solution, prepared as per manufacturers instructions. Degassed 30 mg ml^{-1} cetrimide solution was the second receptor phase solution utilised.

7.4.3 Depth profiling

Skin permeation experiments were carried out using glass Franz-type diffusion cells (see Chapter 2, Section 2.1) with the donor chambers of the cells dosed with either 1ml (approximately 0.8g) or 30μl (approximately 30mg) of each formulation +/- ketoprofen. The 30μl doses were applied to the skin then massaged into the skin ten times using a circular motion of a glass rod. A total of six replicates were carried out for each formulation. Samples were stored at –20°C prior to analysis. The skin was removed from the cell and excess formulation was removed from the top of the skin prior to tape stripping as detailed in Chapter 2 section 2.6.1.

7.5 Analytical Methods

7.5.1 HPLC analysis

An aliquot of extraction fluid was taken from each vial and EPA, 15-HEPE and ketoprofen were assayed as Chapter 2, Section 2.2.3, 2.3 and 2.4 respectively.

7.5.2 Data analysis

HPLC area data was used to determine the concentration (μg cm^{-2}) of compound and this data was plotted against tape strip number.

Statistical analyses were carried out using Instat 3 for Macintosh (GraphPad Software, Inc), where non-parametric Mann-Whitney tests were employed to determine differences between specific pairs of formulations.

7.6 Results and Discussion

7.6.1 Preliminary study

Table 7.2 shows the average mass of both EPA and DHA following incubation under different conditions. Following incubation with skin the levels of EPA and DHA detectable were markedly reduced, by up to approximately 0.5mg, demonstrating that fish oil permeating porcine skin is susceptible to metabolism and or cell incorporation.

Table 7.2 Average EPA and DHA content from skin metabolism study

Sample	Average EPA content (mg)	Average DHA content (mg)
Fish oil and skin	1.066	0.979
Fish oil, cetrimide, skin	1.070	0.944
Fish oil	1.686	1.648
Fish oil and cetrimide	1.570	1.493
Skin	not detected	not detected
Skin and cetrimide	not detected	not detected

7.6.2 Depth profile analysis of EPA – cetrimide receptor phase

The depth profile analysis for EPA using freshly excised porcine skin and the cetrimide receptor phase (non growth media (NGM)) is seen in Figure 7.1. Typical profiles were produced, whereby the amounts of EPA localised diminished with increasing depth (Heard et al 2003b). The profile shows the mass cm^{-2} (± S.E.M) extracted from the groups of tape strips 1 – 30 and the remaining heat separated epidermis and dermis of the skin section.

It must be noted that no 15-HEPE was detected, suggesting either that the skin had ceased to be viable before the experiment or the cetrimide receptor phase solution was adversely affecting metabolic processes within the skin. The results show a slight difference in permeation of the formulations containing ketoprofen and those which do not. A lower concentration of EPA can be expected when ketoprofen is present as there is 2.5% less EPA in the applied dose. The difference in the 30µl dose and 1ml dose is as expected, in that the 1ml dose shows significantly greater permeation ($p=0.0387$). This is a common feature of finite versus infinite dosing. However the presence of ketoprofen in the formulation in the 30µl dose enhances the permeation of EPA through the skin to the lower levels of the epidermis compared to that of the formulation containing no ketoprofen, as illustrated by the increased concentration of EPA in strips 10 to the epidermis. This enhancement, however, is deemed not significant ($p=0.5899$).

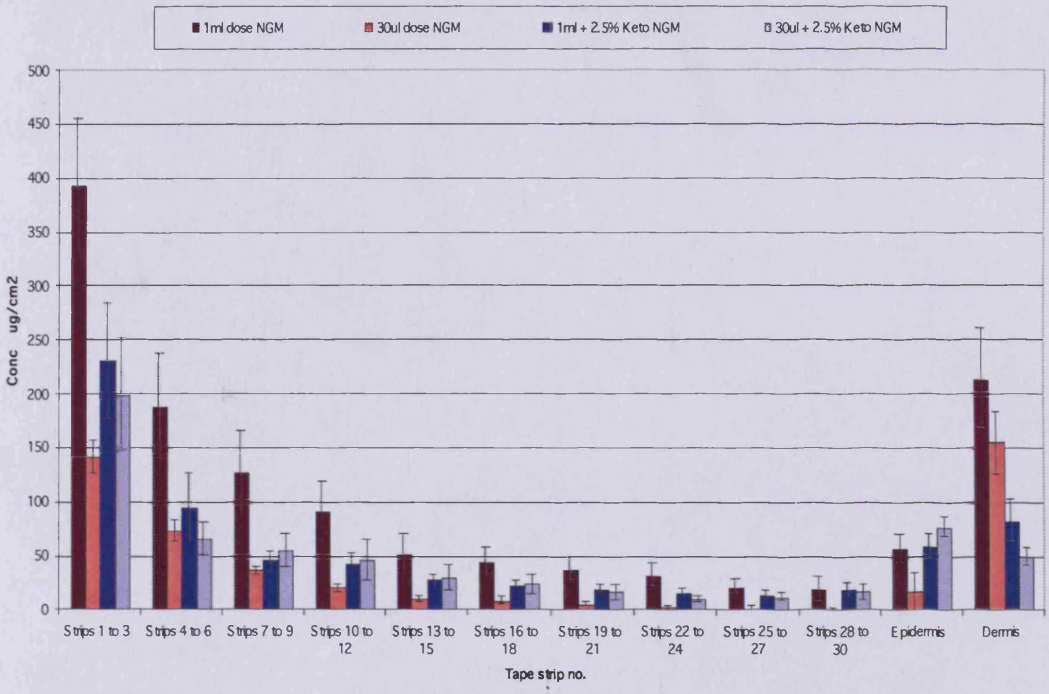


Figure 7.1 Comparison of NGM samples (n=6 ± s.e.m.)

A clearer comparison of doses can be made when examining the percentage of the applied dose that permeated (see Figure 7.2). This takes into account the presence and the true effect of the ketoprofen upon the permeation of EPA.

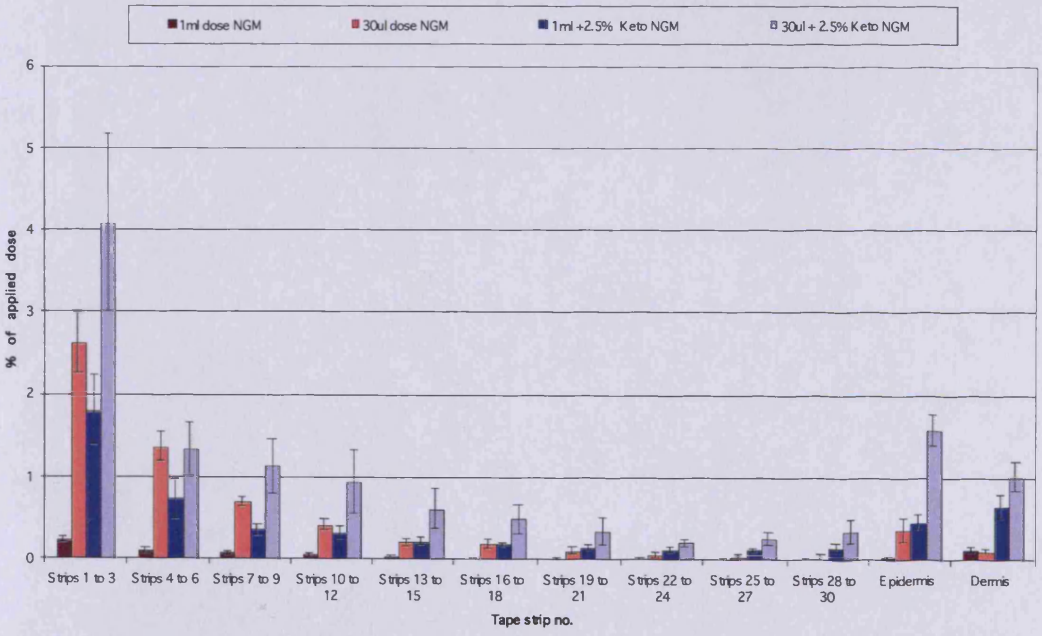


Figure 7.2 Percentage of applied dose comparison of NGM samples (n=6 ± s.e.m.)

When considering percentage values, it can be seen that proportionately more EPA localised from the 30µl dose containing ketoprofen than from the 1ml dose containing ketoprofen equating to an average of 0.6% of the applied dose. This may be related to the massaging protocol employed when applying the 30µl dose. Massaging enhances the uptake into skin, possibly assisted by mechanical erosion of the SC.

7.6.3 Depth profile analysis of EPA – Growth media sustained skin

The results using the GMS skin show that there was no significant difference ($p>0.10$) between any of the doses (see Figure 7.3). There was however an overall reduction in the concentration of EPA detected. A direct comparison between the GMS and NGM samples, indicated that the amounts of EPA differed by up to 200µg cm^{-2} in the 1ml infinite doses compared to the same dose with the NGM samples, producing a difference of 0.05 - 0.1% of the applied dose thus indicating a significant breakdown of EPA during the permeation process. This is thought to be due to the greater metabolic activity in the skin as an effect of growth media sustaining the skin, as the other aspects of the experiment were identical.

The formulation which exhibited the greatest permeation was the infinite dose containing ketoprofen. This is to be expected as, if a ketoprofen-EPA complex exists, this would affect the metabolism of EPA by the LOX enzymes including, as in this case, the 15-LOX iso-form.

There does not appear to be much difference in the concentrations of EPA in the skin between the other formulations or doses. This could be due to a saturation of the LOX metabolic pathway of EPA, perhaps even at a 30µl dose. Once again a clearer trend can be seen when the percentage of the applied dose is compared. (Figure 7.4)

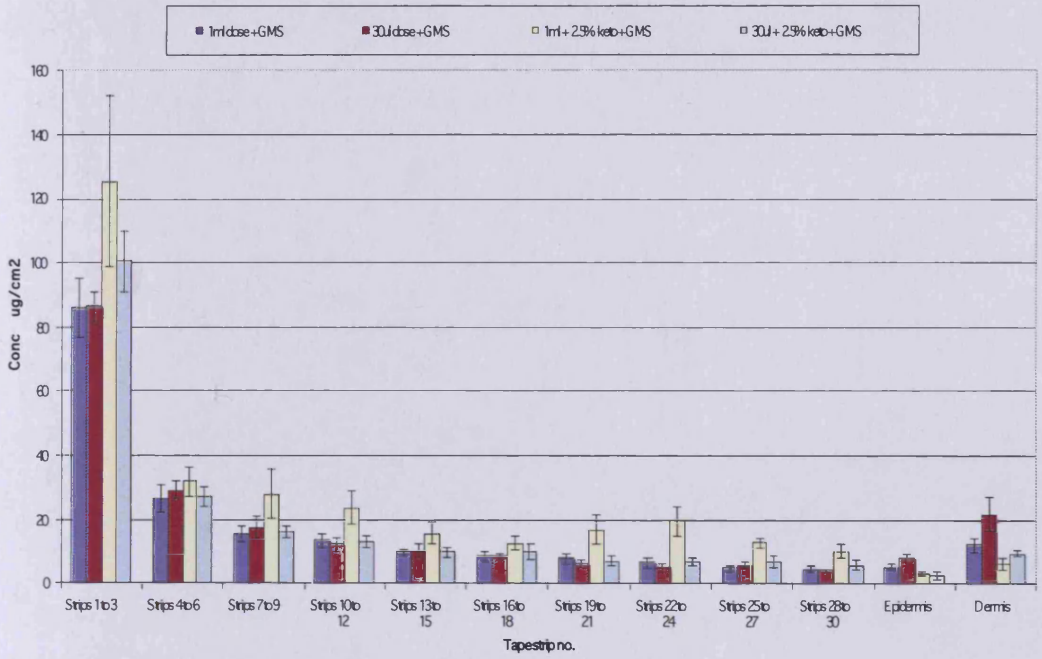


Figure 7.3 Comparison of GMS skin samples (n=6 ± s.e.m.)

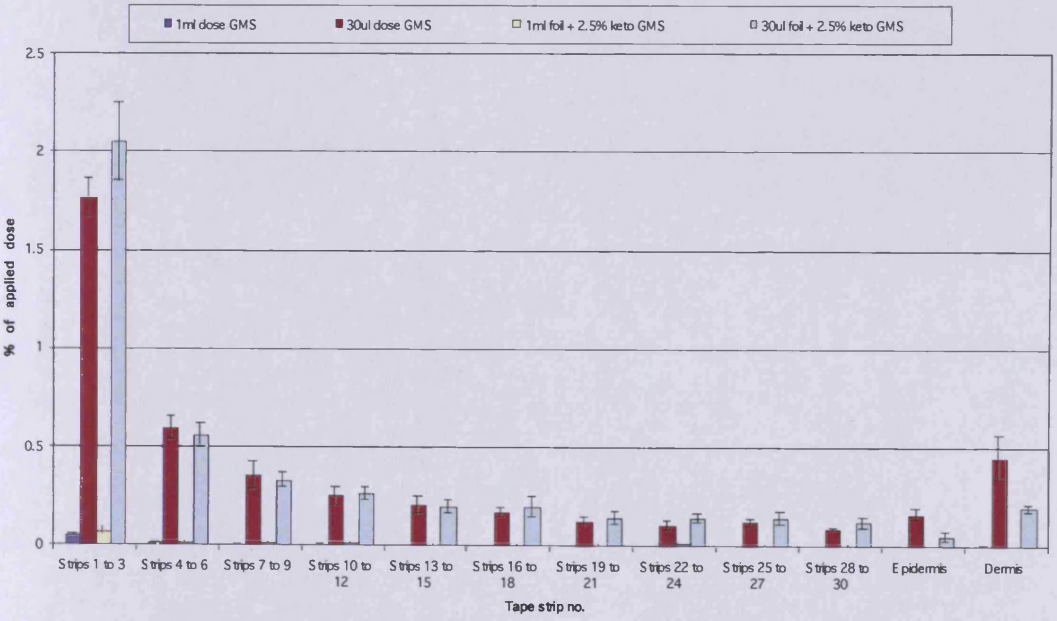


Figure 7.4 Percentage of applied dose comparison of GMS samples (n=6 ± s.e.m.)

7.6.4 Depth profile analysis of ketoprofen

The depth profiles for ketoprofen in all doses are compared below in Table 7.3. The majority of the ketoprofen is found in the dermis of all samples after 24 hours of permeation (highlighted). It is clear that the greatest concentration of ketoprofen is present when a 1ml dose is used with the NGM cetrimide receptor phase solution. The GMS samples show a vastly reduced concentration of ketoprofen in remaining epidermis and dermis, indicating its utilization within the skin either from an EPA-ketoprofen complex or in its role as an inhibitor of COX enzymes.

Table 7.3 Depth profile of ketoprofen in all samples

	Concentration (μg cm^{-2})			
	1ml GMS	**30ul GMS**	**1ml NGM**	**30ul NGM**
Strips 1 to 3	9.81	9.04	22.42	3.25
Strips 4 to 6	2.97	2.28	6.10	1.27
Strips 7 to 9	2.25	1.61	4.09	1.01
Strips 10 to 12	2.00	1.44	3.15	0.85
Strips 13 to 15	1.66	1.20	2.24	0.73
Strips 16 to 18	2.00	0.70	1.86	0.57
Strips 19 to 21	1.61	0.52	1.95	0.47
Strips 22 to 24	1.71	0.51	1.63	0.46
Strips 25 to 27	1.74	0.67	1.87	0.53
Strips 28 to 30	1.33	0.50	1.42	0.31
Epidermis	3.60	2.66	42.96	8.24
Dermis	29.83	26.21	206.16	28.57

Figure 7.5 shows the molar ratios of EPA:ketoprofen as a function of depth within the skin. The average molar ratios for 1ml GMS, 30μl GMS and 1ml NGM samples was approximately 10:1. This increases sharply in the 30μl NGM sample to approximately 34:1. This may be due to the readiness and ease of permeation through less metabolically active skin to the dermis, leaving a greater proportion of EPA in the epidermal layers. The molar ratio of EPA:ketoprofen within the formulation was approximately 6:1 which is less than that found within the skin. This may be due to the metabolism of the fish oil triacylglycerols to free fatty acids possibly allowing greater association.

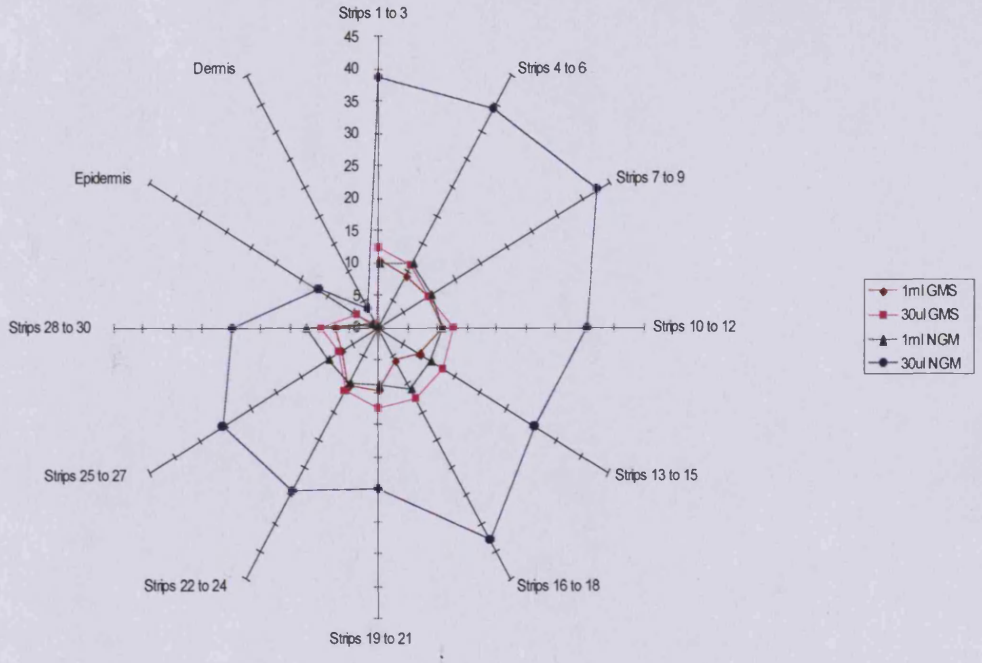


Figure 7.5 Molar ratios of EPA:ketoprofen as a function of depth

7.6.5 Analysis of 15–HEPE – Growth media sustained skin

Unlike with NGM, Figure 7.6 shows that 15-HEPE was being generated in the skin when GMS was used as receptor phase. This suggests:

1. The skin was indeed viable during the experiment
2. The cetrimide receptor phase may have had an adverse affect upon the skin in the NGM samples.

The presence of this metabolite is itself indicative of a high metabolic activity within the GMS skin samples. The distribution of 15-HEPE is consistent with the hypothesis that the presence of ketoprofen is one reason for the increased permeation of EPA, i.e. as a consequence of forming a permeation complex and thus inhibiting the metabolism of EPA by 15-LOX.

As expected the greatest formation of 15-HEPE was seen with 1ml of fish oil. The change to a finite dose resulted in less metabolite being produced, again as expected. The addition of ketoprofen into the formulation resulted in an even lower concentration of metabolite being produced. As only ketoprofen, fish oil and BHA (fixed at 0.05%

w/w) were present, this reduction in metabolism of EPA from fish oil must have been due to the ketoprofen as BHA was present in all formulations. The slight increase in the overall production of 15-HEPE as the tape strip number increased was due to the proximity of the EPA to the highly metabolically active basal layer of the epidermis. It also appears that there was a reservoir effect in the remainder of the epidermis after strip number 30 and a higher proportion within the dermis, implying that permeation of 15-HEPE through the epidermis into the dermis occurred. It is possible that some metabolite was generated in the dermis, although it is more likely that 15-HEPE was accumulating at the skin / receptor phase interface.

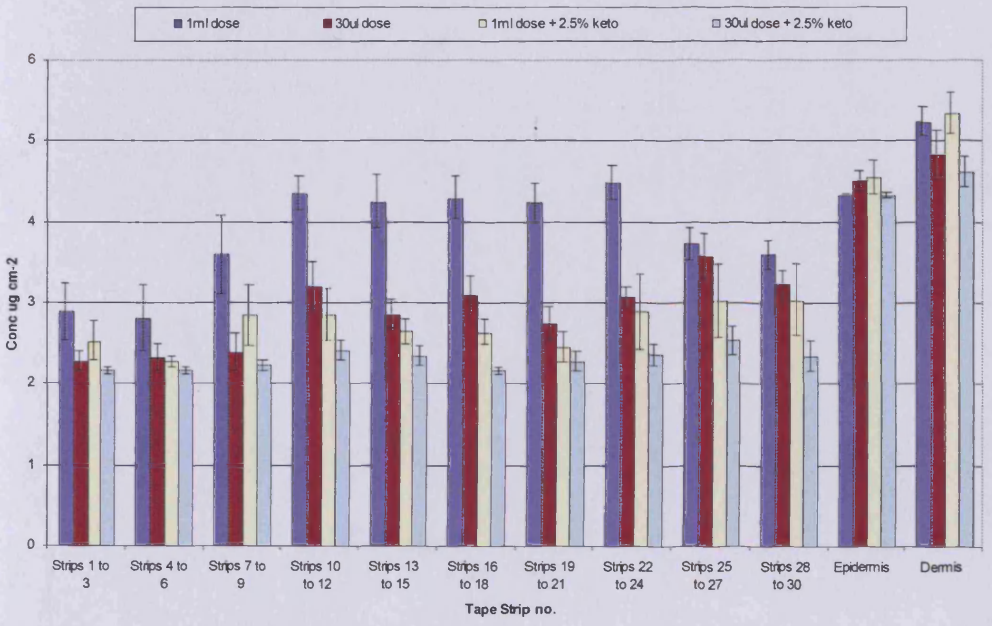


Figure 7.6 Concentration of 15-HEPE as a function of depth (n=6 ± s.e.m.)

The production of 15-HEPE was clearly dependent on the concentration of EPA within the skin. Figure 7.7 illustrates the concentration of 15-HEPE as a percentage of the EPA present at that particular point within the skin. It is, in effect, the percentage of EPA converted to 15-HEPE at that point within the skin. The shape of the profile corresponds well with the activity of the layers of the epidermis. A greater conversion of EPA to 15-HEPE is seen as the proximity to the highly metabolic basal layer of the skin is increased. The presence of ketoprofen was also seen to exhibit an inhibitory effect upon the metabolism of EPA to 15-HEPE up to the remainder of the epidermis

and the dermis. This difference between the upper layers of the epidermis and remainder of the skin may be explained by the permeation and accumulation of 15-HEPE within the lower layers of the skin. The inhibited permeation of 15-HEPE into the receptor phase may result in a reservoir of the compound within the dermis and remaining epidermis. However, the action of ketoprofen in the metabolically active layers of the epidermis is significant, especially when comparing infinite doses of the formulation. As ketoprofen is known to act on the COX enzymes and not LOX this is a surprising inference when considering the reduction seen in concentration of LOX produced 15-HEPE. It is likely that some degree of complexation takes place between EPA and ketoprofen leading to less free EPA available to be metabolised. 15-HEPE does not account for all the metabolised EPA however as other catabolic enzymes such as COX are present and will also be metabolising EPA.

Also evident during the permeation process is a plateau of metabolism. After the first three strips the values for both infinite and finite formulations, + ketoprofen and the formulations –ketoprofen, appear to have become similar and not significantly different ($p>0.10$ in all comparisons) indicating a metabolic saturation level within the skin. Once reached, the permeation of EPA exceeds the metabolism and a constant concentration of metabolite and EPA is seen. Such flooding of the particular metabolic pathway, COX or LOX, permits the permeation of further molecules of EPA in an unconverted state. The concentration of ketoprofen present in the formulations plays an important role in flooding the COX pathway, as if the pathway is competitively inhibited by ketoprofen, this creates the opportunity for EPA permeation.

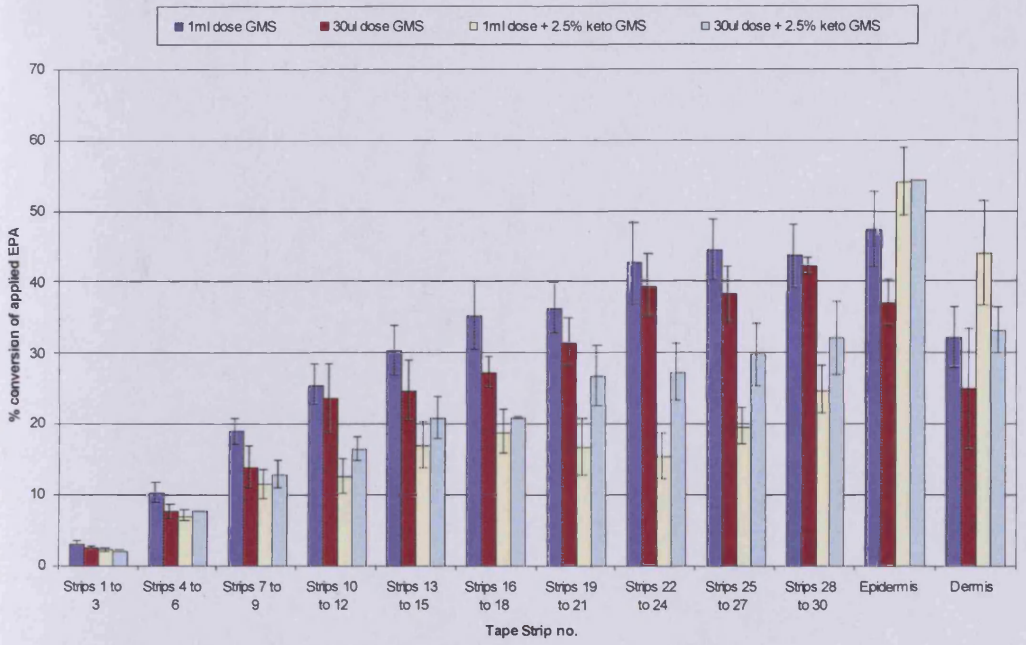


Figure 7.7 Concentration of 15-HEPE as a percentage of EPA at specific depth (± s.e.m.)

It is not appropriate to compare finite and infinite dosing, as the thermodynamic effects are different, introducing an extra factor in permeation and leading to iniquitous comparisons being made. Figure 7.8 shows the comparison of the 1ml doses of both formulations with both receptor phase solutions.

Both NGM results show an increased percentage of EPA. It is evident that the presence of ketoprofen is having an effect on the permeation of EPA, be it in the GMS samples as explained or in the NGM samples. This alludes to the possibility of another mechanism in operation within the formulation that enables ketoprofen to enhance the permeation of EPA. This phenomenon is examined in Chapters 8 and 9.

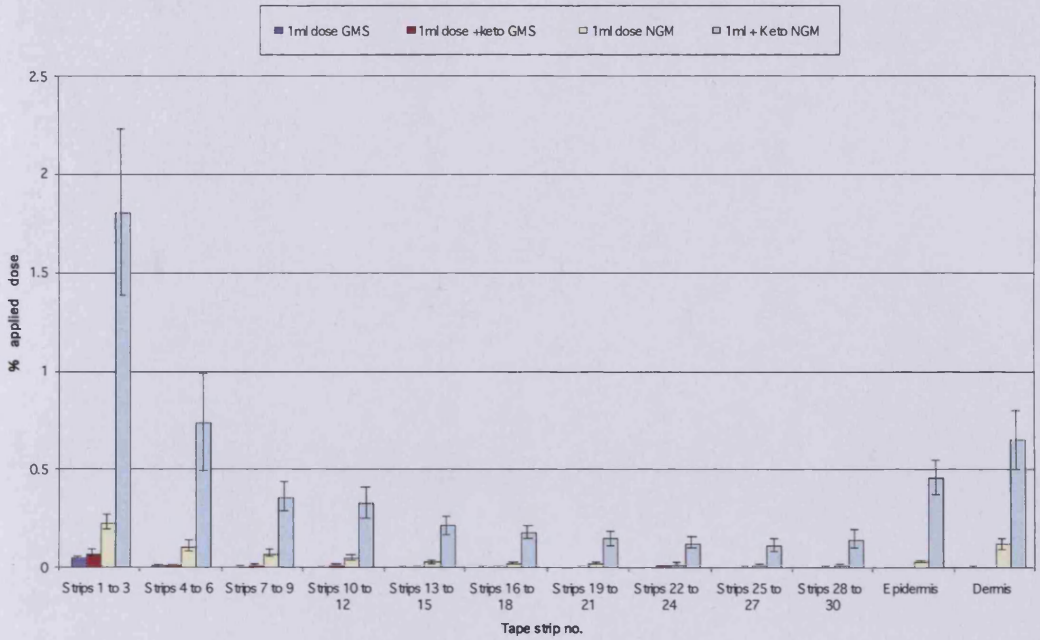


Figure 7.8 Comparison of percentage of applied dose of 1ml formulations, GMS and NGM (± s.e.m.)

The data from the finite doses are compared in Figure 7.9. The finite dose comparison shows a logical pattern of permeation. The lowest permeation was seen with the 30µl dose without ketoprofen with the viable, metabolically active, GMS skin. When ketoprofen was added inhibition of metabolism by 15-LOX was increased permitting more EPA to permeate unchanged. With the NGM skin, a greater concentration of EPA permeated as the skin was not viable. The presence of ketoprofen once again enhanced the permeation of EPA as in the 1ml doses.

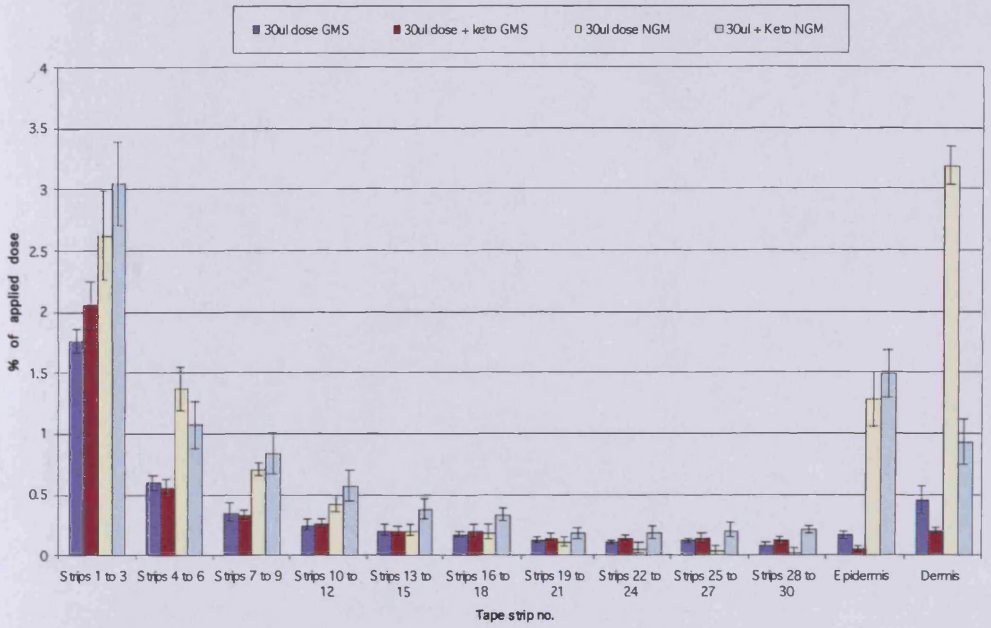


Figure 7.9 Comparison of percentage of applied dose of 30µl formulations, GMS and NGM (± s.e.m.)

7.7 Conclusions

Several conclusions can be drawn from this investigation. Firstly, it has been shown that metabolism within the skin (in this case via 15-LOX) is unfavourable to the transcutaneous permeation of EPA. The ability of ketoprofen to enhance the permeation of EPA through the skin does negate this to a certain degree, but it must still be considered when designing further EPA permeation experiments. However, metabolism of EPA in the transcutaneous treatment of arthritis does not detract from the system, as 15-HEPE has also been shown to be a highly effective anti-inflammatory.

Evident within this chapter is the importance of ketoprofen for enhanced EPA permeation in both growth media sustained skin and growth media absent skin. The data from this study has suggested a further explanation for the enhancement demonstrated in Chapter 4 and by Thomas and Heard, (2005). The presence of ketoprofen in the formulation appears to decrease the 15-LOX metabolism of EPA and resulting conversion to its metabolite, 15-HEPE. However, if ketoprofen is inhibiting the action of COX then more EPA is left in an unaltered state upon which the LOX

enzymes can act. This should then cause an increase in 15-HEPE production, but this was not seen to be the case. If the metabolism of COX is hindered and the 15-LOX pathway becomes saturated, more EPA is left free to permeate. This would explain greater overall permeation but not the influence of ketoprofen on the metabolism by 15-LOX as seen in this chapter.

An additional explanation for the enhancing ability of ketoprofen is therefore required. 15-LOX is just one enzyme prevalent in the epidermis and it may be the case that other enzymes are just as important in the metabolism of EPA. Thus the link between ketoprofen, enzyme pathways and transdermal permeation of EPA appears to be only part of the solution as even in non-viable skin ketoprofen has a beneficial enhancing effect.

A possible complexation between ketòprofen and EPA could go some way to explaining the result. This complexation could cause a drag effect by ketoprofen on EPA allowing greater permeation. The co-permeation of drugs and of drug / solvent complexes is a phenomenon currently under scrutiny (Heard et al., 2006) and this data could be another example of such an occurrence.

The third conclusion is that the choice of receptor phase solution in skin permeation and specifically skin metabolism studies is a crucial aspect when designing and carrying out such an experiment. As stated in Haberland et al., (2005) the slightest change of receptor phase constituents can have a drastic effect on the viability of the skin cells. This study demonstrates that cetrimide, a widely used receptor phase, is not a good choice for this category of work and that a growth medium should be used in the majority of skin diffusion experiments to try and mimic *in-vivo* conditions as closely as possible. That said, this issue must be balanced alongside the necessity to provide sink conditions in skin experimentation.

Chapter 8

Probing the phenomenon of permeation enhancement of fish oil/EPA by ketoprofen

ii NMR spectroscopy and molecular graphics

8.1 Introduction

The co-permeation of compounds is a common feature of topical formulations containing more than one active ingredient, although the ability of one compound to enhance the other's permeation has not been fully investigated. It can be hypothesised that drugs within a formulation are not distinct entities in discrete solvation cages and that some degree of complexation takes place depending on the nature of the compounds and solvent in question. The nature of solvation itself is the complexation of solvent and solute (Figure 8.1) and hydrogen bonding between molecules and water is the fundamental factor of water being one of the most widely used solvents for organic molecules.

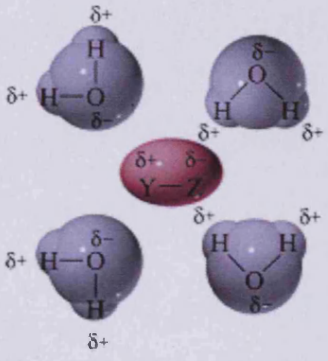


Figure 8.1 Hypothetical solvation of a polar compound by water

The complexation that could occur between molecules within a solution would have a follow-on effect on the permeation of these molecules through the skin, both thermodynamically and physically. This is on the proviso that complete dissociation does not occur beforehand. The permeation enhancement of compounds by chemical means and by careful selection of solvents is a field that has had more attention but still leaves certain aspects of permeation unanswered. For example, the implicit assumption made by many that a drug will permeate leaving the solvent behind in the vehicle or stratum corneum has recently been shown to be inaccurate (Thomas and Heard, 2004; Heard et al., 2006). It is assumed that either the solvent would have, or should have evaporated if volatile enough or, the stratum corneum has been efficient enough to stop any further permeation into the underlying dermis. It has been shown that ethanol and 1,8-cineole used as enhancers can permeate the skin in a dose dependent manner and enhance permeation of solute in a similar manner (Heard et al., 2006). Such

considerations highlight the importance of understanding the fate of enhancers and solvents. Mechanisms of enhancement are often the subject of publications Williams and Barry, (2004), Williams et al, (2006), Yamane et al., (1995) but the fate of solvents and enhancers is discounted or it is assumed that the enhancer has no further effect on the skin or upon permeation and is "washed out" of the stratum corneum leaving the skin to return to its original state (Williams and Barry, 2004).

In Chapter 4, the *in-vitro* transcutaneous delivery of EPA and ketoprofen from a series of formulations containing a fixed 2.5% ketoprofen and various concentrations of the enhancer 1,8-cineole was examined. It was found that the dose dependent enhancement of ketoprofen by 1,8-cineole was not replicated with EPA. A hypothesis for this apparent enhancement was stated in that ketoprofen may have been preferentially solvated by 1,8-cineole and that the loss of solvent from the formulation thermodynamically aided ketoprofen permeation. The permeation of EPA was enhanced by the presence of ketoprofen and similarly the permeation of ketoprofen was enhanced by the presence of fish oil. This synergistic enhancing effect was again demonstrated throughout Chapter 6 leading to the proposal of an EPA / ketoprofen complex forming within the formulation and permeating through the dermis.

Chapter 7 discussed the inhibition of COX by ketoprofen, which could provide a mechanism for a greater concentration of EPA to permeate. It was shown that the presence of ketoprofen in a formulation increased the presence of the EPA throughout the skin and conversely lowered the presence of the 15-LOX metabolite of EPA, 15-HEPE. This suggests two main hypotheses concerning the permeation enhancement of EPA by ketoprofen:

1. Formation of an EPA-ketoprofen complex and corresponding drag effect of ketoprofen on EPA
2. Inhibition of enzymes that act upon EPA by ketoprofen resulting in the less demanding passage of EPA through the skin, investigated in Chapter 9

In this chapter the formation of an EPA-ketoprofen complex is investigated using NMR to determine complexation involving possible π-π interactions between EPA and the

aromatic regions of ketoprofen. As a further insight into possible complexes, a series of molecular models were constructed and binding energies of these complexes were calculated.

8.2 Probing $\pi - \pi$ Interactions

8.2.1 Molecular orbitals and π-π interactions

A molecular orbital is the discrete energy and region of space around the nuclei of the atoms in a molecule in which an electron is found. The four prominent electron orbitals that can exist on individual atoms are s, p, d and f orbitals. The shapes of these orbitals are shown in Figure 8.2. The number notation signifies the energy of orbital and also corresponds to the distance of the electron from the nucleus of the atom with 1 being the highest energy and closest to the atom.

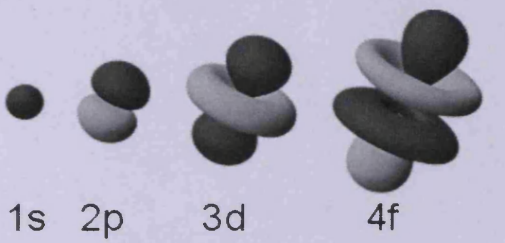


Figure 8.2 s, p, d and f atomic orbitals (http://en.wikipedia.org/wiki/Chemistry)

These orbitals give rise to distinct molecular orbitals including sigma (σ) and pi (π). Of importance in this chapter's work are π molecular orbitals which are commonly found between alkene carbons. A π orbital is a molecular orbital formed by side-by-side overlap of atomic p orbitals (see Figure 8.3), in which the electron density is found above and below the bond axis. As EPA is a polyunsaturated fatty acid, several alkene bonded carbons exist and similarly several π molecular orbitals.

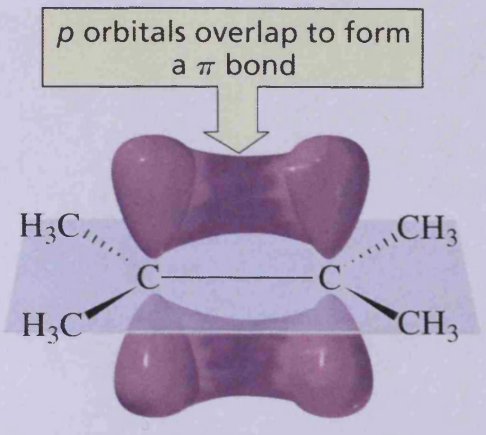


Figure 8.3 π molecular orbital formed by ethene (http://wps.prenhall.com/)

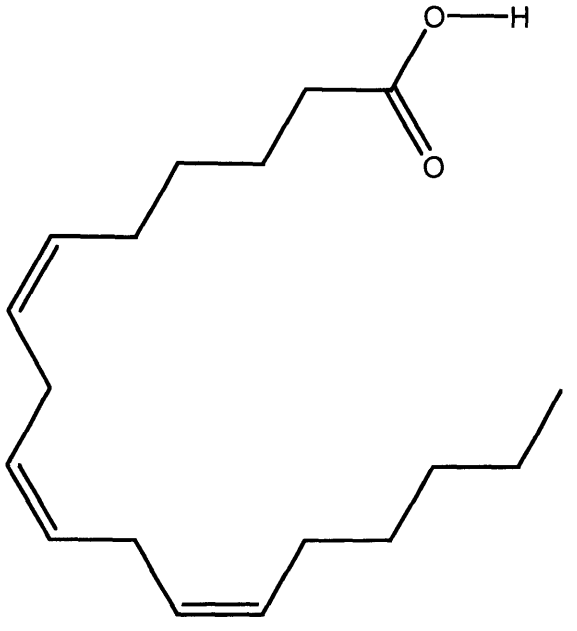
Once these π molecular orbitals are formed it is possible to achieve π molecular orbital interactions between different molecules, i.e. two π molecular orbitals on different molecules interact and loosely bond.

Examination of the structures concerned in this thesis, EPA and ketoprofen, indicated that the complexation of the two compounds could involve π–π orbital interaction or attraction via hydrogen bonds as opposed to a reaction forming a single compound as, during HPLC analysis of tape strips and receptor phase solution, the appearance of a complex is not seen. This means the complex must disassemble relatively easily under HPLC conditions and indicates that a π-π / hydrogen bond interaction is likely. Attractive interactions between π systems represent a significant factor within biological systems (Heard et al., 2003). To date the main focus of such research has been the benzene dimer due to its ease of analysis (Harris et al., 2003). The interaction energies of this dimer configuration are comparable to weak hydrogen bonds at approximately 10-15 kJ mol^{-1} (Harris et al., 2003). In this thesis the interactions of much bigger molecules are examined, namely the unsaturated regions of EPA and the aromatic regions of ketoprofen. The theory of similar interactions have been investigated by Hunter and Sanders who have published a significant work on π–π interactions and established several rules when considering such attractions. They also illustrate how π–π interactions are fundamental to DNA helix formation and to the intercalation of drugs into a DNA helix via face to face π stacked complexes with the aromatic DNA bases (Hunter and Sanderson, 1990).

The nature of the molecular interactions involved in two structurally similar compounds to this work has recently been examined (Heard et al., 2005): tamoxifen, a hormonal treatment for oestrogen receptor positive (ER+) cancer, and GLA, an *n*-6 polyunsaturated fatty acid with anti-cancer properties. From the structures of the illustrated compounds (Figure 8.4 and 8.5) it is apparent that there is the possibility of π-π orbital attraction between the aromatic moieties of ketoprofen and regions of unsaturation that exist in polyunsaturated fatty acids, as exemplified by Heard et al. (2005) when examining the addition of borage oil (a natural oil containing a high percentage of GLA) to tamoxifen in Miglyol 812N (a synthetic oil containing purely saturated fatty acids used as control). Using a similar method, ^{1}H NMR spectroscopy was used to probe modulation of ketoprofen aromatic proton signals in the presence of varying concentrations of fish oil. As this thesis involves the study of EPA from fish oil (a blend of different PUFAs), standards of EPA and DHA free acids, the main constituents of fish oil, were analysed to examine the effect of individual fatty acids.

To further elucidate if any interactions took place a series of molecular modelling experiments were carried out by Dr. Jamie Platts, Department of Chemistry, Cardiff University. These models simulate the formation of the most stable and therefore thermodynamically favoured complex that could be found within a given system between two compounds. By examination of such models the atoms within the adjacent molecules that are most affected by any complexation can be deduced.

(A)



(B)

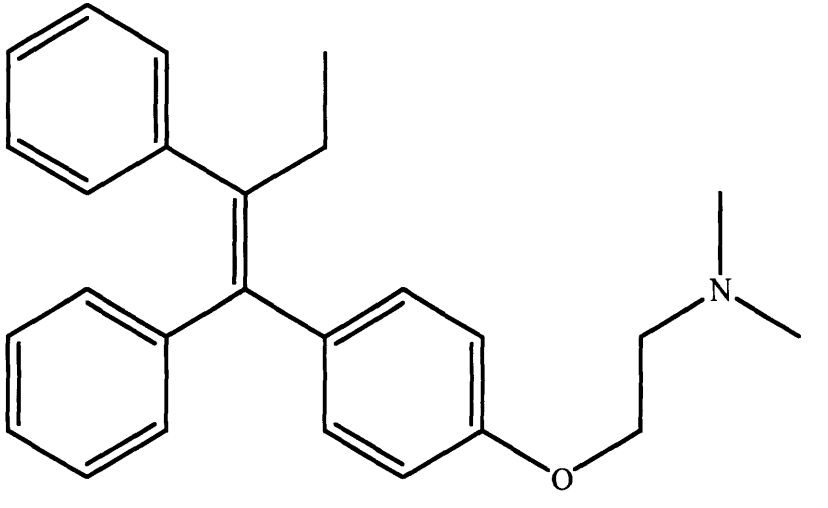
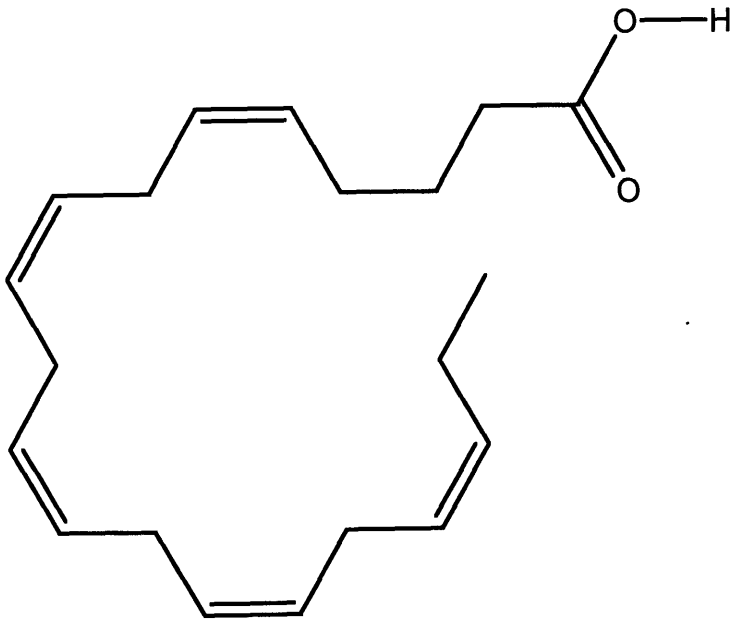


Figure 8.4 (A) γ-linolenic acid, (B) tamoxifen

(A)



(B)

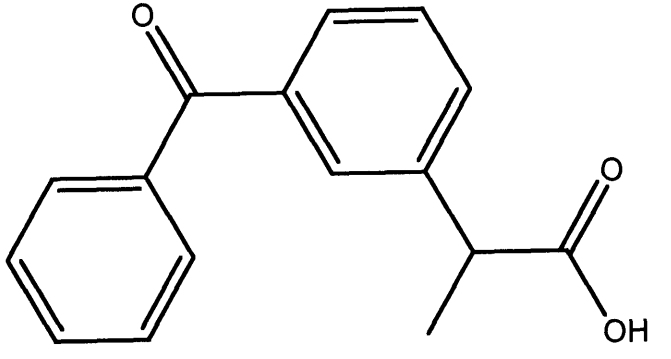


Figure 8.5 (A) cis, 5,8,11,14,17-eicosapentaenoic acid (EPA), (B) ketoprofen

8.3 Methods

Detailed methods for ^{1}H NMR spectral modulation are found in Chapter 2, Section 2.8. Ketoprofen proton assignments are illustrated in Figure 8.6 (assigned using the Spectral Database for Organic Compounds, (SDBS).

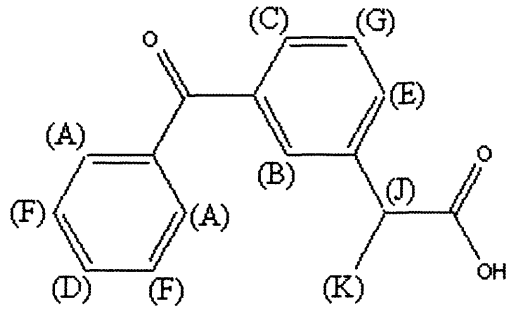


Figure 8.6 Structure of ketoprofen with assigned protons

Figure 8.7 is an example of the aromatic region of the ketoprofen spectrum produced from 2.5% ketoprofen in Miglyol 812N with the assigned protons and their relevant positions within the spectrum.

As the region affected by π–π bonding is the aromatic region of ketoprofen, this is the region which has been the focus of these studies. Assignments (J) and (K) represent a single proton and a methyl group respectively (three equivalent protons). Neither signal is in the aromatic region having shifts of approximately 3.8 and 1.5ppm respectively and has therefore been omitted from analysis.

8.3.2 Molecular Modelling

The detailed method for the molecular modelling and binding energy calculations can be found in Chapter 2, Section 2.9.

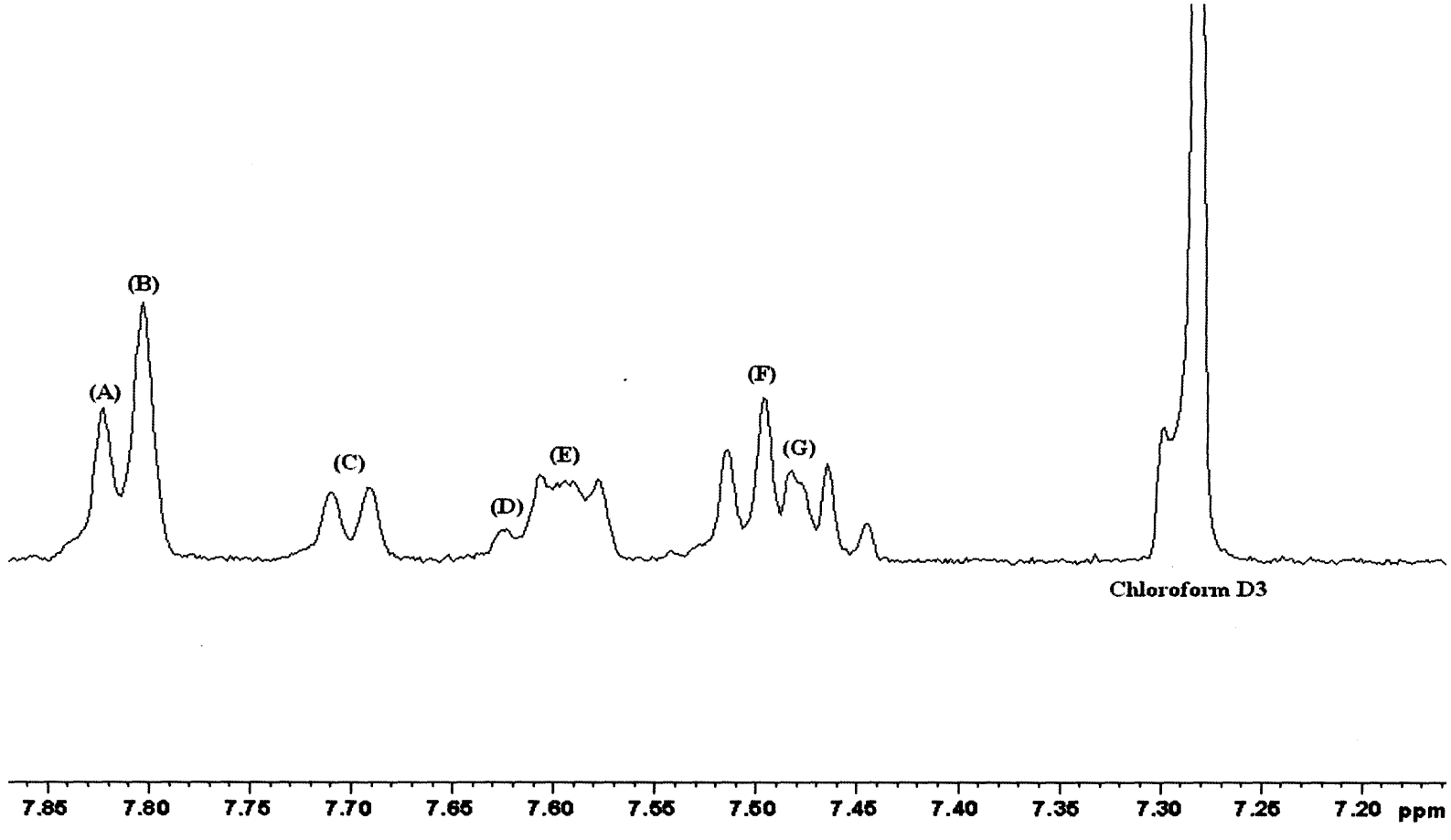


Figure 8.7 NMR spectrum of ketoprofen aromatic region showing proton assignments

8.4 Results and Discussion

8.4.1 Fish oil and Miglyol Formulations

NMR spectra have been shown to be highly sensitive to local chemical environment and the technique has been used previously to probe π-π interactions where such processes are manifested as up or downfield shifts depending on the magnitude of shielding / deshielding modulation (Kelly et al., 2001). Figure 8.8 shows the modulation of the ketoprofen aromatic protons signals in the different formulations prepared in Section 8.3 and it is clear that addition of fish oil to ketoprofen in Miglyol 812N resulted in dose-dependent downfield chemical shifts of signals from aromatic protons on the ketoprofen structure. The data shown is a result of several ^{1}H NMR experiments involving these compounds and the shift data, although seemingly minor, represents a significant, reproducible pattern.

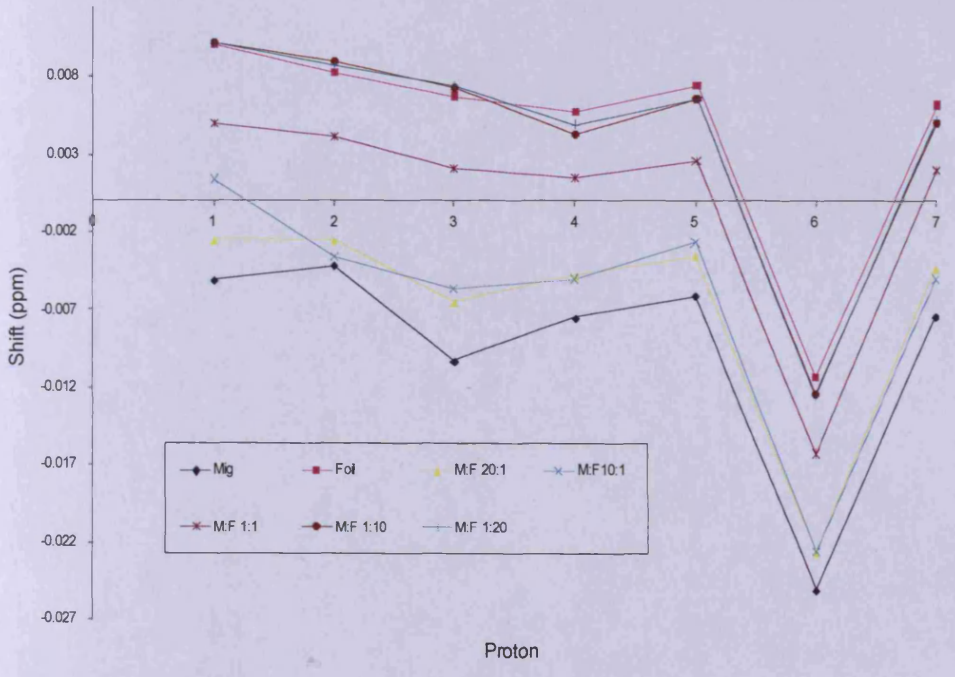


Figure 8.8 Chemical shifts of ketoprofen aromatic protons in fish oil / Miglyol formulations

From examination of the NMR spectra it would appear that the proton most susceptible to shift when in the presence of fish oil or Miglyol is proton (F), which are *meta* to the

carbonyl group on the unsubstituted phenyl ring – this susceptibility is logical as the carboxylic acid group would cause more steric and chemical hindrance to a potential π–π interaction. A single EPA molecule or EPA present on a triacylglycerol would experience difficulty in bonding in any way to the carboxylate side whereas the free benzene ring, which is also likely to be in a different plane, represents a more attractive option for any possible interaction.

8.4.1.1 Molecular modelling of EPA triglyceride / ketoprofen complex

Figure 8.9 shows the lowest energy geometry located for the complex of an EPA triglyceride with ketoprofen, which would have a molecular weight of approximately 1163. The highlighted protons (F) are the most noticeably affected in the NMR spectra. This is unexpected as these protons appear to be furthest away from the triglyceride structure and would have the least attractive / repulsive force applied of all the ketoprofen protons. This complex contains a hydrogen bond between the carboxylic acid group of ketoprofen and a carbonyl of the triglyceride, contributing to the calculated binding energy of 95.4 $kJmol^{-1}$. This is nearly a ten fold increase compared to the benzene dimer and indicates that this complex is very strongly bound and highly likely to form. This large binding energy and resulting stability makes it understandable how this complex could permeate the skin as a whole, as there is no obvious mechanism in the passage through the skin that could supply sufficient energy to cause dissociation. These results strengthen the hypothesis of permeation enhancement of EPA by ketoprofen stated in earlier chapters.

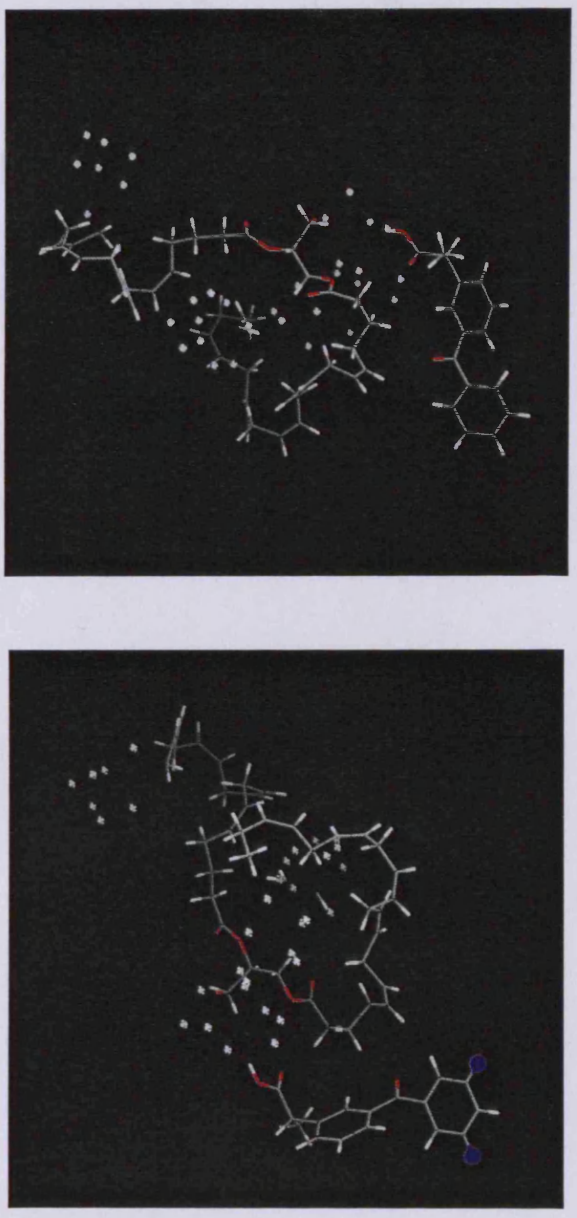


Figure 8.9 EPA triglyceride / ketoprofen complex

8.4.2 EPA and Miglyol formulations

^{1}H NMR was carried out using differing concentrations of pure EPA free fatty acid and dose-dependent downfield shifts were again observed (Figure 8.10). As with fish oil, proton (F) appeared to be most susceptible to shifting, indicating that this effect may be due to individual fatty acids within the fish oil.

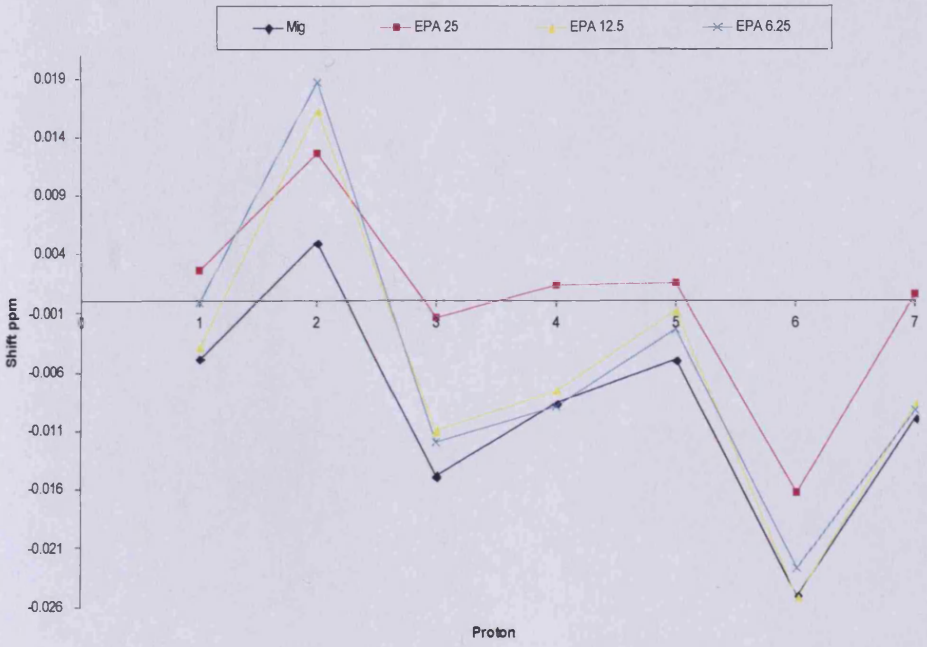


Figure 8.10 Chemical shifts of ketoprofen aromatic protons in EPA / Miglyol formulations

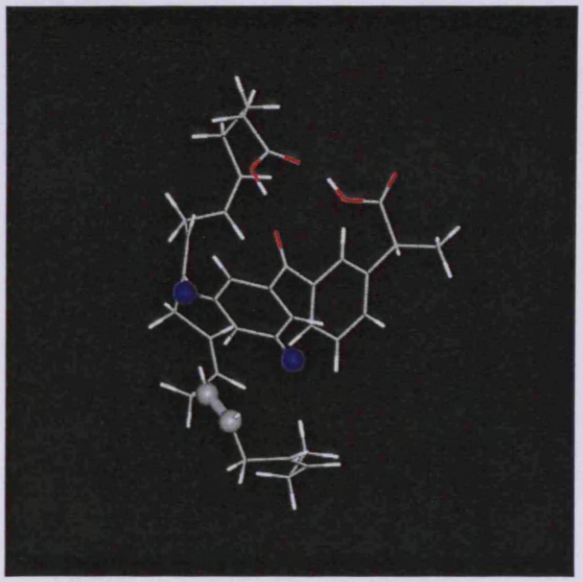
The main difference between the free fatty acid and the triacylglycerol appears to be the effect upon the (B) proton of ketoprofen. This proton lies on the opposite ring to proton (F), *ortho-* to both carbonyl and carboxylic acid groups. This difference in the shifts may be due to the lesser steric hindrance of the free fatty acid, allowing it to approach closer to ketoprofen and hence exert a greater effect on other protons, specifically (B). Alternatively, free EPA may be susceptible to dimerisation between pairs of carboxylate groups unlike in the triglyceride, which may be partly responsible for the differences in the spectra.

8.4.2.1 Molecular modelling of EPA / ketoprofen complex

The molecular modelling in Figure 8.11 once again supports the evidence of complexation provided by the NMR spectra. The closest protons to the EPA structure are protons (F) (Figure 8.11 (a)) and also highlighted are the carbon atoms most likely to be involved in this π–π bonding. The binding data for an EPA free fatty acid and ketoprofen (molecular weight approximately 556) is 157.0 kJ mol^{-1}, a substantial complexation energy much greater than the triglyceride / ketoprofen complex. The

orientation of the groups involved in this interaction is such that π... π stacking seems unlikely, and this might be better described as C—H...π contact. Proton (B) is highlighted (Figure 8.11 (b)), and can be seen to be within a region of electronegativity where the carboxylic acid group of both ketoprofen and EPA and the carbonyl group are present. This could explain the difference in shift of the proton not seen with the triglyceride as the complex differs in shape in this area.

(a)



(b)

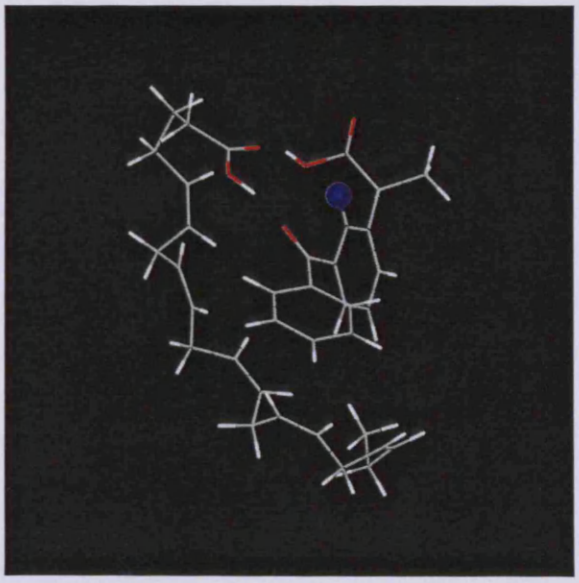


Figure 8.11 EPA free fatty acid / ketoprofen complex, (a) protons (F) with likely interaction, (b) proton (B) in electronegative environment

8.4.3 DHA and Miglyol Formulations

The formulations containing DHA and Miglyol follow a similar trend, with a large shift difference seen on protons (B) and (F) (Figure 8.12). DHA at a level of 6.25mg ml^{-1} also effects proton (E). As DHA has an additional two carbon atoms within the alkene chain it is possible that they are responsible for the effect on this proton. At higher concentrations DHA is not able to fit as easily around the ketoprofen molecule due to competition for space and so the affect is not as marked.

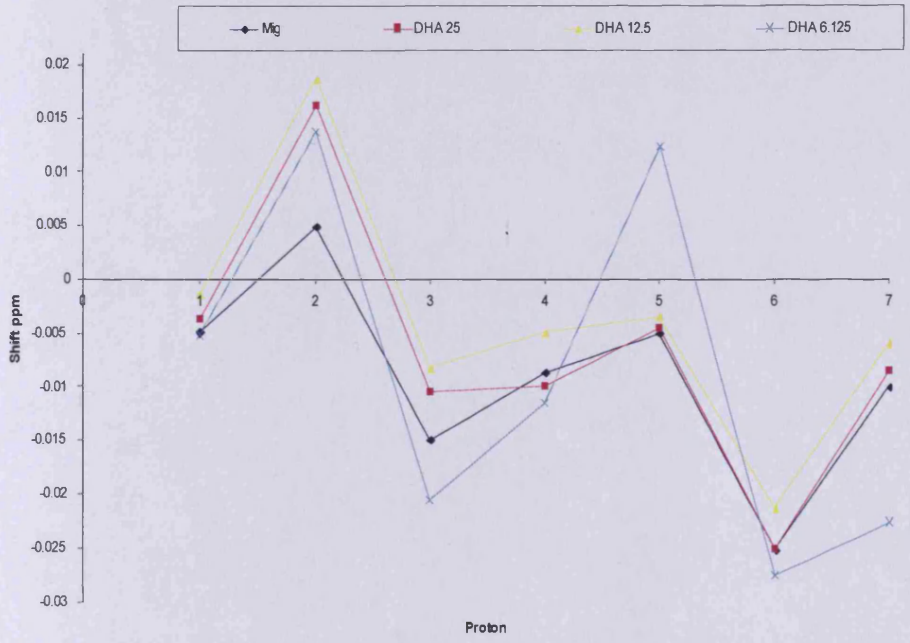


Figure 8.12 Chemical shifts of ketoprofen aromatic protons in DHA / Miglyol formulations

As EPA has been the main focus of this thesis molecular modelling using DHA was not carried out.

8.4.3.1 Miglyol / ketoprofen molecular modelling

To compare the binding energies and structures of the EPA / ketoprofen complex and the control formulation, the Miglyol / ketoprofen complex was also modelled, approximate molecular weight 764, (Figure 8.14). The binding energy for the synthetic triglyceride is 109.1 kJ mol^{-1}. This also represents a high energy binding complex and

indicates that even in the presence of such a strongly bound complex EPA as a free fatty acid or triglyceride can displace another compound to form a stronger complex thus further providing evidence of an EPA / ketoprofen complex able to permeate skin intact.

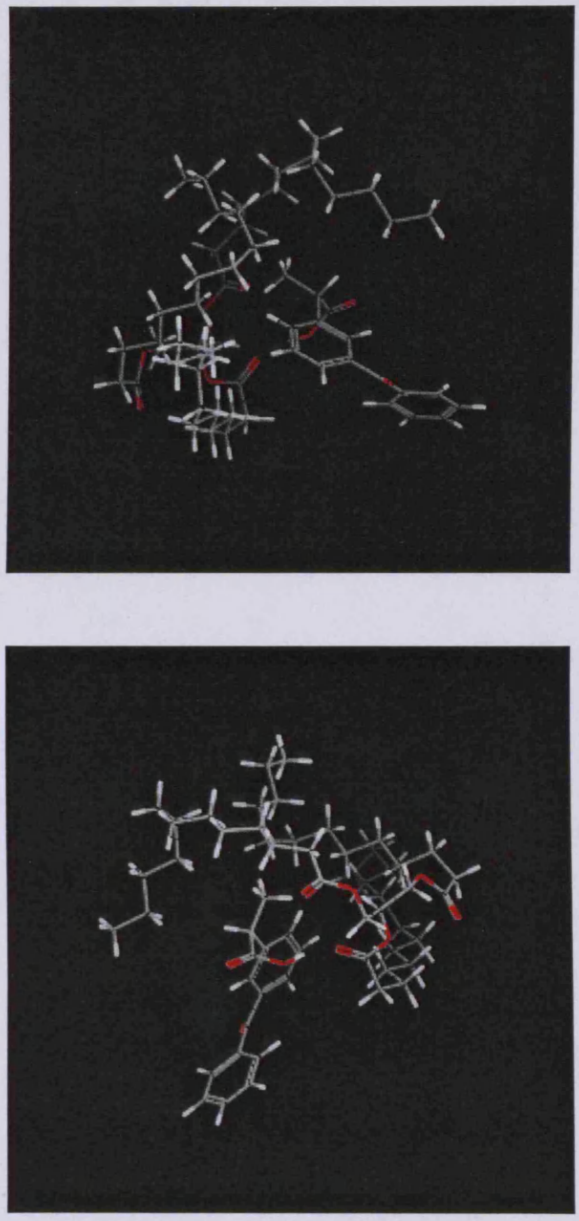


Figure 8.14 Miglyol / ketoprofen complex

8.5 Conclusions

It is clear that the ketoprofen ^{1}H NMR shifts are distinctly different in the presence of fish oil and Miglyol. Also clear is the difference between the test and the control media, containing no unsaturated moieties. Furthermore, the same general pattern of shifts is observed when pure EPA and DHA were used in place of fish oil.

The binding energies of the complexes are also indicative of a strongly bound complex that could possibly endure the rigours of permeation through the skin. The enhancement of EPA by ketoprofen can also be attributed to the complexation process. It was seen in Chapter 4 that very little EPA permeated from a fish oil vehicle alone, underpinning the need for ketoprofen in the formulation. The results explain these findings and those of Chapter 7. If the complex forms within the formulation then a dual enhancing effect could be at work. The lipophilic EPA triglyceride / fatty acid permeates the lipophilic SC taking the more hydrophilic ketoprofen with it. Then in the more hydrophilic layers of the viable epidermis and dermis the reverse occurs. Ketoprofen permeates taking its π bound EPA with it. The Log P values for the complexes could not be determined, however if calculated, they could indicate a further explanation for the synergistic enhancement as well as the likely form of the complex that is able to permeate the skin.

It appears that the molecular weights of the complexes have no significant effect upon their binding energies and but it is the unsaturation of the EPA chains that has the over-riding effect, again supporting the notion of bonding by a π-π / C-H....π interaction. The binding energy of tamoxifen and GLA has also been found to be considerably greater than the benzene dimer structure at 91.2kJ mol^{-1}. This binding energy is also considerable and has implications in for further work using these two compounds.

The possibility of the complex permeating the skin intact has been shown by the magnitude of the binding energies of the complexes. However, interactions with fatty acids and other molecules in the skin are likely but these interactions must be studied to determine their individual binding energies and to determine whether these possible

complex energies are stronger than those of the EPA / ketoprofen complex. The issue of the complex dissociating when in contact with epidermal enzymes is investigated and discussed in detail in Chapter 9.

In summary, the geometries of these complexes show strong O—H...O hydrogen bonds in all cases, and in the case of ketoprofen complex with free EPA there is also some evidence of C—H...π and/or π - π interactions, giving rise to regiospecifically solvated complexes. Permeation of these intact complexes across skin could, at least in part, account for increased permeation reported for mixtures of ketoprofen with fish oils, EPA or DHA.

Chapter 9

Probing the phenomenon of permeation enhancement of fish oil/EPA by ketoprofen

iii. Influence of fish oil / ketoprofen on epidermal COX-2 and LOX

9.1 Immunocytochemistry (ICC) of epidermal COX-2 and LOX enzymes

The epidermis is the main site for metabolism and chemical conversion processes when considering the permeation of drugs / molecules through the skin. Once the barrier of the SC has been overcome permeating molecules then encounter the epidermal enzymatic systems. Most phase 1 and phase 2 reactions, including oxidation, hydrolysis and methylation can occur within the epidermis, though discussed in Williams, (2003) at a level of less than 10% of the liver. Inflammatory processes such as psoriasis and psoriatic arthritis require and are propagated by the same enzymes involved in other arthritic conditions, namely COX-2 and 5-LOX. This provides a good model for arthritic conditions in deeper tissue as well as indicating the fate of the compounds as they permeate the skin.

Chapter 6 demonstrated that when using viable skin metabolism of EPA to its 15-LOX metabolite 15-HEPE did indeed occur. A clear pattern was also seen as the metabolism of EPA to 15-HEPE increased as EPA neared the basal layer. The addition of ketoprofen to the formulation impeded this conversion. Chapter 8 indicated that an EPA / ketoprofen complex is possible and this may be the reason for the decrease in metabolism. The study in Chapter 8 showed the relationship between EPA and ketoprofen in a formulation and possibly throughout permeation. The interaction of EPA and ketoprofen with the main inflammatory enzymes, however, was still unknown and the effect of EPA on ketoprofen and vice versa when in the presence of these enzymes was still unclear.

This chapter investigates the effect of EPA and ketoprofen on the COX-2 and LOX enzymes within porcine skin. Incorporated with the data from Chapters 7 and 8 this should then elucidate mechanism of EPA permeation enhancement by ketoprofen and also show the ability of such a formulation to inhibit these enzymes and thus indicate the efficacy of such a formulation.

Immunocytochemistry (ICC) is a method employed to qualitatively determine the activity of particular enzymes in a given system. In this case the skin will be set, cut and stained to

show the presence and activity of COX-2 and LOX enzymes at given time points throughout a normal skin permeation experiment.

9.2 Methods

The method for the staining process is described in Chapter 2, Section 2.7.

9.3 Results

9.3.1 ICC analysis for COX-2

The primary antibody used for this analysis was COX-2 antibody #4842 (purchased from Cell Signalling Technology) which was specific for endogenous levels of total COX-2.

9.3.1.1 Analysis of formulation containing 2.5% ketoprofen

Figure 9.1 illustrates the staining seen at the specified time-points. It is very clear that the presence of a ketoprofen and EPA formulation dramatically reduced the stain at all time-points. This is indicative of a large reduction in the COX-2 enzyme present in the skin. (A) is the control sample where no formulation had been applied. The darkness of the epidermis is in stark contrast to the lower dermis which is almost transparent due to the lack of metabolically and enzymatically active cells. The small amount of staining in the epidermis is to be expected as the skin had recently been removed from the ear and small amounts of COX-2 have already been expressed in response to trauma.

At 8 hours the skin with no formulation applied (B) shows heavy staining, especially in the more biologically active basal layer of the epidermis. After removal of the skin from the ear it is apparent that an inflammatory response had been triggered. The damage to the skin upon removal from the ear would initiate this inflammatory response with the production of cytokines and COX-2 and eventually the metabolism of AA to continue the inflammatory cascade. The stratum corneum is labelled.

It can be seen in (D) that 8 hours after administration of the formulation the staining is significantly less than the control. It also appears to be less than the 0 hour control indicating a substantial inhibition of COX-2. The formulation is likely to be acting in two ways; inhibition of the induced COX-2 by ketoprofen, followed by a down regulation of inflammatory cytokines caused by the increase in ratio of anti-inflammatory to pro-inflammatory mediators as a result of COX-2 metabolism of EPA instead of endogenous AA. The active basal layer also appears to be subdued in its COX-2 production suggesting the rapid permeation of ketoprofen through the rest of the epidermis.

After 24 hours (C) the COX-2 production would seem to have reached a maximum as the stain throughout the epidermis is of an equal distribution. The top layers of the epidermis appear to be the darker regions of the skin. This could be due to the progression and accumulation of active COX-2 generated by the lower layers of the epidermis, through to the upper stratum spinosum and stratum granulosum.

Even after 24 hours the staining seen after formulation application (E) is noticeably reduced, compared to the equivalent control sample (C). Some staining can be seen in the top layers of the epidermis. After 24 hours the skin is likely to be nearing a necrotic state and a slight darkening of the stain, compared to control, is to be expected. The difference between the test and control confirms just how extensively ketoprofen and EPA inhibit and even cause a decrease in the induction of COX-2 enzymes. The considerable effect seen by the formulation is also an indication of what would occur after transcutaneous permeation through to the joint synovium and then within the synovial fluid. If this effect is repeated within these tissues, the benefit of such a formulation is self-evident.

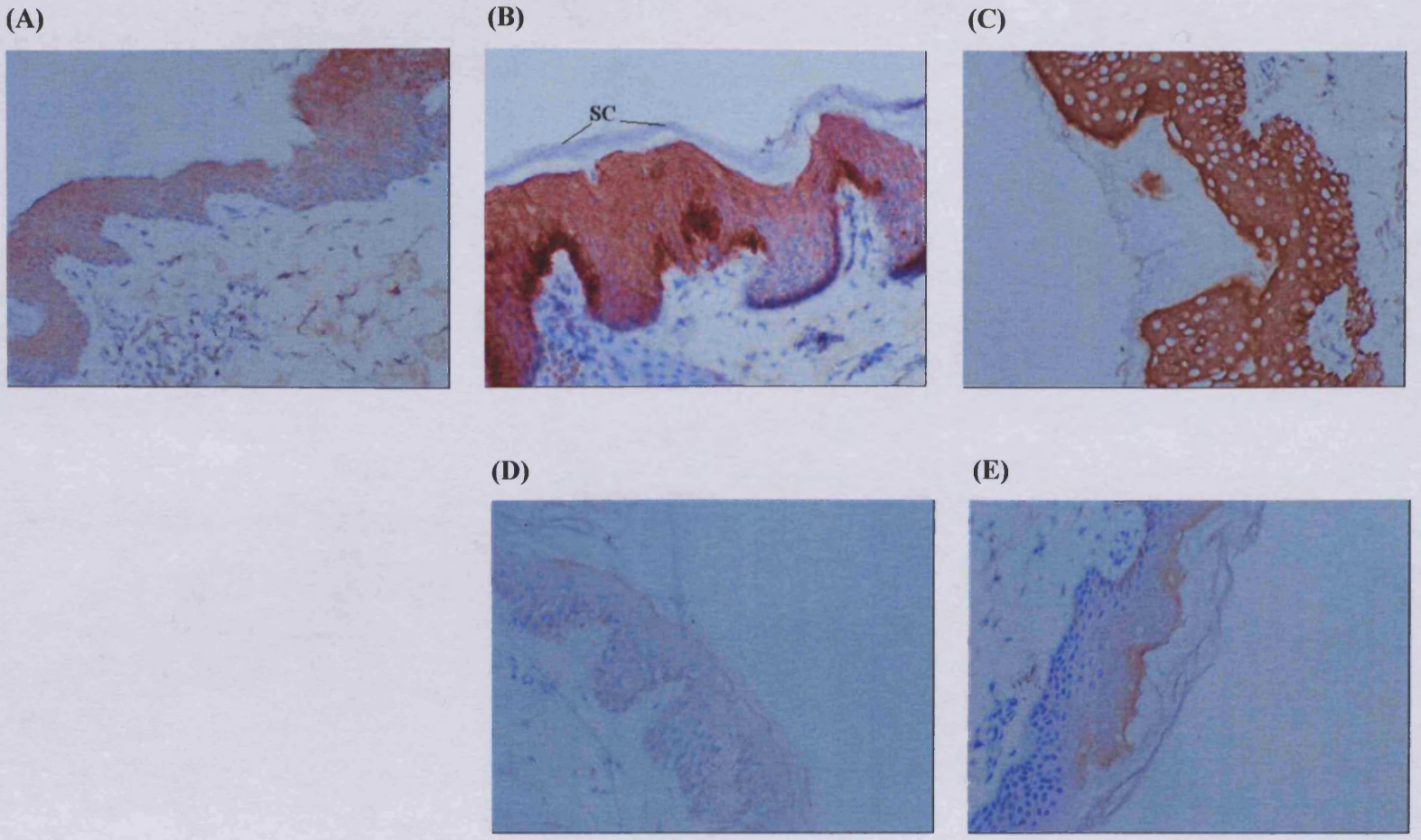


Figure 9.1 COX-2 staining from a formulation containing 2.5% ketoprofen w/w in a fish oil vehicle. (A) 0 hour control, (B) 8 hour control, (C) 24 hour control, (D) 8 hour test sample, (E) 24 hour test sample

9.3.1.2 COX-2 analysis of fish oil formulation

Figure 9.2 shows the COX-2 staining for the formulation of fish oil alone. The 0 hour control sample (A) shows limited staining, similar to the 0 hour control of Figure 9.1. Again it is evident by the degree of staining that COX-2 has been induced in the short time post excision. The 8 hour control (B) was heavily stained compared to the 0 hour control (A) due to the inflammatory process occurring for 8 hours. In contrast, the 8 hour test sample (D) had much reduced staining compared to (B). A possible explanation is that EPA from the fish oil actively competed with AA, affecting COX-2 induction via its ability to inhibit cytokine release and COX-2 gene expression.

Samples (C) and (E) did not follow the trend seen thus far. The 24 hour control (C) showed a decrease in the staining due to decreased concentration of COX-2 present. The 24 test sample (E) is heavily stained, more so than the equivalent control sample (C). The decrease in the COX-2 staining seen in 24 hour control could be due to the short lifespan of the skin sample and after the initial peak at 8 hours the skin has become necrotic, the enzymes denatured and their activity and availability to interact with the antibody also ceased. Another cause could be the switch of inflammatory pathway to the LOX system. This would lead to a decrease in the production of COX-2 and in the reduced staining seen in the sample. The dark staining seen in the test sample (E) could be due to an increase in COX-2 production via the converse mechanism, inhibition of LOX enzymes causing a switch to the COX pathway. As EPA was limited within the skin due to other enzymatic processes this pathway may have become more active. When ketoprofen was present it was possible to inhibit the excess COX-2 produced and so explaining the difference in staining between ketoprofen containing and ketoprofen free formulations.

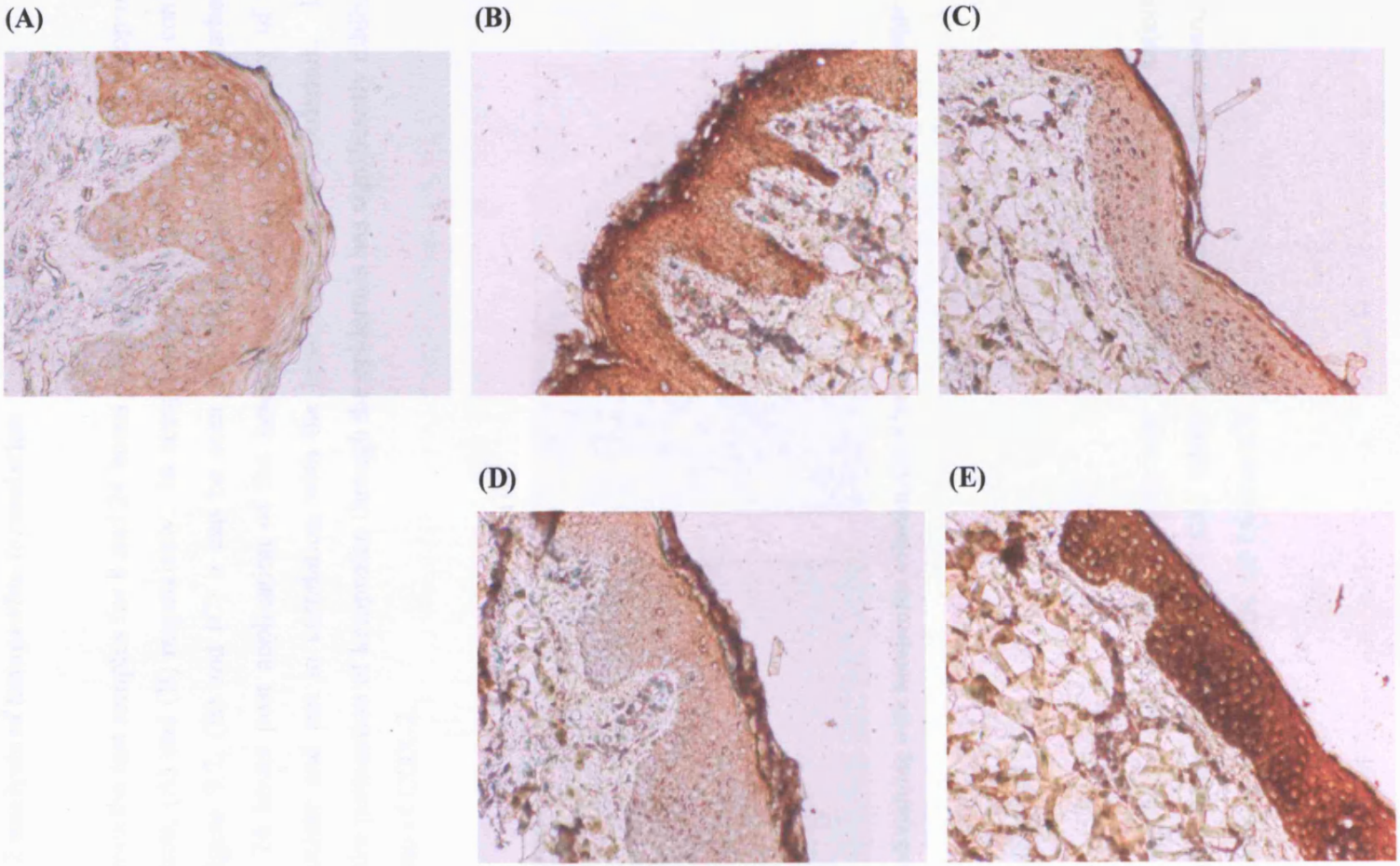


Figure 9.2 COX-2 staining with fish oil formulation, (A) 0 hour control, (B) 8 hour control, (C) 24 hour control, (D) 8 hour test sample, (E) 24 hour test sample

9.3.1.3 COX-2 analysis of ketoprofen formulation

Figure 9.3 shows the test samples for 8 and 24 hours post application of a 2.5% ketoprofen in water solution, (A) and (B) respectively. In comparison to both the equivalent control samples in Figure 9.2, (B) and (C) it can be seen that the staining has been markedly reduced. At 24 hours post application of the ketoprofen solution the majority of the staining is nuclear and not in correlation with the basal layer of the epidermis. This suggests that the permeation of ketoprofen through the epidermis has significantly inhibited the proliferation of COX-2.

(A) (B)

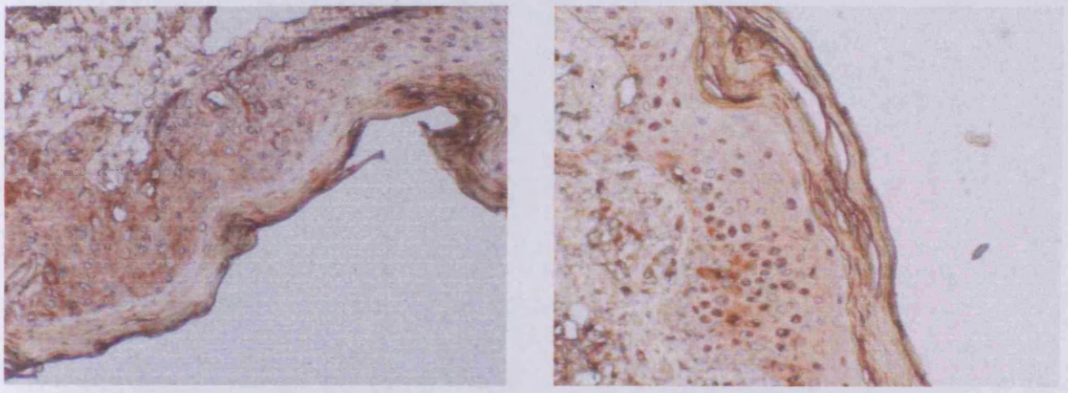


Figure 9.3 COX-2 staining with ketoprofen solution, (A) 8 hour test sample, (B) 24 hour test sample

9.3.2 ICC analysis of LOX

The primary antibody used for this analysis was 5-lipoxygenase polyclonal antiserum (purchased from Cayman Chemical, USA) which had cross-reactivity for human, rat, murine, hamster and porcine 5-LOX, 12-LOX and 15-LOX.

9.3.2.1 Analysis of formulation containing 2.5% ketoprofen

The control sample shown in Figure 9.4 (A) shows a relatively high degree of LOX present throughout the skin, again localised at the basal layer of the epidermis (highlighted). A certain concentration of LOX enzymes including 5-LOX and 15-LOX is likely to have been induced in response to the trauma caused by excision but the majority is likely to be due to endogenous enzymes. The degree of staining is due to all LOX enzymes being stained, not just a particular iso-form but this non-specificity is not a problem as 5-LOX is of particular interest due to the inflammatory connotations associated with its induction. 15-LOX is also of interest as this is said to be the most prevalent form of LOX within skin (Miller and Ziboh, 1998).

Eight hours post application of the 2.5% ketoprofen in fish oil formulation (C) the LOX enzymes present in the skin appear to have been inhibited relative to the 8 hour control (A). The degree of staining between the two 8 hour samples is very different. The EPA present appeared to diminish the stain of LOX either by inhibiting the staining because of its agonistic effect on the LOX enzyme or due to a negative feedback caused by reduced inflammatory mediators and cytokines. The presence of ketoprofen could have aided this negative feedback in its inhibition of COX. However, if a ketoprofen formulation was used alone, a greater degree of LOX induction would be expected to take place to compensate for one inflammatory pathway being inhibited. This would imply that EPA is having the greater effect in causing the reduction of LOX staining via its metabolism, as opposed to a reduction in inflammatory mediators assisted by ketoprofen. The basal layer was again the darkest region of staining and can be explained by the permeation rate of EPA as, after only 8 hours, EPA would still be in short supply at this lower epidermal layer. After a further 16 hours a difference between the test and control samples was again evident (B and D). Heavy staining in the control sample (B), particularly focused at the basal layer, was expected as, after 24 hours, the inflammatory process is in full progress. The test sample was slightly darker than the 8 hour test, again as expected as only EPA acts upon LOX and

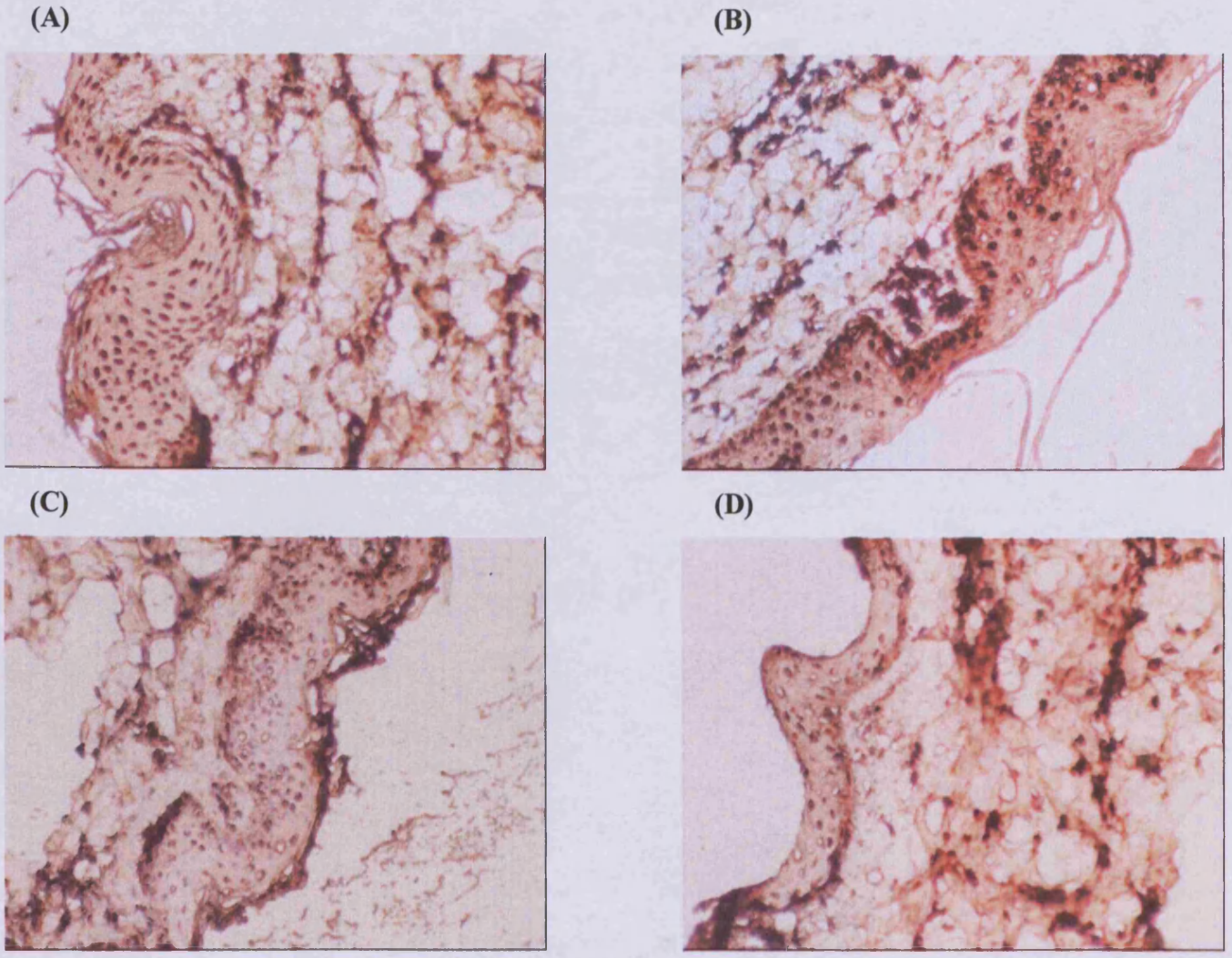


Figure 9.4 LOX staining of formulation containing 2.5% ketoprofen w/w in fish oil vehicle, (A) 8 hour control, (B) 24 hour control, (C) 8 hour test sample, (D) 24 hour test sample

the inhibition of COX by ketoprofen and EPA would not only deplete available EPA but may also encourage LOX induction.

9.3.2.2 Analysis of fish oil formulation

Figure 9.5 shows the LOX staining for a fish oil formulation containing no ketoprofen. (A) represents the endogenous LOX enzymes present. After 8 hours (B) the staining became slightly darker indicating the production of LOX enzymes and was now more evident between the keratinocytes in the extra-cellular space. The addition of fish oil to the skin caused reduction in the concentration of LOX enzymes as illustrated by (D). The staining in (D) was comparable to that of the control section (A). It is apparent that the presence of fish oil, and in particular EPA from fish oil is having a marked affect upon the production of LOX enzymes.

After 24 hours (C) the degree of staining has increased and again evident was the dark staining in the basal layer of the epidermis. Post-application of a fish oil formulation (E) the staining was reduced. It is interesting that the staining was quite light until the basal layer where the staining became darker nearer the metabolically active cells. This pattern of staining seems to indicate the route of permeation of EPA through the skin and the inhibition of LOX along the way. Also of note is that the significant difference between the basal layer and the rest of the epidermis was not as noticeable in the equivalent time-point (D) in Figure 9.3. This signifies the permeation enhancement of EPA by ketoprofen through the epidermis to the basal layer within 24 hours: the enhancement allowed EPA to exert its effect on the LOX enzymes produced in the basal cells, supporting the theory of EPA permeation enhancement via formation of a ketoprofen / EPA complex discussed in Chapter 8. Once the ketoprofen–EPA complex has reached a potential site of action i.e. enzyme, the formation of the complex seems to have no detrimental effect upon the action of either EPA or ketoprofen. The ability of the EPA and ketoprofen to dissociate from this

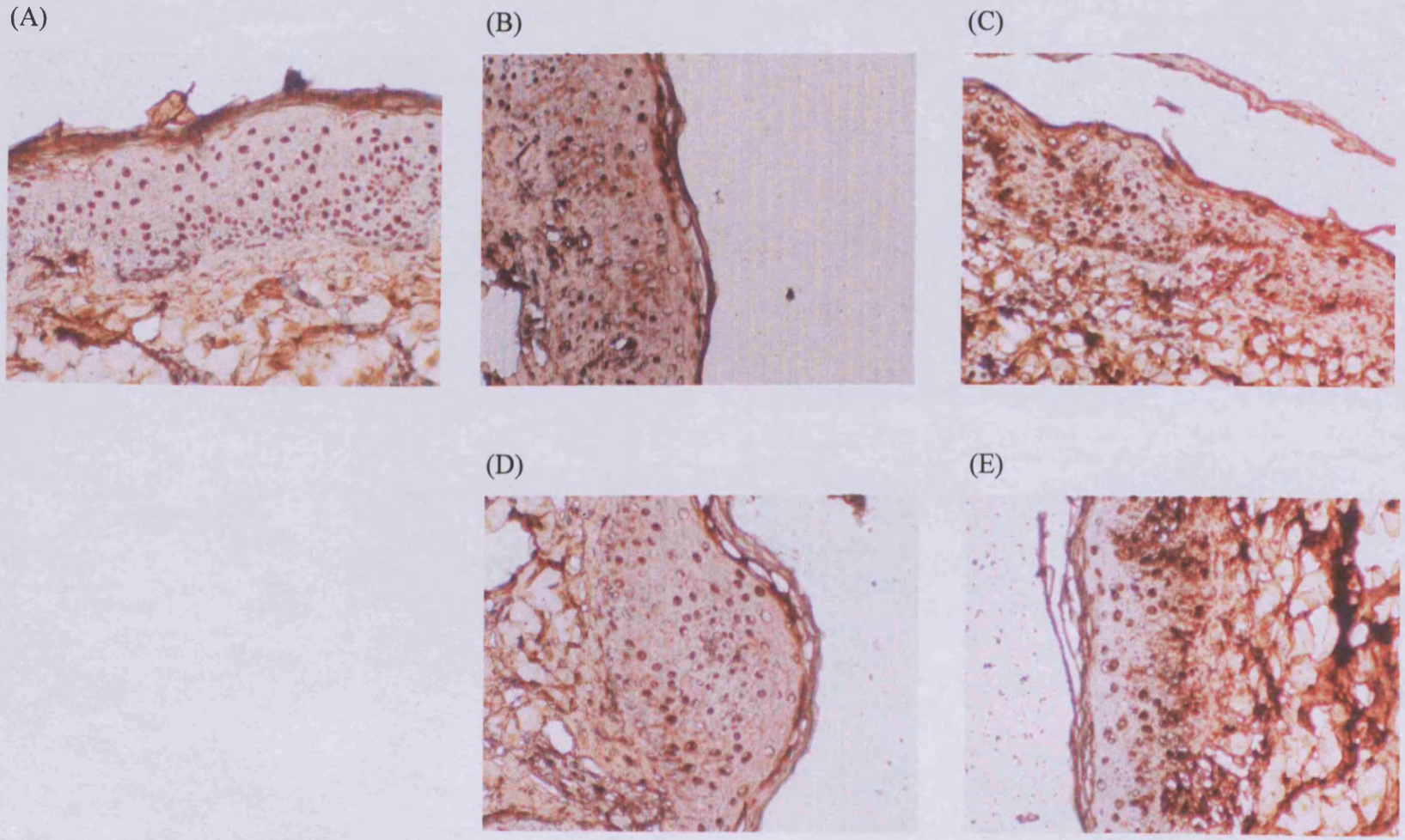


Figure 9.5 LOX staining with fish oil formulation, (A) 0 hour control, (B) 8 hour control, (C) 24 hour control, (D) 8 hour test sample, (E) 24 hour test sample

complex and act upon enzymes is a significant finding as this would need to occur throughout the permeation process when encountering inflammatory mediators.

9.3.2.3 LOX analysis of ketoprofen solution

Figure 9.6 shows the 8 hour and 24 hour LOX analysis for the ketoprofen solution, (A) and (B) respectively. As ketoprofen was not expected to be active upon LOX the staining seen in both (A) and (B) can be expected. After 8 hours the intercellular space within the epidermis is very darkly stained and is comparable to the control (B) in Figure 9.5, interestingly after 24 hours the darker regions of staining are the keratinocyte nuclei. This suggests that after 24 hours the LOX present has permeated into the keratinocytes, possibly to further up-regulate its proliferation and that of other inflammatory mediators.

(A) (B)

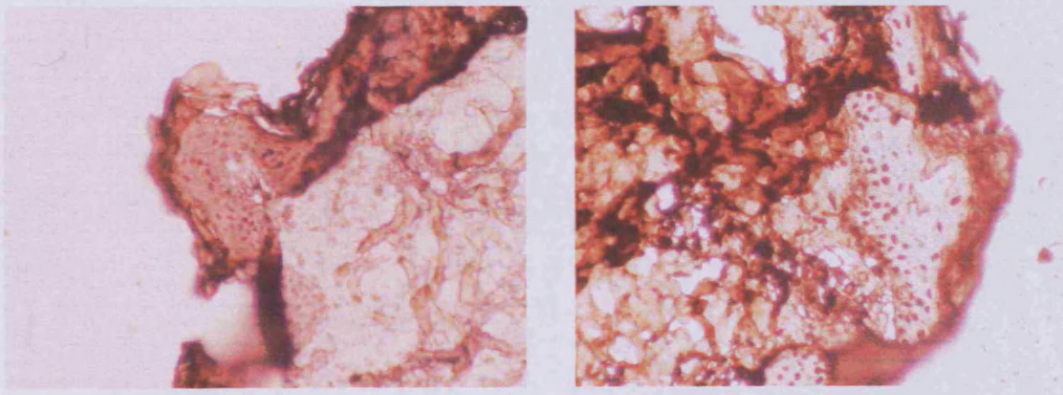


Figure 9.6 Lox analysis for ketoprofen solution, (A) 8 hour test sample, (B) 24 hour test sample

9.4 Discussion

Examination of the data in this chapter alludes to several possible mechanisms concerning the possible action of EPA and ketoprofen upon inflammatory enzymes, namely COX-2

and LOX. It has been shown that a formulation containing ketoprofen, along with fish oil (Figure 9.1) had a greater influence on COX-2 staining than either ketoprofen (Figure 9.3) or fish oil alone (Figure 9.2). A result that was expected as both compounds act as inhibitors of COX-2 within its active site. The synergistic action upon the overall COX-2 staining by EPA and ketoprofen can be explained by their separate inhibitory actions. Ketoprofen blocks the enzyme and therefore inhibits its activity and inflammatory action. EPA acts as an agonist of COX-2 and so occupies the active site and prevents staining and also AA metabolism. EPA also utilises its ability to suppress the gene expression of COX-2 and so the COX-2 that would usually be expressed in such inflammatory conditions, is diminished resulting in reduced staining even after 24 hours.

The LOX staining was again as expected with ketoprofen (Figure 9.6) showing no effect on LOX staining / activity and EPA (Figure 9.5) diminishing the LOX stain up to 24 hours after application of formulation. With ketoprofen present the staining seen was not appreciably different. Within this formulation the inhibition of COX-2 by ketoprofen would allow a greater concentration of EPA available to inhibit LOX. This must also be balanced with the induction and up-regulation of the individual inflammatory pathways. If COX-2 inflammation is reduced it is likely that the LOX inflammatory pathway will compensate. EPA once again can slow this, by its inhibition of the expression of inflammatory cytokines and mediators which up-regulate LOX.

Chapter 8 demonstrated a strong possibility of a ketoprofen / EPA complex that may be strongly bound enough to permeate the skin intact. It appears that this complex does not stop the binding to enzymes within the skin as the presence of ketoprofen does not severely affect the ability of EPA to inhibit LOX. Chapter 7, however, indicates that the presence of ketoprofen inhibits the formation of the 15-LOX metabolite of EPA, 15-HEPE. This inconsistency could be explained by the difference in analysis of the chapters. The presence of ketoprofen was significant when *quantifying* 15-HEPE formation but not significant when *qualitatively* analysing the enzyme staining studying three possible LOX iso-forms.

9.5 Conclusion

The anti-inflammatory action of a ketoprofen / EPA formulation has been demonstrated and the efficacy of such a treatment for inflammatory disorders has been shown. As ketoprofen and EPA permeate further through an inflamed joint the same inflammatory processes would be occurring and this chapter has shown that the formulation can adequately deal with such processes. The formulation has shown the ability to act on the two main inflammatory mediators over a period of 24 hours as it permeates the skin and this can be used as an inflammatory model for deeper tissues.

The formation of a ketoprofen / EPA complex appears not to interfere significantly with the anti-inflammatory action which is a requirement as the formulation permeates through inflamed tissue. This complex has been found to jointly enhance the permeation of ketoprofen and EPA through the skin (Chapters 3 and 4), so with the enhancement of permeation coupled with the apparent dissociation of the complex when required holds great promise for further development of the two-pronged dual action anti-arthritis therapy discussed in Chapter 1.

Chapter 10

General Discussion

10.1 General discussion

The objective of this thesis was to probe the development of a formulation that could deliver EPA (the major bioactive polyunsaturated fatty acid of fish oil) and ketoprofen, a non-steroidal anti-inflammatory drug transcutaneously to provide a dual action therapy to combat the two main mechanisms of inflammation seen in arthritic conditions. During this development investigations were carried out to assess the ability of these agents to permeate full-thickness skin and underlying tissues. The action of the formulation within skin during the permeation process was also investigated to determine if the two active compounds had an effect upon each other as they permeated.

The work showed that transcutaneous delivery of EPA and ketoprofen was possible although only as a basic formulation. The formulation used in Chapter 3 provided negligible amounts of EPA permeation across the skin; however these formulations contained a maximum of 20% (w/w) fish oil in a thickened gel formulation. It was found within this study that the presence of HPC as thickener had a retarding effect on the permeation of EPA from fish oil, but seemingly posed no such issues for ketoprofen. Chapter 3 also highlighted the permeation enhancement of ketoprofen by different concentrations of fish oil in the formulation. There was a direct correlation between the concentration of fish oil within the formulation and the permeation of ketoprofen and this enhancement was at first thought to occur by the disruption of the lipid bilayers within the SC, as previously described by Barry (1987). The un-thickened formulation employed in Chapter 4 used fish oil alone as the delivery vehicle for ketoprofen at 2.5% w/w. This was more successful as the permeation of both EPA and DHA from fish oil (in addition to ketoprofen) was observed. Included within these formulations were different concentrations of the terpene enhancer 1,8-cineole. Ketoprofen once again exhibited a concentration dependent increase in permeation whereas EPA and DHA from fish oil appeared to be impaired by its presence. In a fish oil/ ketoprofen formulation the presence of the fish oil seemed to enhance the permeation of ketoprofen compared to an aqueous control. However, an unexpected outcome from the permeation studies was that the presence of ketoprofen also enhanced the permeation of EPA and DHA from the fish oil vehicle. Perhaps most surprising is the data that

indicated that the permeation of EPA and DHA was *dependent* upon the presence of ketoprofen within the formulation. This inference cannot be explained by conventional skin theory as the smaller less lipophilic ketoprofen would have been expected to permeate independently of the triglyceride-containing fish oil. The enhancement of ketoprofen by 1,8-cineole, whereas no enhancement of fish oil occurred, was explained by selective solvation within the formulation. As the 1,8-cineole selectively solvated ketoprofen, no enhancement of the permeation of EPA and DHA occurred, due to reasons that became clearer within Chapter 8.

Subsequent to the successful *in-vitro* permeation of EPA/DHA and ketoprofen through full thickness skin, it was demonstrated in Chapter 5 that these compounds were able to penetrate deeper, i.e. into the synovial fluid of a porcine joint capsule using the basic non-thickened formulation. In Chapter 6 a novel transcutaneous delivery model was developed and used to provide preliminary data for the uptake of EPA into an *ex-vivo* cartilage ex-plant post transcutaneous permeation. Unfortunately, this experiment didn't provide any further information as to the efficacy of the formulation against the biological inflammatory response. However, Curtis et al., (2000) had proved the efficacy of EPA supplementation to arthritic cartilage employing treatments of a similar concentration to the ones found to have permeated into the receptor phase solution and incorporated within the cartilage ex-plant.

In summary, the early chapters (3-6) indicate that a simple ketoprofen in fish oil product could be beneficial for the treatment of arthritic conditions. When considered together, these *in-vitro* experiments have shown the formulation has the potential to deliver EPA and ketoprofen through the (whole) skin, the joint capsule and potentially taken up by joint cartilage. The prime factor that remains to be considered is dermal clearance from the skin into the systemic circulation. This could reduce the amounts of compound ultimately bioavailable to the cartilage, although of the proportion of compounds in the system following 'inadvertent' transdermal permeation a certain amount would be delivered to the capsule.

A major outcome of the skin permeation data was the apparent enhancement of fish oil / EPA permeation by ketoprofen. Chapters 7, 8 and 9 can be considered as a collective investigation into this phenomenon and the two main hypotheses concerned firstly, the formation of a π–π ketoprofen / EPA complex – the existence of which was strongly supported by the NMR/molecular modelling work of Chapter 8. Secondly, the ketoprofen inhibition of epidermal enzymes active upon EPA as discussed in Chapter 7 and 9.

During the work it was found that both theories were plausible. Chapter 7 demonstrated that the presence of ketoprofen in both media sustained skin and skin with no growth media enhanced the permeation of EPA through the skin. This was thought in part to be due the inhibition of epidermal COX by the ketoprofen. Both EPA and ketoprofen act upon these enzymes and so if ketoprofen preferentially binds with COX then this leaves more EPA un-metabolised and free to permeate. It was also discovered that ketoprofen caused a decrease in the production of 15-HEPE, the 15-LOX metabolite of EPA. As ketoprofen does not act upon LOX enzymes another mechanism must be in action. The possible complexation of EPA with ketoprofen was then investigated in Chapter 8. The modulation of ketoprofen ^{1}H NMR spectra from different formulations containing varying concentrations of fish oil and a control saturated triglyceride enabled the hypothesis of the existence of a possible complex. Molecular modelling of the systems (the different formulations) discovered that complexes of considerable binding energies could indeed form. If this was the case then the previous results seen concerning the permeation enhancement of EPA by ketoprofen could be attributed to this complex. Once the complex is formed the triglyceride / free fatty acid could aid the permeation of its associated ketoprofen into the lipophilic SC via the drag effect. Once permeated the more hydrophilic ketoprofen could aid the permeation of the triglyceride / free fatty acid through the epidermis again via the drag effect. This could explain the synergistic permeation enhancement seen with these compounds. The formation of this complex however did not appear to reduce the effect the compounds can have upon inflammatory enzymes, as Chapter 9 demonstrated that the formulation is capable of inhibiting both COX-2 and LOX enzymes within the skin (either the complexes dissociate in situ, or

bind to the binding sites intact). Such inhibition would also be seen throughout the inflamed tissue as the formulation permeates.

10.2 Future work

The development of a commercial formulation has been firmly supported by the data in this thesis. It may be possible to enhance the permeation of both ketoprofen and EPA via formulation modulation although the amount of fish oil within the formulation would need to be high – however the optimum (target) dose within the joint capsule needs to be established. When considering a formulation the issue of odour arising from the use of fish oil must also be considered. The use of 1,8-cineole was found to have an odour masking effect however the effect on the permeation was not favourable. The ideal would involve a permeation enhancer which works for both EPA and ketoprofen with dual role of odour masking. The use of thickeners in any formulation would also need to be addressed as the use of HPC, a well recognised thickener in pharmaceutical formulations proved to retard the permeation of EPA.

In-vivo work on animals (e.g. arthritic rat) could also be undertaken to determine the efficacy of the formulation with an arthritic model. Ultimately, any clinical benefit would need to be established via an appropriate clinical trial.

10.3 Concluding remarks

The hypothesis that a novel dual-action, transcutaneous anti-arthritic formulation can be developed has been achieved. All major stages of the delivery into a joint have been investigated and at each stage the formulations have indicated success in its purpose. The theory of vehicle-drug complexation has also been developed with strong evidence that complexation and synergistic permeation enhancement can take place between the two actives, which may have major ramifications for currently accepted wisdom in the field of topical drug delivery.

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