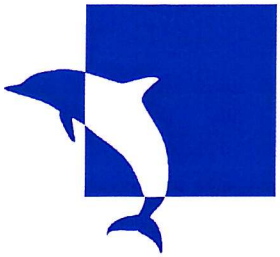

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Evaluation of Microdialysis as a Tool for Studying Percutaneous Drug Absorption and Cutaneous Metabolism.

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Doctor of Philosophy

The Faculty of Medicine, Health and Biological Sciences
Clinical Pharmacology

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ABSTRACT

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCES
CLINICAL PHARMACOLOGY GROUP

Doctor of Philosophy

EVALUATION OF MICRODIALYSIS AS A TOOL FOR STUDYING PERCUTANEOUS DRUG ABSORPTION AND CUTANEOUS METABOLISM.

By Warren E. Keene

Cutaneous microdialysis is a method used to sample the extra-cellular fluid in dermal tissue. It uses a semi-permeable fibre, which is implanted in the dermis parallel to the skins surface. The fibre is perfused with a sterile physiological solution, which is collected at set periods. Low molecular weight compounds are able to diffuse freely into or out of the fibre in the direction of the concentration gradient. Once fibres have been implanted and are perfused, drug solutions can be applied to the skin surface directly above.

Methyl salicylate (MeS) was the initial model drug.
Occlusion of the drug site had no effect on the tissue Cmax but gave an increased AUC. Blood flow restriction using noradrenaline resulted in an increased Cmax and an increased AUC. Analysis for salicylic acid (the major metabolite) found higher concentrations than could be explained by the concentration present in the applied formulation, indicating that MeS was metabolised as it penetrated the skin.
Comparisons of two vehicle formulations, one containing ethanol the other propylene glycol, showed that ethanol enhanced the transdermal absorption of methyl salicylate relative to the propylene glycol formulation.
The transdermal absorption of salicylic acid and methyl salicylate was compared, salicylic acid gave the highest dermal concentrations despite being less lipophillic than methyl salicylate.
MeS was introduced directly into the dermis via a microdialysis fibre in order to compare the metabolic activity of the epidermis to the dermis. Technical difficulties were encountered but the data indicated that MeS is metabolised in the dermis though to a lesser degree than in the epidermis.

Ketoprofen was used to study the dialysis of highly protein bound drug, it has a literature protein binding value of 95%. Two fibres were used, a 2kDa fibre, which only dialysed free drug, and a 3MDa fibre, which dialysed both free and protein bound drug. The 3MDa fibres dialysed a higher concentration of ketoprofen however the increase was due to the increased efficiency of 3MDa fibres to dialyse free drug. The protein bound portion of the drug was not dialysed in an appreciable amount *in vivo* unlike the *in vitro* experiments. The studies were repeated using periods of zero flow in the fibres halfway through the study period. Protein levels were found to significantly reduce during dialysis but returned to basal levels immediately after the stop flow period there was also an increased concentration of ketoprofen in the samples after the period of zero flow from the 3kDa fibres but not from the 2kDa fibres. The microdialysis fibre was removing mobile protein from the surrounding tissue faster than it could be replaced, so only free drug was being sampled. However after the period of zero flow both the tissue concentration of protein and protein bound ketoprofen had returned to normal tissue levels.

Fenbufen and biphenylacetic acid are a pro-drug and its active metabolite both were applied topically. Biphenylacetic acid was found to penetrate to the dermis in greater concentrations. Biphenylacetic acid is the least lipophillic of the two compounds and this probably allows it to partition out of the stratum corneum and into the viable epidermis to a greater extent. Very low concentrations of biphenylacetic acid were detected after topical application of fenbufen indicating that there may be some minor β-oxidation activity in the upper layers of the skin. However the level of activity is not great enough to generate a therapeutic concentration of biphenylacetic acid. An unknown metabolite was detected following fenbufen application, which could not be identified but most likely was an oxidation product possibly γ-hydroxy-4-biphenyl butyric acid.

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1 Introduction

1.1 Skin: Structure and Function

The skin is one of the largest organs in the human body, in adults it can weigh between 4.5–5 kg and has a surface area of approximately $2m^2$ (Tortura *et al,* 1996). Human skin has an average thickness of 3mm. It consists of three main layers, the epidermis (only 60μm thick) at the surface, then the dermis and finally the subcutaneous layer, see figure 1.1. As well as supplying the obvious barrier function, skin also has several other important functions that will be discussed later.

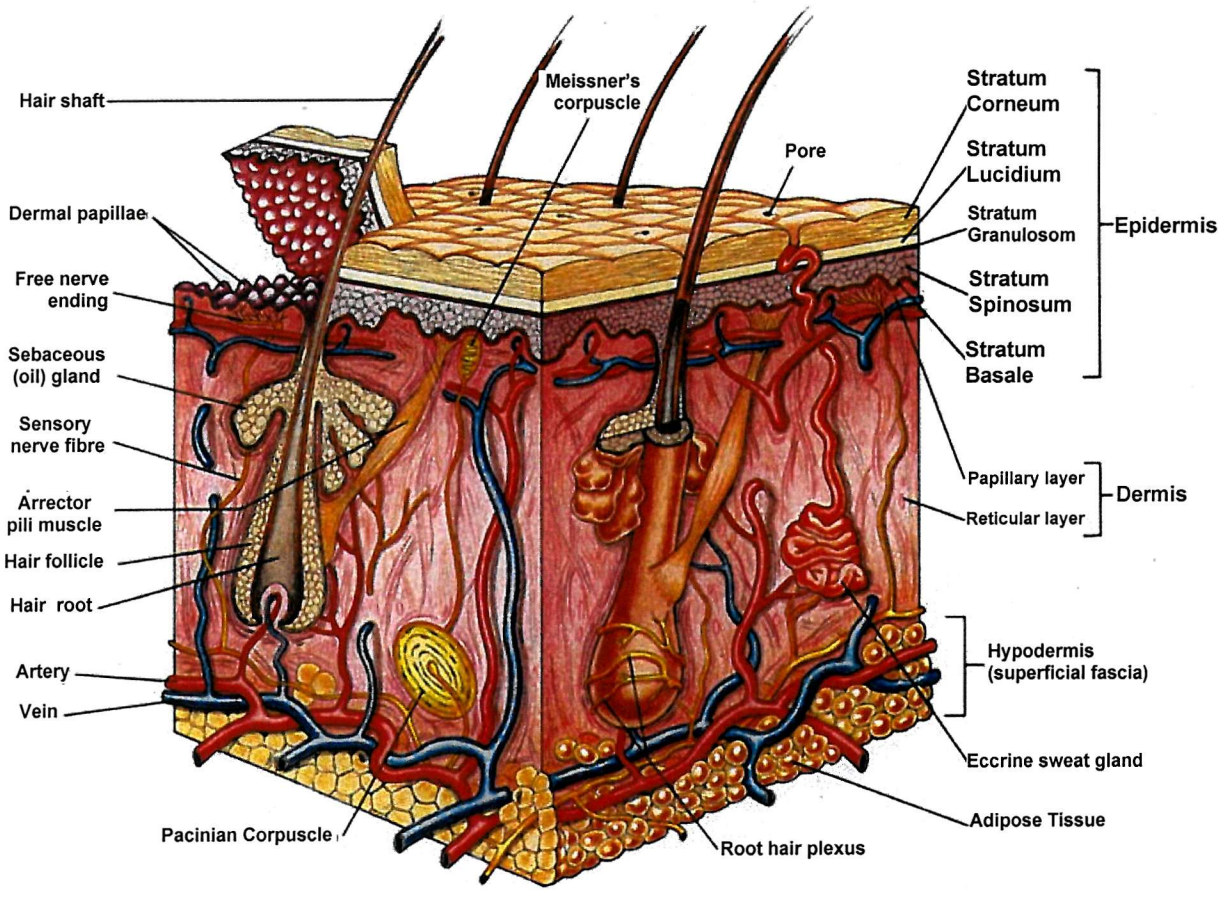

Figure 1.1 : Structure of the skin (Tortura *et al,* 1996)

1.1.1 Epidermis

The surface layer of the skin or epidermis consists primarily of stratified squamous epithelia and contains four principle cell types:

Keratinocytes- Approximately 80% of the epidermis by volume is made up of keratinocytes. It is these cells that give the skin its barrier properties, this is mostly due to the protein keratin which is produced in large quantities within the cells (Elias, 1983). The keratinisation of the cells as they develop makes the skin waterproof and protective against heat, light, micro-organisms and a wide range of chemicals. The keratinocytes in the epidermis are not uniform but layered, with the most differentiated cells at the surface (i.e. containing the most keratin and almost no internal structure) and the undifferentiated stem cells at the base. The stem cells are constantly multiplying and dividing producing keratinocytes which slowly migrate to the surface. As they rise the internal structures of the cell are broken down and eventually replaced with keratin. Also lamellar bodies are created which are secreted by exocytosis during the end stage of differentiation. Once excreted the lamellar bodies form a lipid bilayer surrounding the keratinocytes. Once the cells reach the surface they remain there until abrasion causes the cell remnants to be shed and they are replaced from deeper layers. Keratinocyte differentiation is normally divided into 5 steps resulting in 5 layers, and takes approximately 12-24 days in adult humans (Eckhart, 1989; Elias, 1983; Wiechers 1989):

1) Stratum basale- A single layer of stem cells that is constantly dividing to produce keratinocytes. Melanocytes and Merkel cells (see later) are also to be found within this layer
2) Stratum spinosum- The spinosum contains up to 10 layers of polyhedral cells which are tightly joined to each other by

junctions called desmosomes. The cells begin to shrink and flatten and have a spikey appearance caused by the increased number of desmosomes.

3) Stratum granulosum- The 3-5 layers of cells in the granulosum contain granules of keratohyalin which is a precursor to keratin. Also lamellar bodies are formed and increasing numbers of filaments cross the cytoplasm. At this stage in differentiation the nuclei of the cells are beginning to be broken down and they start to die. The shrinking and flattening first seen in the stratum spinosum continues.
4) Stratum lucidum- The lucidum is usually only detectable in thick skin such as on the soles of the feet or the palm. The 3-5 layers of cells are flat and dead and contain droplets of a clear substance (eleidin), which is the final pre-cursor to keratin.
5) Stratum corneum- The 25-30 layers of cells in the corneum are flat, dead and completely filled with keratin and surrounded by a thick lipid-protein envelope. The cells are joined laterally by specialised desmosomes. They provide the main barrier to all external hazards (Elias, 1988).

Keratin formation- Keratin is one the most important proteins when discussing the barrier properties of the skin. It supplies the keratinocytes with the required strength and rigidity to withstand the constant interaction with the outside environment. The first step in keratin formation is the production keratohyalin this is a small histidine and cysteine rich protein. Cells very early on in the keratinisation process begin to produce this in large quantities, however it is mostly clearly seen in the stratum granulosum. The small granules that give it its name contain mostly keratohyalin. The keratohyalin is converted to a protein called eleidin, this protein appears

to have a very short half-life and can normally only be seen in the palms and soles of the feet in the stratum lucidium as droplets of a clear liquid. Eleidin is rapidly converted to keratin, which in epithelial tissue exists as 20 different types. They can be grouped into two classes; type 1 which are broadly acidic molecules and type 2 which are basic to neutral molecules. All 20 types have the same basic structure, a central rod consisting of approximately 300 amino acids with a globular head at each end. The central rod consists of 4 lengths of alpha helix separated by regions of non-helical protein. Keratin molecules dimerise but only between different types, keratin dimers then form heterotetramers, which are the basic unit of a keratin filament, see figure 1.1a. In all cells keratin filaments form the cytoskeleton but in differentiated keratinocytes they form the majority of the cell's constituents.

Melanocytes- Melanocytes are found in the stratum basal but have projections which branch out into the upper layers, their purpose is to produce and transfer granules of melanin into keratinocytes. The melanin forms a protective barrier around the nucleus protecting it from UV light (Burkitt *et al.*, 1993).

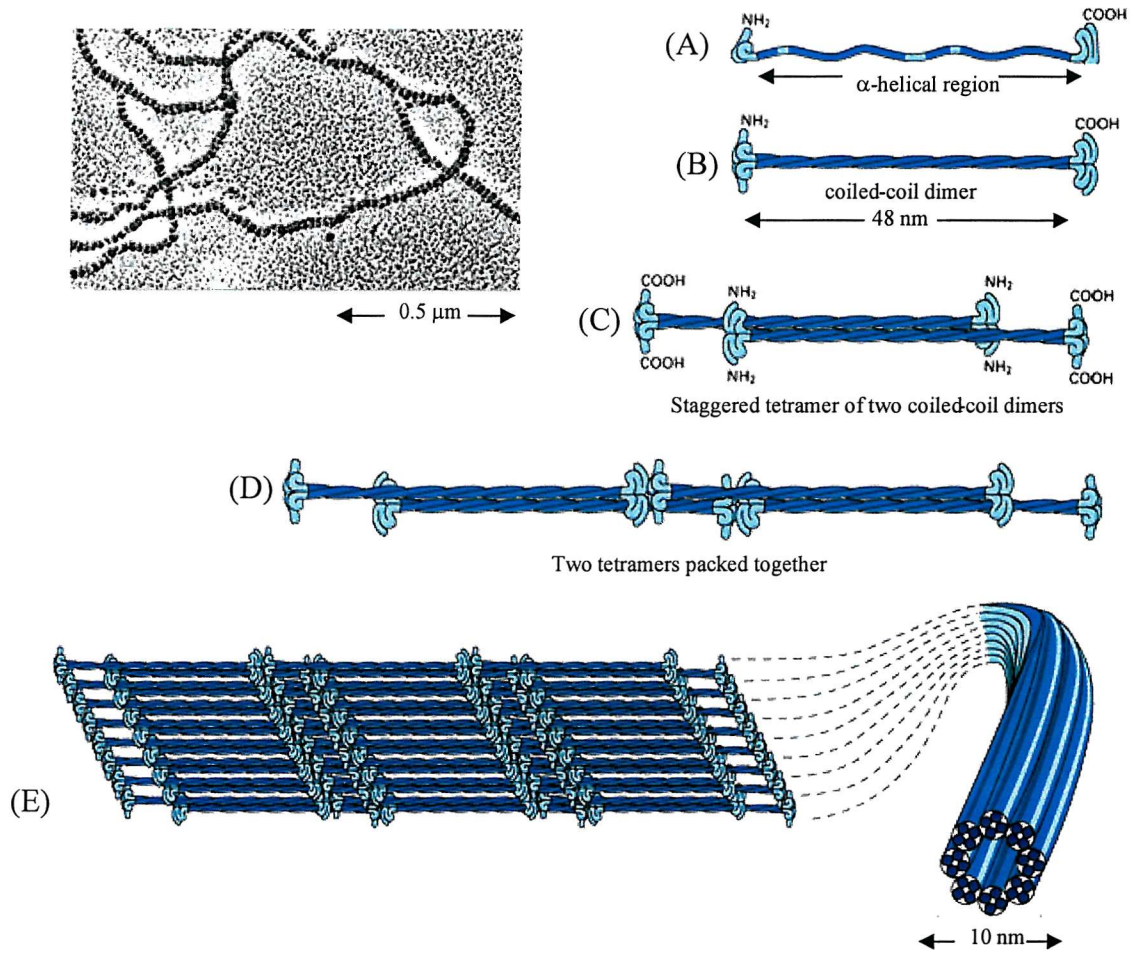

Fig. 1.1a: Steps in the formation of keratin filaments. a) single keratin molecule, b) dimmer of type I and type II keratin, c) keratin heterotetramer, d) and e) keratin filaments. (Albert, 1994)

Langerhan's cell- The Langerhan's cells are produced in the bone marrow and migrate to the epidermis where they interact with white blood cells to aid immune responses (Silberberg *et al.*, 1976a).

Merkel cells- These cells are located in the stratum basal and are attached to the keratinocytes via desmosomes. They make contact with the flattened portion of the ending of a sensory neuron and are thought to aid the sensation of touch (Tortura *et al,* 1996).

1.1.2 Dermis

The second layer of the skin is the dermis, it is mostly connective tissue containing collagen and elastin fibres. The upper papillary dermis has a high concentration of a glycosaminoglycan matrix whereas the lower reticular dermis contains more collagen and elastin. These compounds are secreted by fibroblasts, which is the main cell type of the layer. Also present are macrophages, lymphocytes and mast cells.

The main skin appendages are embedded within the dermis. Hair follicles, sebaceous glands, sweat glands, nerves, lymphatic drainage as well as blood vessels and the papillary plexus are all located in the dermis.

1.1.3 Subcutaneous Layer

Below the dermis is the subcutaneous layer or hypodermis this comprised mainly of adipose and connective tissue. This layer contains numerous nerve endings called lamellated corpuscles, which are sensitive to touch, there is also a network of arteries and veins that lie at the base of the subcutaneous layer. From these arteries a fine, dense capillary network extends through the dermis up to the dermo-epidermal junction where it forms a plexus that mirrors the papillae structure of the interface. The blood then enters the arterial-venous loops and is returned to the subcutaneous veins via post capillaries, venules and veins. The capillary network is particularly dense around the skin appendages i.e. hair follicles and sebaceous glands.

1.1.4 Skin Function

As well as providing a barrier to all external factors, the skin has several other important functions (Tortura *et al,* 1996).

1) Barrier: The skin acts as an effective physical barrier against both chemical and biological agents. This is mostly due to the stratum corneum the uppermost layer of the skin. It is highly hydrophobic as it consists of dead, flat, keratinised cells in a lipid-rich medium.
2) Blood reservoir: The blood volume in the skin varies according to the physical activity of the individual. This combined with the blood flow can have a major impact on the concentration of topically applied drugs in the skin. The blood vessel system contained within the dermis is the major entry point for xenobiotics into the systemic circulation.
3) Regulation of body temperature: Regulation is via sweat glands and the extensive network of blood vessels contained within the dermis and hypodermis. The blood flow in the skin can vary enormously depending on the body temperature.
4) Sensation: Nerve endings and receptors are present which allow the sensations of temperature, touch, pressure and pain.
5) Excretion: Sweat is the vehicle for loss for ions and certain organic compounds.
6) Immunity: Langerhan's cells, macrophages and lymphocytes are present in the skin all of which are involved in the immune response
7) Synthesis of vitamin D: UV light activates a precursor molecule that is then modified in the liver and kidneys.

1.2 Percutaneous Absorption

As has been mentioned earlier the skin acts a barrier to all external insults whether chemical, microbiological or physical. As far as chemical insult is concerned the skin was first thought to be an almost total barrier for all chemicals, however early last century Shwenkenbecker (1904) proposed that lipid soluble substances could penetrate more readily than water soluble compounds. It is now known that many substances permeate through skin, and percutaneous absorption has been recognised as an important area of study (Feldman & Maibach, 1966, 1969, 1970). Knowledge of percutaneous absorption and the subsequent fate of compounds within the skin layers is necessary from two different aspects:

1) The accidental exposure to harmful substances and risk assessment.
2) The use of topically applied therapeutic drugs.

The use of topically applied drugs for both systemic and local action has become an increasingly important field, with the use of transdermal patches joining the more traditional ointments and lotions. Two of the more recent drugs to be used transdermally are nicotine in nicotine replacement therapy for smokers and estradiol for hormone replacement therapy, see table 1.1.

Drug	Use	Point of Action	Application Method
Nicotine	Nicotine Replacement	Systemic	Transdermal Patch
Fentanyl	Pain Relief	Systemic	Transdermal Patch
Estradiol	HRT	Systemic	Transdermal Patch
Lidocaine	Local Anaesthesia	Local	Cream or lotion
NSAID's	Pain Relief	Local	Cream or lotion
Steroids	Anti-inflammatory	Local	Cream or lotion
Aspirin	Anti-platelet action	Systemic	Transdermal Patch

Table 1.1 Common topically applied drugs

Transdermal application has several benefits:

1) Steady-state pharmacokinetics: Transdermal absorption is a constant process (zero-order) allowing a continual delivery of drug resulting in a steady-state concentration at the therapeutic level.
2) Increased dose at site of application: Topically applied lotions and creams have the potential to deliver a large dose at the site of application (i.e. for muscle pain or skin diseases). This can also reduce the chance of developing side effects, because the concentrations in the rest of the body will be much reduced in comparison due to dilution in the systemic circulation (Cross *et al.*, 1998)
3) Reduction of toxic effects: Both oral and hypodermic delivery of drugs gives a large bolus dose which can sometimes lead to systemic concentrations exceeding the therapeutic level and reaching a toxic concentration. As transdermal absorption delivers compound at a constant rate the bolus effect is removed. Also oral administration leads to a high concentration of the drug in the stomach, which can lead to unwanted side effects as in the case of aspirin (Pohle *et al*, 2001).
4) Reduction of first pass metabolism: After an oral dose the majority of the absorbed drug will pass directly to the liver via the hepatic portal vein. Once in the liver it is exposed to a wide variety of metabolising enzymes which may result in some or all of the compound being inactivated.

Despite these benefits there are several disadvantages to transdermal absorption that limits its use to a relatively small number of compounds.

1) Skin penetration: Many drugs do not have the right properties to allow them to penetrate the skin in appreciable amounts.
2) Steady-state concentration: Those drugs that do penetrate the skin may result in local or systemic concentrations that are below the therapeutic level.
3) Skin reactions: The topical application of some drugs can lead to skin reactions that cause discomfort and pain.

The majority of transdermal research is focused on how to increase the penetration of drug by modifying the skin, drug or application method (Wester *et al*, 1993).

Percutaneous absorption is often split into three distinct phases (Schaefer & Jamoulle, 1988):

1) The penetration phase- entry of the compound into the stratum corneum.
2) The permeation phase- diffusion through viable epidermis into the dermis.
3) The resorption phase- uptake of the substance through the vascular system.

However this depiction of transdermal absorption is somewhat too idealistic and the three phases are not so separated and will overlap considerably and factors affecting one phase can be just as important in the later phases.

1.2.1 Movement of drug from vehicle to stratum corneum

The first stage is the partition of the drug from the application vehicle into the stratum corneum (Scheuplein, 1969; Barry 1992). This is dependent on several factors: the concentration of the applied drug, the vehicle in which the drug is contained and the physicochemical properties of the drug.
For optimal absorption, these three factors all have to be modified to achieve the best partition of the drug from the vehicle into the stratum corneum. The higher the concentration of the applied drug, the higher the concentration achieved in the stratum corneum (Maibach & Feldman, 1969. Dupuis *et al.* 1984). However constantly increasing the applied concentration to achieve the required skin concentration is impractical as it increases the cost of the preparation, the increased concentration may lead to toxic effects or sensitisation reactions, or it may be difficult to achieve the requisite concentration. The final distribution of the drug between the formulation and skin is ultimately controlled by the partition coefficient between the upper layers of the skin and the drug vehicle (Potts & Guy, 1992).

Partition Coefficient

The partition coefficient is directly related to the compounds lipophilicity. The stratum corneum is a lipid rich environment. Therefore, in order for the partition coefficient to favour the skin, the applied compound must be lipophilic. The best measurement of lipophilicity, as well as a good guide to the absorption of a compound, is the octanol/water partition coefficient (P_{ow}) (Potts & Guy, 1992). P_{ow} represents the ratio of a dissolved substance between equal volumes of octanol and water when mixed and allowed to reach equilibrium, the higher the value the greater the affinity for octanol and the greater the lipophilicity. Compounds with high P_{ow} values tend to have high percutaneous absorption. However the relationship is not linear and the greatest absorption occurs with a P_{ow} of approximately three, and above this value the absorption of compounds reduces significantly (Katz & Shaikh, 1965; Scheuplein, 1976). Compounds with a P_{ow} value over $\approx$ 3 have an increasing affinity for the stratum corneum; and the reason why absorption decreases beyond this point is that the point of equilibrium is shifted too far towards the stratum corneum. Compounds with a high lipophilicity are unable

to partition into the relatively aqueous lower epidermis and dermis and form a reservoir of drug within the stratum corneum (Hewitt *et al*, 1993). This reservoir effect has been shown to occur for a number of compounds such as fluocinolone acetonide (corticosteroid) (Vickers, 1963), 4-chloro-2-methylphenoxyacetic acid (herbicide) (Kolmodin-Hedman *et al*, 1983) and several aromatic amines (methylene-bis-2-chloroaniline and methylenedianiline) (Hewitt *et al*, 1993). The main characteristic of these compounds is that a low systemic concentration is achieved during topical application but the concentration can be maintained for up to two weeks after the compound has been removed because the reservoir in the stratum corneum slowly diffuses into the epidermis.

An ideal vehicle for application would be one which has a low solubility for the drug compared to the skin surface, moving therefore the equilibrium for diffusion towards the skin and allowing a high percentage of the applied drug to penetrate. As the drug concentration in the vehicle decreases and the skin concentration rises an equilibrium is reached and the flux of drug through the surface of the skin reduces. Several approaches have been taken to keep flux high:

i) Use of a super saturated solution of the drug which would maximise the amount of the drug that can penetrate before equilibrium is reached (Hadgraft, 1999).

ii) Use a modified vehicle that contains two solvents, one is highly soluble to the drug but also volatile, the other has a much lower solubility to the drug but is less volatile at body temperature.When the formulation is applied the volatile solvent begins to evaporate potentially increasing the concentration slightly and more importantly reducing the solubility of the vehicle for the drug. This constantly shifts the point of equilibrium further towards the skin, thereby maintaining a high rate of flux even though the vehicle concentration may eventually be less than the tissue concentration

Another important factor to consider is the ability of the drug to diffuse through the vehicle itself. If the diffusion rate is low, especially in the case of highly viscose formulations, then the layer of vehicle adjacent to the skin may become depleted of drug reducing the flux to zero. Ideally the vehicle should have a low viscosity, however this has to be balanced with the ease of application and the quantity that is required to be applied.

1.2.2 The movement of drug through the stratum corneum

Once the compound has partitioned into the skin there are two routes that have been suggested for the movement of compounds through the skin barrier, the transcellular and the intercellular route (Barry, 1987, 1992, Scheuplein, 1967). The compounds taking the intercellular route must move around the keratinocytes through the lipid rich intercellular region, which is arranged in a multiple lipid bi-layer (Weichers, 1989; Hadgraft, 1999). This region is formed from lamellar bodies, which are exocytosed from keratinocytes just before reaching the stratum corneum. The composition of the stratum corneum lipids is an approximate 1:1:1 molar ratio of cholesterol, long chain free fatty acids and ceramides (Elias, 1983). In contrast to the high phospholipid content of most viable cells the stratum corneum contains almost none at all. It appears that this lipid rich medium is an important part of the stratum corneum's barrier function, skin which has had the intercellular lipids extracted from it has shown dramatic decrease in its barrier function (Matoltsy *et al*, 1968; Anderson and Rayker, 1989). This route is considered to be the more important of the two because both lipophilic and hydrophilic compounds can take advantage of it (Elias *et al.*, 1981; Bodde *et al*, 1991), see figure 1.2, below. The transcellular route requires the compound to partition into and out of the cell membrane of keratinocytes. The interior of these cells is packed with keratin, which is a more aqueous environment than the intercellular region, this route is more likely to be important for hydrophilic compounds (Barry,1991). Neither of these routes is mutually exclusive and most topically applied compounds would probably be able to take advantage of both routes

There is a third route that has been suggested which bypasses the stratum corneum altogether, which is via the skin appendages i.e. the sebaceous glands, hair follicles and sweat pores. They are thought to be especially important for large polar molecules as well as being an alternative route for most compounds (Tregear, 1961; Schaefer & Redelmeier, 1996). These appendages can act as shunts moving compounds through the epidermis directly to the dermis. One study using haired and hairless mice showed that the absorption of benzo[a]pyrene was 3-4 times greater in the haired mice (Kao *et al.*, 1988). In humans the absorption of methyl nicotinate was

demonstrated to vary with the follicle density of the application site with forehead > forearm > palm (Tur *et al*, 1991). The compounds coumarin and griseofulvin penetrate the skin almost solely by the appendageal route, possibly by using the sebum as a vehicle (Ritschel *et al.*, 1989).

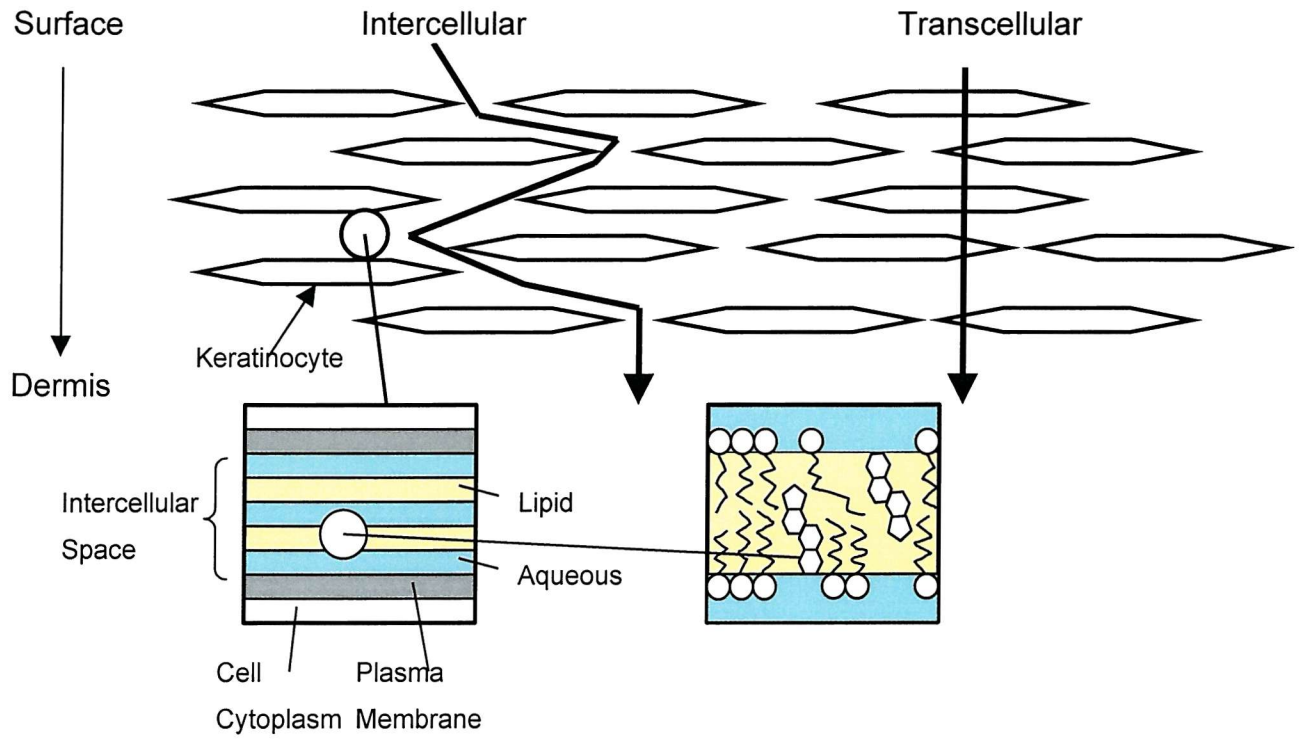

Figure 1.2. Illustration of the intercellular and transcellular routes and the lipid bilayer arrangement between the cells of the stratum corneum

Irrespective of which route is used, the movement of a compound through the stratum corneum (which is the main penetration barrier) can be described by Fick's first law of diffusion (Guy & Hadgraft, 1988), see equations 1, 2 and 3.

$$\mathbf{J = K_p * \Delta C} \qquad \text{(eqn. 1)}$$

J = rate of flux at steady-state (mass of drug per unit area per time)

K_p = permeability constant

ΔC = concentration gradient across stratum corneum

$$K_p = (K_m * D) / l \qquad \text{(eqn. 2)}$$

K_m = partition coefficient of penetrant between stratum corneum and vehicle.

D = diffusion constant (unit area per time)

l = diffusion path length, stratum corneum thickness. (cm)

If equations one and two are combined:

$$J = (K_m * D * \Delta C) / l \qquad \text{(eqn. 3)}$$

Equation three highlights the important factors for stratum corneum flux. Increasing the drug solubility in the stratum corneum or decreasing the solubility in the vehicle can increase the flux. The flux can also be increased by raising the diffusion rate within the stratum corneum or by applying a higher concentration of drug (Bach & Lippold, 1998).

The factors affecting the movement of drug through the upper layers of the skin can be split into several areas:

i) Chemical:

In general absorption tends to favour small, uncharged compounds with a high lipophilicity. Fick's law states that flux is proportional to the partition and diffusion coefficients of the applied compound. For a high diffusion coefficient the compounds must have a minimal interaction with the tissue medium, therefore small compounds have a higher rate of flux as they are less impeded by tissue structures. Charged compounds will interact strongly with other charged species and can even induce dipoles in relatively uncharged species thereby reducing the diffusion coefficient (Idson, 1983; Pugh *et al*, 1996; Roberts *et al*, 1996; Roberts *et al*, 1998; Goosen C *et al*, 1998).

ii) Concentration

As has been already stated earlier, Fick's law clearly shows that increasing the concentration of the applied formulation results in a greater flux through

the stratum corneum (Idson, 1983; Schaefer & Redelmeier, 1996). Maximising the concentration by using super-saturated solutions gives the highest possible flux until equilibrium is reached (Bach & Lippold, 1998) .

iii) Vehicle

The vehicle can affect the overall flux of the applied compound in three ways, firstly the partition coefficient of the drug between the vehicle and the skin governs the position of the equilibrium, and therefore the flux through the stratum corneum. Secondly if the diffusion rate of the compound within the vehicle is low a depleted layer of vehicle may form adjacent to the skin reducing the flux. Thirdly the presence of a penetration enhancer, such as oleic acid, ethanol or dimethyl sulphoxide (Guy *et al*, 1990) can increase the diffusion rate of applied compounds through the stratum corneum and the epidermis. The enhancement is caused by disrupting the rigid lipid matrix in the intercellular space (Tanojo *et al*, 1998; Barry, 1987; Taguchi *et al*, 1999). The lipid matrix naturally takes the form of a bilayer, the absorption of polar compounds such as water or ethanol causes the aqueous regions to increase and disrupts the close packing of the polar head groups of the lipids (Barry, 1991), both actions serve to give absorbed compounds greater freedom of movement within the intercellular space and hence increase the rate of diffusion. The same is true for non-polar enhancers however in this case it is the close packing of the hydrophobic tales that is disrupted leading to an increased rate of diffusion.

iv) Occlusion

Occlusion of the skin with dressings or preparations results in an increased hydration state and temperature, and has been shown to aid the penetration of various compounds (Behl *et al*, 1980; Bucks *et al*, 1988; Ryatt *et al*, 1988). In general the absorption of lipophilic compounds is enhanced with occlusion more than hydrophilic though the difference is often marginal, and there are exceptions such as hydrocortisone and certain phenols, which show no enhancement despite being lipophilic. The enhancement is a combination of the hydration and temperature changes, an increase in temperature automatically results in increased rates of diffusion (Kligman, 1983). Hydrated/occluded skin can contain up to 50% more water than control skin and causes it to be significantly thicker (Blank *et al*, 1964; Treffel *et al*, 1992).

The increase in thickness results from the keratinocytes swelling due to hydrated keratin and increasing the water content of the intercellular matrix. The rigid structure of the lipid matrix supplies the skin with much of its barrier properties (Scheuplein,1965), and an increased water content increases disorder in the bilayer structure increasing the rate of diffusion for compounds travelling the intercellular route. This would explain the increased absorption of lipophilic compounds as the intercellular route is considered to be their primary path. Hydrophilic compounds are thought to utilize the transcellular route primarily; hydration of the relatively aqueous keratin interior is unlikely to increase cellular penetration, which explains the reduced enhancement with occlusion.

1.2.3 Physiological factors affecting permeation

Once drug has entered the skin a lot of factors can influence the drugs permeation characteristics. These can vary widely between people, formulation and manner of application, leading to large inter-individual variations, which will be shown in the results chapters. Possibly the most important factor in terms of inter-individual variation is the physiological differences between subjects.

i) Anatomical site

The physiology of human skin varies according to anatomical site with differences in skin structure, the thickness of the epidermis and the number and distribution of skin appendages, which have an impact on the permeation of applied compounds (Qiao & Riviere, 1995). The thickness of the epidermis ranges from 10μm for eyelids to 1mm on the palms and the soles of the feet (Scheuplein & Bronaugh, 1983). Variation in hair follicle and eccrine sweat gland density can also be an important factor for those compounds that depend on the appendageal route. The regional variation in the content and composition of the intercellular lipid has also been shown to be a contributory factor in the permeation of compounds through the stratum corneum. These factors have been proven to have an effect by several studies:-

The rank order of permeation as shown by the presence of salicylic acid in the urine after application of 25% methyl salicylate to 50cm^2 skin under occlusive dressing was, sole < heel < instep < forearm < abdomen. There was 4-fold difference between abdomen and sole of the feet (Roberts *et al.*, 1982). Absorption of ^{14}C hydrocortisone showed a similar pattern with sole < palm < forearm < < back < axillary < forehead < jaw < scrotal skin. There was a 42-fold difference between the sole and scrotal skin (Feldman & Maibach 1967). Comparison of absorption of benzoic acid, benzoic acid sodium salt, caffeine and acetylsalicylic acid by skin strip and excretion methods, showed that the forehead was twice as permeable than the arm or abdomen (Rougier *et al.*, 1987)

The main trend seems to follow skin thickness with areas with thickest stratum corneum having the lowest permeation, such as the sole and palm. However there has also been reported a correlation between trans-epidermal water loss (TEWL) and percutaneous penetration (Lotte *et al.*, 1987), the TEWL represents the level of skin hydration and density of sweat glands. This highlights both the potential for the appendageal route and the importance of skin hydration in the perturbation of intercellular lipid.

ii) Age

Age does not appear to have a great influence on the percutaneous penetration of many compounds. The general trend is that adult human skin is less permeable than either infant skin or the elderly (Christophers & Kligman, 1965. Behl, 1985). The changes are most likely attributable to alterations in skin structure with increase in age such as a reduced hydration state, increasing thickness of skin and variations in the lipid content of the stratum corneum.

iii)Ethnic Group

The few studies that have been performed have not demonstrated any substantial difference between different ethnic groups (Lotte *et al.*, 1993). One study showed a slightly higher permeability to nicotine in caucasian skin compared to darker skin types and suggested that the difference was related to melanin content (Berardesca *et al*, 1990). A later study showed that the effectiveness of EMLA and the penetration of fluocinolone acetonide were both reduced in black skin relative to caucasian skin (Berardesca *et al*, 1996).

iv) Gender

No substantial difference in skin permeability has been demonstrated between the two sexes in adult humans (Roy and Flynn, 1990; Reed *et al*, 1995).

v) Drug protein binding

In the discussion above the percutaneous movement of drug has been considered purely from the aspect of unbound free drug. However nearly all drugs interact with constituents of cells in order for them to exert an effect and for a large number this means binding to proteins. As well as binding to their target protein, many drugs are bound by non-specific binding sites on proteins, the most important of which is albumin (Rang, Dale & Ritter 3rd Ed.; Bickel, 1986; Benfeldt & Groth, 1998). There are other proteins that can bind a wide variety of compounds such as β-globulin and glycoproteins but they are present in much lower concentrations (Sjoholm, 1986). The percentage of protein bound drug at therapeutic concentrations stated in published literature is always given in terms of plasma albumin. The albumin concentration in extra cellular fluid (ECF) in human skin is on average $^2/_3$ the plasma value (Bert *et al*, 1986). Even with this reduced concentration the molar concentration of available binding sites far exceeds the typical molar concentration of a topically applied compound. As the normal plasma concentration of albumin is 0.6mmol/l and each protein has two binding sites, there are 1.2 mmol/l of binding sites. In skin ECF this figure will reduce to approximately 0.8mmol/l. A typical tissue concentration of topically applied drug (in the studies in this thesis) is approximately 0.03mmol/l, over a 20-fold difference. Under these conditions the percentage of bound drug is independent of both the drug and protein concentration and is dependent solely on the affinity of the binding site for the compound. Therefore the plasma albumin binding values for compounds will be approximately the same for skin ECF albumin.

Once a drug molecule is bound to albumin, it is much larger; most topically applied drugs are no more than 500 daltons, a typical albumin molecule is > 30,000 daltons. This increase in size and mass will reduce the diffusion constant of the drug and according to Ficks law (eqn. 3) will reduce the total drug flux. However protein binding reduces the concentration of free drug and therefore increases the concentration gradient with respect to the skin surface which in turn will increase drug flux according to Fick's law. If the compound is at least moderately lipophilic then the drug-protein complex will have a greater polarity and water solubility than the unbound molecule. This will move the

partition equilibrium towards favouring a more aqueous environment such as the lower epidermis and dermis, which again would increase flux. At this time the exact balance of these opposing forces is not known.

1.2.4 Factors causing the removal of drug from the epidermis/dermis

Once a compound has reached the lower epidermis and dermis, diffusion is no longer the only process taking place. Several other factors come in to play which actively lower the concentration by removing the compound from the skin.

A) Vasculature Uptake

Possibly the most important removal process is entry into the systemic blood supply via the dermal vasculature. The epidermis itself has no blood supply and relies on diffusion for its nutritional requirements, in comparison the dermis has an extensive vascular network that consists of two plexuses one at the epidermal/dermal junction and the other at the interface between the dermis and the subcutaneous layer. The vasculature at the epidermal/dermal junction also extends up into the dermal papillae and is exceptionally dense around skin appendages. The blood vessels act as a sink for any absorbed compounds, because the systemic concentration of any topically applied compound will always be far less than the skin concentration. Therefore there will be diffusion into the blood vessels within the dermis and the blood flow will prevent any build up of concentration within the vessel.

The importance of blood flow was highlighted by two separate experiments; the first compared the dermal clearance of 7 different steroids (Siddiqui *et al*, 1989). The steroids were applied in chambers to the backs of living, anaesthetised rats and dead rats after the epidermis had been removed, and the disappearance from the chambers measured over time. The dermal clearance was found to be 2-5 fold higher in the living rats. Different researchers using 7 non-steroidal anti-inflammatory drugs (NSAID's) repeated the study and also found a marked increase in dermal clearance in the living animals, the greatest difference was for salicylic acid, which gave a 5 fold increase (Singh & Roberts, 1993). The researchers in the NSAID study repeated it to look at the effects of vasoconstriction by adding epinephrine to the chamber fluid. The resulting dermal clearances lay between the values for the live and dead animals and in comparison with the live animals epinephrine

resulted in increased local tissue drug concentrations down to 6mm below the site of application (Singh & Roberts, 1994).

Cutaneous blood flow is clearly an important factor when considering the dermal drug concentration and the depth of penetration, and vasoconstrictors can be used to modify the blood flow and increase local tissue concentration and depth of penetration.

B) Metabolism

Once a topically applied compound reaches the viable epidermis it is exposed to the metabolic activity of the skin which can actively modify a wide range of compounds leading to inactivation or in a few cases lead to the activation of originally harmless substances, e.g. benzo[a]pyrene. Both phase I and phase II metabolism has been shown to exist in skin (Pendlington *et al*, 1994; Van de Sandt *et al*, 1993) though generally to a far lower extent than that seen in the liver, see table 1.2.

The principle site for xenobiotic metabolism in the skin is the epidermal keratinocytes partly because they are the most abundant cell type in the skin but also because keratinocytes are in an ongoing state of differentiation and the internal structure is in the process of massive change requiring a wide range of metabolic reactions.

	Phase I metabolism	Phase II metabolism
Oxidation:	Hydroxylation	Glucuronidation
	Deamination	
	Dealkylation	Sulfation
	Epoxidation	
		Glutathione conjugation
Hydrolysis:	Ester hydrolysis	
	Epoxide hydrolysis	Methylation
Reduction		Acetylation

Table 1.2. Cutaneous metabolic reactions.

i) Phase I Metabolism

Phase I metabolism is primarily thought to act as preparation for phase II reactions in that they either uncover or add a chemical group on which phase II reactions can occur.

Oxidation:

Mixed function oxidases (MFO) are possibly the most important group of metabolising enzymes as they catalyse a wide range of reactions for a range of compounds. At their simplest these enzymes catalyse the insertion of a single atom of oxygen into the substrate, subsequent rearrangement or decomposition gives the final end product, see figure 1.3. All the reactions require the presence of molecular oxygen as well as NADPH, which drives the reaction by being simultaneously reduced.

$$\textbf{Substrate (RH)} + O_2 + \textbf{NADPH} + H^+ \Rightarrow \textbf{Product (ROH)} + H_2O + \textbf{NADP}^+$$

Figure 1.3: Reaction catalysed by mixed function oxidases.

The largest family of enzymes in the MFO are the cytochromes P450 which are capable of metabolising both endogenous compounds and xenobiotics. Cytochrome P450 enzymes are further divided into sub groups by comparing their gene sequences, P450 enzymes with than 40% homology are assigned to different families CYP1, CYP2 etc. If the homology is 40–50% then they are assigned to different subfamilies CYP2A, CYP2B etc., enzymes with a homology greater than 55% are classified as members of the same subfamily CYP2A1, CYP2A2 etc. The P450 enzymes mainly involved in xenobiotic metabolism are CYP1, 2 and 3.

CYP1

This family contains the subtypes CYP1A1 and CYP1A2 and are over 70% homologous they are primarily responsible for the oxidation of polycyclic aromatic hydrocarbons and are often termed aryl hydrocarbon hydroxylases (AHH). So far only CYP1A1 has been detected in human epidermis using

substrates such as benzo[a]pyrene and 7-ethoxyresorufin (Bickers *et al*, 1982). The basal level of activity of CYP1A1 in epidermal tissue is generally low in comparison to liver, typically less than 5%. However, pre-treatment with polycyclic aromatic hydrocarbons (benzo[a]pyrene, β-napthoflavone) can induce an increase in the activity by 2-6 fold (Bickers *et al*, 1985; Merk *et al*, 1984; Phan *et al*, 1990). Although the oxidation of polycyclic aromatic hydrocarbons (PAH) is primarily a detoxification reaction the outcome is also the formation of mutagenic epoxides which are capable of binding to DNA and causing mutations which lead to tumour formation (Bickers & Mukhtar, 1991). Several studies have been performed to identify the distribution of CYP1A1 within the skin. Several researchers have isolated epidermal, dermal and skin appendage (sebaceous gland, hair follicle) tissue and tested each for AHH activity. In general the epidermis and the epidermal-origin appendages express the highest AHH activity and dermal tissue has the lowest activity (Wiebec *et al*, 1975; Akin & Norred, 1976; Coombes *et al*,1983; Das *et al*, 1986). There have also been studies to determine the enzyme activity within sub-populations of cells within the epidermis in order to determine the effect of differentiation. One study used flow cytometry to separate the cells according to morphological differences, and split cells into two groups, a high CYP1A1 content and a low CYP1A1 content (Stauber *et al*, 1995). The low group was found to be predominately basal cells and the high content group was predominately differentiated cells. Another study separated the cells using centrifugation on a Percoll gradient, on the principle that cell buoyant density decreases with differentiation (Reiner, 1992). The basal level of CYP1A1 activity was found to increase with the level of differentiation. If the skin was pre-treated with an inducer the activity in all cells was higher however the order was reversed, and the basal cells had the highest activity.

In conclusion CYP1A1 activity is present in the skin though at a far lower level than the liver, activity is far higher in the epidermis and epidermal-origin appendages than in the dermis. Thus the activity within the epidermis increases with the level of differentiation of the keratinocytes however the ability of the keratinocytes to respond to induction is reduced with the level of differentiation.

CYP2

The CYP2 family contains the greatest number of forms of cytochrome P450 and the largest range of substrate specificity. The only form with detectable activity in skin tissue is CYP2B, often referred to as the phenobarbital-inducible cytochrome P450. The activity was detected in human epidermis microsomes using 7-pentoxyresorufin and 7-benzoxyresorufin as substrates (Pham, 1990). The CYP2B family has been found to be active towards cyclophosphamide and ifosfamide (anticancer drugs) as well as nicotine, aflatoxin B_1 and 6-aminochrysene (Chang *et al*, 1993).

CYP3

Only CYP3A has been detected using monoclonal antibodies in human skin, in the epidermis, the sebaceous glands and in mast cells within the dermis (Murray *et al*, 1988).

Hydrolysis:

Both ester and epoxide hydrolases have been detected in human skin. Ester hydrolases (see figure 1.4) are of two types A and B; A is an arylesterase whilst B is further split into cholinesterases and carboxylesterases. Human epidermis has been shown to be active towards corticosteroid esters (Tauber & Rost, 1987) and a number of corboxylesterases have been identified in subcutaneous fat and keratinocytes using organophosphate inhibition and substrate specificity. Esterases are very stable and activity has been measured in human cadaver skin even after freezing (Jiang *et al*, 1996)

$$\mathbf{R\text{-}C(O)O\text{-}R + H_2O \Rightarrow RCOOH + ROH}$$

Figure 1.4: Reaction catalysed by ester hydrolases.

Epoxide hydrolase converts epoxides into diols, see figure 1.5. Epoxides are mainly PAH's that have undergone initial metabolism by MFO's specifically the CYP1A family. Epoxide hydrolase has been detected in human epidermis (Pham *et al*, 1989) and the cutaneous levels of microsomal enzymes vary between 3-28% of the hepatic values (Mukhtar & Bickers, 1981) Epoxide

hydrolase activity was also detected in human hair follicles using benzo[a]pyrene epoxides (Del Tito *et al*, 1983)

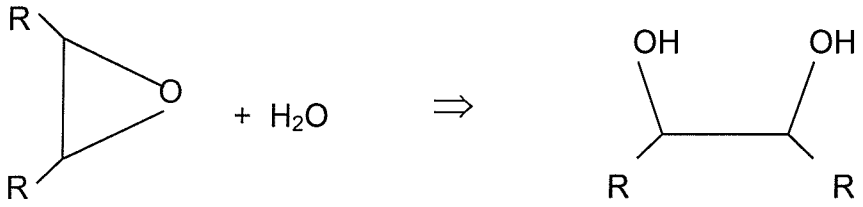

Figure 1.5: Reaction catalysed by epoxide hydrolases.

Reduction:

Human skin has a reductive capacity that is mainly used to reduce steroids and free radicals that are generated within the tissue. NADPH cytochrome C reductase has been observed in human epidermis (Pohl *et al*, 1976). Testosterone 5α-reductase has been shown to reduce both testosterone and progesterone in human skin (Pham *et al*, 1990). Microsomal and cytosolic azoreductases have been identified in the skin using 1-phenylazo-phenol and phenylazo-2-napthol as substrates (Collier *et al*, 1993). Oxygen free radicals are reduced in the skin by a combination of catalase, superoxide dismutase and NAD(P)H quinone reductases; at the same time any nitroxide radicals generated are reduced by oxidoreductases (Fuchs *et al*, 1989).

ii) Phase II Metabolism

Phase II metabolism is the usually the final step in xenobiotic metabolism as a method of detoxification. The aim of xenobiotic metabolism/detoxification is to increase the polarity and/or size of the compound to aid its excretion via the urine or bile. Although phase I metabolism sometimes results in a compound that is water soluble which can be excreted readily, normally phase II metabolism is required. Phase II enzymes are conjugative enzymes i.e. they add moieties to the functional group formed in phase I reactions resulting in large water soluble molecules that are easily excretable via the urine or bile.

Glucuronidation:

Glucuronidation is essentially the transfer of glucuronic acid, an oxidised form of glucose, from uridine diphosphate glucuronic acid to the target compound via a functional group which can be a hydroxyl, carboxyl, nitrogen or sulphur group. The enzymes responsible for the catalysis of these reactions are called UDP-glucuronyl transferases (UGT's). They are membrane bound and found in the endoplasmic reticulum of cells in several tissues including skin. There are two human UGT subgroups, isoforms from both families have been shown to metabolise a broad range of phenolic substrates and therefore they are important in the metabolism of many xenobiotic compounds.

Several studies have demonstrated glucuronidation activity in human skin, Pham *et al* (1990) demonstrated that reconstituted human epidermis glucuronidated 1-naphthol and 4-nitrophenol.

Sulphation:

Sulphation as the name implies is the transfer of a sulphate group from 3′-phosphoadenosine-5′-phosphosulphate (PAPS) to the target molecule catalysed by sulfotransferases (ST), see figure 1.6.

ROH + PAPS ⇒ RS + PAP

Figure 1.6: Reaction catalysed by sulphur transferases.

There are three families of ST's, phenol ST (PST), hydroxysteroid ST (HST) and oestrogen ST (OST) and they are important for endogenous steroid metabolism. They are known to activate several compounds, they are responsible for the creation of the ultimate mutagen for several carcinogens including benzo[a]pyrene. The functional group that they attack is generally a hydroxyl and simple phenols are good substrates.

Human scalp skin is capable of activating minoxidil, a hair growth stimulant, by sulphation, with the activity located in keratinocytes in the hair follicles (Anderson *et al*, 1998). Other studies have shown that human skin is capable of sulphating dopamine, p-nitrophenol and triclosan as well as minoxidil,

although the rate is approximately 10-20 fold less than liver (Moss *et al*, 1996).

Glutathione conjugation:

Glutathione is a tripeptide consisting of glutamic acid, cysteine and glycine. It has many roles including anti-oxidant, cysteine reservoir, prostaglandin synthesis and conjugation of many xenobiotics. Glutathione conjugation is mediated by glutathione-S-transferases (GST) which catalyses the reaction between the nucleophilic sulphur group on the cysteine with an electrophilic functional group on the target substrate, see figure 1.7. The resulting glutathione conjugate can either be excreted directly, usually in the bile, or it can undergo further metabolism creating a mercapturic acid derivative, which is excreted mainly in urine.

$$\mathbf{RX + GSH \Rightarrow R\text{-}S\text{-}G + XH}$$

Figure 1.7: Reaction catalysed by glutathione-S-transferases.

GST activity was first demonstrated in human skin using styrene oxide and 3-methylcholanthrene-11,12-oxide as substrates (Mukhtar & Bresnik, 1976). When 1-chloro-2,4-dinitrobenzene was used as a substrate the area of highest activity was found to be the sebaceous gland. The epidermal basal cells were also found to have GST when β-naphthoflavone was used, pre-treatment was also found to induce activity by 2-fold (Coombes *et al*, 1983).

1.3 Methods of Measuring Cutaneous Drug Concentration and Metabolism

Many methods have been employed to measure transdermal drug absorption and cutaneous metabolism and comprise both *in vitro* and *in vivo* studies. None of the methods are ideal and they have different advantages and disadvantages. In general human *in vivo* studies will generate the most relevant data however they can be expensive and time consuming *In vitro* studies are quicker and cheaper but the data produced may not be as relevant to the *in vivo* situation.

1.3.1 *In vivo* methods

A) Skin stripping

A drug is topically applied and allowed to penetrate the stratum corneum and diffuse through the epidermis. The time allowed for this will vary according to drug used and site of application. Once the allotted time for absorption has passed all drug remaining on the surface is carefully removed. By repeatedly applying adhesive tape to the application site and pulling it off sharply the surface layers of the skin can be removed sequentially. The drug can be separated from the tape using an organic solvent extraction method and analysed. This study can be performed using multiple sites with different exposure times to give an experimental time course. However, skin stripping is an invasive technique and often painful. There are also problems with contamination if the surface of the skin is not cleaned thoroughly and more than one layer may be removed in a single tape removal. (Pershing *et al*, 1992; Wester *et al*, 1989; Surber, 1996)

B) Skin Blisters (Brunn *et al*, 1991; Surber *et al*, 1993)

This involves the separation of the epidermis from the dermis along the basale lamina by the application of prolonged suction on the skin surface to create a small liquid filled blister. Drug can be applied topically to the blister or systemically before, during or after formation. The blister fluid can then be sampled at various time points and analysed and the absorption profile of the

drug calculated. The epidermal blister roof can also be sampled and analysed for drug concentration. However this method does not examine the contribution of the entire skin thickness to the absorption profile and factors such as blood flow and partition into the dermis from the epidermis are removed.

C) Skin Biopsy (Surber, 1996)

Small discs of full thickness skin can be removed both before and after the topical application of drug. The skin sections can either be separated into layers via chemical treatment or frozen and cut into layers using a cryotome. The individual layers can then be analysed for drug content giving an accurate drug concentration at varying depths. However this is an invasive technique and in order to achieve an absorption time-profile, multiple biopsies would have to be taken from each volunteer representing different exposure times.

D) Microdialysis

(See below)

E) Blood and Excreta Collection (Wester & Maibach, 1989)

One of the most extensively used methods for determining percutaneous absorption is the collection and analysis of blood and excreta following the topical application of a radio-labelled compound. The compound is normally labelled using carbon-14 or tritium because plasma and excreta levels of absorbed drug are often well below assay detection limits, however scintillation detection allows the measurement of minute quantities. Although this method is extremely good for determining the systemic bioavailability and the route of excretion it gives little information about the absorption rates at the site of application.

1.3.2 *In vitro* Methods

A) Isolated Perfused Skin (Behrendt *et al*, 1989)

Animal skin such as a porcine skin flap or an isolated rabbit ear can be removed from the animal and the underlying vasculature perfused with oxygenated buffer to ensure the viability of the tissue. Compounds of interest can then be applied topically and the perfusion buffer can be sampled and analysed. This allows the effect of the whole skin on absorption to be studied including metabolism, however the data are derived from an animal model and may have limited relevance to the absorption through human skin.

B) Full Thickness Skin Culture (Steinstrasser *et al*, 1995)

Excised skin discs (either whole skin or separated layers) are cultured in a suitable medium in an organ culture dish; the skin is kept epidermal side up and the surface of the skin remains above the level of the medium. The drug can then be applied either onto the surface of the skin or (more normally) added directly to the medium. The culture medium can then be sampled and analysed to detect the formation of metabolites. This method is useful for measurement of whole or partial skin metabolism but would not give useful data for absorption rates.

C) Diffusion Chambers Using Full and Partial Skin Preparations (Bronnaugh *et al*, 1989)

Whole or partial skin can be used with diffusion chambers to measure transdermal absorption rates. The diffusion chamber is maintained at body temperature and consists of a receptor and donator chamber with the skin mounted between them. The receptor chamber is filled with a physiological buffer solution designed to keep the skin viable and to mimic tissue tonicity. The donator chamber is either filled with a drug solution or the drug is applied in its normal vehicle directly to the skin surface. The receptor fluid is either continually circulated to represent blood flow, or static and stirred. Throughout the study, samples of the receptor fluid are taken and replaced with fresh medium. Analysis will give data on absorption rates and if the skin is kept viable also on skin metabolism. Full thickness skin is often used in these

studies, however this may not be ideal as the contribution of the dermis may not reflect the *in vivo* situation where compounds are taken up into blood vessels just below the epidermal-dermal junction.

D) Cell Culture

Epidermal cell cultures can be used to study metabolic activity, cells can either be obtained from whole skin by breaking it down enzymatically using either proteinase or trypsin or using a transformed cell line such as SVK 14. When using cells obtained from whole skin it is possible to separate them according to their state of differentiation, the separated cells are added to a PERCOL density gradient solution and centrifuged. The density of keratinocytes changes with the degree of keratinisation so the cell suspension forms several layers each containing cells from a different stage of development. Each layer can be individually studied to determine the level of activity contributed by different layers of the epidermis.

The level of differentiation in a cell culture can also controlled by modifying the level of calcium ions available to the cells. A keratinocyte cell culture containing less than 0.04 mM Ca^{2+} will produce cells that are relatively undifferentiated, but raising the concentration to 1.2mM Ca^{2+} can induce the cells to differentiate. The cells can then be separated via PERCOL centrifugation (Reiners *et al.*, 1990)

The use of isolated cells will give data on metabolic activity of the epidermis and by separating cells according to the level of differentiation will allow the relative contribution of the various layers to be estimated. However the degree of activity may not be representative of that observed *in vivo* for several reasons. After separation, cultured cells become less differentiated, leading to a modification of their activity. Possibly more importantly all the cells in suspension will be exposed to the same concentration of drug, however *in vivo* this may not be the case, the drug may not be capable of reaching all layers of cells at the same concentration which would lead to a possible overestimation of the contribution of particular layers.

E) Sub-cellular Fractions (Steinstrasser *et al*, 1995)

Separation of the microsomal and cystolic fractions of epidermal cells can be achieved by first homogenising and then ultracentrifuging the cell suspension. The supernatant contains the soluble cystolic enzymes whereas the resuspended pellet contains the microsomal fraction. The activity of both these fractions can be measured and compared to the activity of the whole homogenate. Although this is a useful method for determining the cellular site of activity and the range of substrates that can be modified by skin, it is not a true reflection of the *in vivo* situation. By disrupting the cell structure, enzymes may be exposed to different concentrations of compounds than they would normally encounter when present in whole skin.

1.4 Microdialysis

Microdialysis is an *in vivo* method for the continuous sampling and analysis of the contents of extra cellular fluid in a target tissue. It was first used to measure compounds in the cerebro-spinal fluid of dogs (Bito *et al*, 1966) but it has now been used in wide range of tissues both in animals and man and both diseased and healthy (Lonnroth,1987; Ben Nun, 1988; Lonnroth, 1991; Hamberger, 1989; Understedt, 1984). Recently the technique has been used in the skin to study both percutaneous absorption of topically applied compounds, endogenous compounds and inflammation (Anderson *et al*, 1991; Petersen *et al*, 1992).

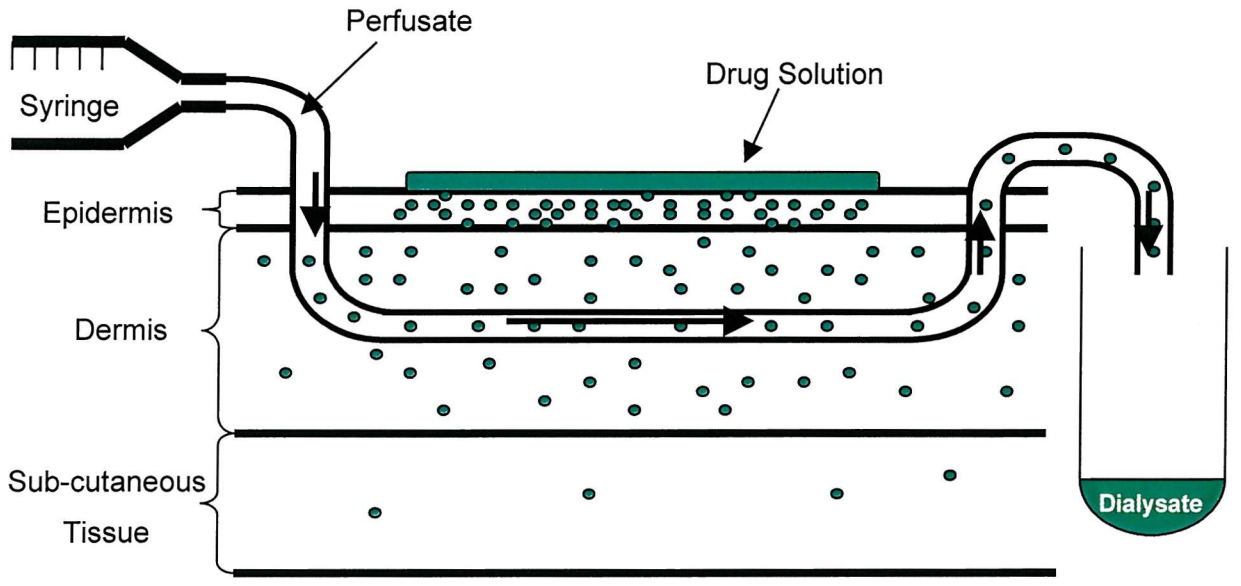

Figure 1.8: Illustration of *in vivo* microdialysis

1.4.1 Principle

Microdialysis works via passive diffusion. A semi-permeable tubular membrane is inserted into the dermis so that it runs parallel to the epidermis. The tubing both enters and exits the skin allowing liquid to be pumped through and collected and is continuously perfused with a physiological buffer solution at a flow rate normally within the range 1-10 μl/min. The probe is completely permeable to small molecules and water but because physiological buffer is used as a perfusate there is no net gain or loss of fluid from the tissue space. Low molecular weight compounds contained within the tissue space or the

perfusate will cross the membrane by passive diffusion according to the concentration gradient (Fick's law), see figure 1.8 and 1.9.

The speed at which equilibrium is reached is governed by several factors:

1) The physico-chemical properties of the compound such as the size and charge (Groth & Jorgensen, 1997).
2) The physico-chemical properties of the membrane such as the charge characteristics of the material, pore size and number of pores per unit area (Flynn,1989; Zhao *et al*, 1995; Amberg & Lindefors, 1989).

At the probe exit, the perfusate, which is now termed the dialysate, is collected and can be analysed. The concentration of the compound within the dialysate will be a reflection of the extra cellular fluid concentration but it will not be the actual concentration. This is because the equilibrium between the perfusate and the extra cellular fluid will usually be incomplete. As the perfusate has a set flow rate it will have a set residence time within the fibre; for example, for a 30 mm fibre with 200μm internal diameter the residence time will vary between 6 and 60 seconds assuming a flow rate between 1 and 10 μl/min. The perfusate will have insufficient time to reach full equilibrium. As the flow rate is constant the concentration in the dialysate ($C_{dialysate}$) will be a constant percentage of that in the tissue space (C_{tissue}). The ratio between the tissue concentration and the dialysate concentration is termed the relative recovery (see equation 4).

$$\text{Relative Recovery} = \frac{C_{dialysate} - C_{perfusate}}{C_{medium/tissue} - C_{perfusat}} \qquad \text{(eqn. 4)}$$

The relative recovery is difficult to determine *in vivo* and normally it is calculated *in vitro* by passing the microdialysis probe through a solution of the compound of interest, analysis of the perfusate, dialysate and bath solution will allow the accurate determination of the relative recovery. Studies have

shown that relative recovery is dependant on some factors but independent of others.

A) Factors influencing relative recovery

Flow rate

The relative recovery is inversely proportional to the flow rate, the higher the flow rate the shorter the residence time within the fibre, therefore less time there is for equilibrium to be reached

Temperature

Diffusion rates are increased at higher temperatures leading to a more rapid establishment of equilibrium and therefore a greater relative recovery.

Compound

The rate of diffusion can be greatly influenced by the physico-chemical properties of the target compound. The relative recovery of the compound is inversely proportional to the molecular weight i.e. the smaller the compound the greater the diffusion rate (Benfeldt, 1999). The structure of the compound will also influence the diffusion rate, the most favourable structure being a globular configuration: any divergence from a spherical form will reduce the rate of diffusion (Benfeldt, 1999). The lipophilicity and the ionisation of the compound will affect the recovery, as both will alter the affinity of the compound for the aqueous perfusate. The extent to which a compound is protein bound within the tissue space will alter the relative recovery; if the compound is highly protein bound and the probe in use does not allow the dialysis of protein then the relative recovery will be low, as only free drug will enter the dialysate.

Membrane

The dialysis probe can have a major influence on the overall relative recovery. The relative recovery will be proportional to the length of the probe for two

reasons. Firstly the residence time of the perfusate within the probe will increase with length and secondly the increased surface area results in a greater number of pores for the compound to diffuse through. A number of different types of dialysis fibre can be used to create a microdialysis probe and they all have different pore and material characteristics. The pore size and distribution vary between the fibre type used: the pore size can vary from 2kDa to 3MDa (Zhao *et al*, 1995) i.e. the smallest pore will allow a 2000 molecular weight compound through and the largest will allow a 3 million molecular weight compound through which would include small proteins, see figure 1.9.

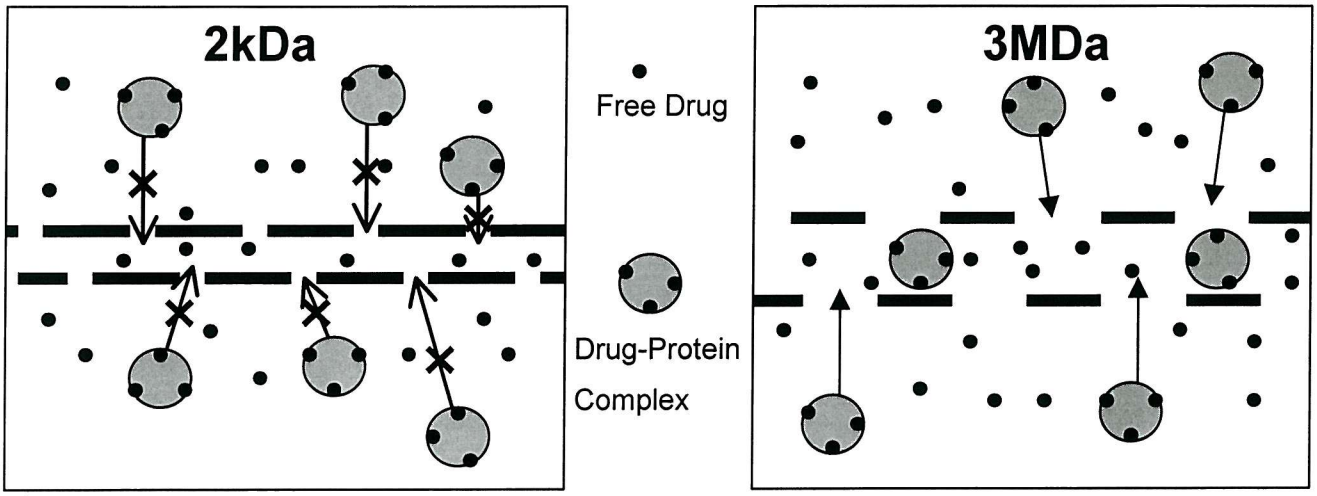

Figure 1.9: Comparison of 2kDa and 3MDa fibre material in relation to protein bound drug.

The larger pored fibres will generally have a higher relative recovery as diffusion is made easier. Possibly more important is the pore size distribution for each fibre; the molecular weight cut-off rating refers to the largest pore size for that fibre and is known as the cut-off point. Due to the construction method the pore size distribution follows an exponential curve with the number of pores increasing as the size decreases. Therefore two fibres with the same cut-off point may have different relative recoveries if the pore distribution varies, the fibre with the greater number of pores larger then the test compound will have the highest relative recovery. The material of the fibre may also influence the diffusion rate; if the material surface contains charged groups it may repel or attract the compound of interest, in the extreme case the compound may become bound to the surface of the fibre.

B) Factors not influencing relative recovery

Compound Concentration

The relative recovery is independent of the concentration gradient.

Blood flow

Blood flow is a major factor in the concentration of drug within the dermis however it does not affect the relative recovery. The blood vessels in the upper layers of the skin are in effect competing with the microdialysis probe to remove the compound of interest from the tissue space and the probe can be considered to act as an artificial blood vessel. With a high skin blood flow there will be a reduction in the tissue concentration as the compound enters the systemic circulation however the relative recovery of the probe in relation to the tissue concentration remains constant.

Probe depth

Like blood flow the probe depth may have a marked effect on the concentration of the compound surrounding the probe but not on the relative recovery. The deeper the probe is inserted into the skin the less drug will penetrate to the tissue surrounding it due to removal by the vasculature, metabolism and dilution in a larger tissue space.

1.4.2 Advantages and disadvantages of microdialysis.

A) Advantages

i. Samples a defined compartment (the extra cellular fluid) rather than entire tissue space as in the case of skin biopsies or the systemic circulation as in blood and excreta sampling.
ii. It can be performed at most anatomical sites and in nearly all tissue types.
iii. It causes minimal damage to the tissue and is less invasive than techniques such as biopsy or skin stripping
iv. Sampling can be continued for extended periods
v. Several probes can be used simultaneously reducing the number of volunteers required and allowing inter- and intra-subject variability to be determined.
vi. Can be used to deliver drug directly to the tissue space or measure tissue concentration after topical application.
vii. Probes can be selected that exclude or allow protein-bound drug to be measured.

B) Disadvantages

i. The low concentrations involved require a high analytical sensitivity.
ii. Highly lipophilic or protein bound compounds are difficult to detect.
iii. Absolute values for absorption are difficult to calculate
iv. Volunteers are required to remain stationary for the duration of the study.

1.5 Drugs Under Study

1.5.1 Methyl salicylate

Methyl salicylate is an antipyretic analgesic that was initially obtained by the distillation of the leaves of *Gaultheria procumbens. Gaultheria procumbens* is a low growing evergreen plant native to the northern Americas; methyl salicylate obtained from this plant is often referred to as oil of wintergreen. Methyl salicylate is an irritant and as such is not used orally but topically for pain relief, either for muscle pain or joint pain. It is available in variety of formulations, which can contain between 10-30% by volume. Its structure is shown below, it has a molecular weight of 152 and it is a colourless liquid with a strong odour. It is only sparingly soluble in water but is miscible in a wide range of organic solvents. It has a P_{ow} value of 3, indicating that it is a highly lipophilic compound. In the body methyl salicylate is rapidly metabolised to salicylic acid by the hydrolysis of the ester group, see figure 1.10.

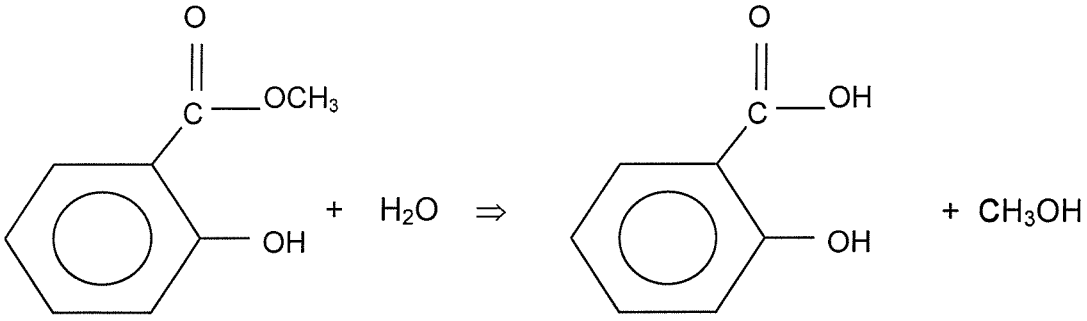

Figure 1.10: Ester hydrolysis of methyl salicylate

Several percutaneous absorption studies have been carried out using methyl salicylate as a model substrate.

A study by Behrendt *et al.* (1989) used an isolated perfused rabbit ear to demonstrate ester cleavage of methyl salicylate. The rate of ester hydrolysis after topical application was 25 times greater than after arterial administration, indicating the presence of esterases within the epidermal layer.

Another study by Megwa *et al.* (1995) examined the depth of penetration of some commercially available salicylates, which included methyl salicylate, in

rat skin. Rat plasma and tissue samples were analysed for both methyl salicylate and its primary metabolite salicylic acid. The study reported that salicylic acid was present in the uppermost layers of the skin and the methyl salicylate:salicylic acid ratio increased in deeper tissue indicating that metabolism to salicylic acid reduced with increasing tissue depth, which is difficult to explain.

Using renal excretion, Roberts *et al* (1982) showed that methyl salicylate is readily absorbed by human skin *in vivo*. It was also shown that the topical availability varied according to anatomical site of application, the rank was abdomen>forearm>instep>heel>plantar. The decrease in availability follows an increase in the relative thickness of the stratum corneum.

In summary, methyl salicylate is readily absorbed by human skin where it undergoes ester hydrolysis to form salicylic acid.

1.5.2 Ketoprofen

Ketoprofen is non-steroidal anti-inflamatory drug with analgesic and antipyretic properties. In anti-inflammatory studies ketoprofen has inhibitory affects on prostaglandin and leukotriene synthesis as well as anti-bradykinin activity. However its mode of action is not fully understood.

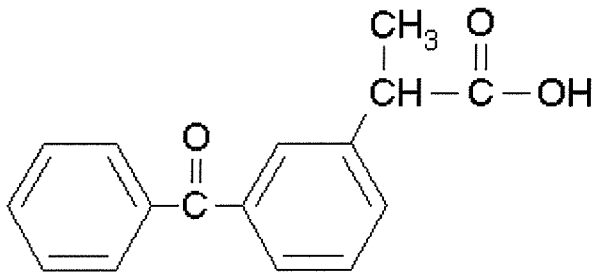

Figure 1.11: Structure of ketoprofen

The chemical name for ketoprofen is 2-(3-benzoylphenyl)-propionic acid and has a molecular weight of 254, see figure 1.11. It has a P_{ow} value of 3, indicating that it is a highly lipophilic compound. It is freely soluble in most organic solvents but practically insoluble in water. Ketoprofen is a racemate

with only the S enantiomer possessing pharmacological action. Blood samples after oral administration show that ketoprofen is >99% bound to plasma protein, mainly albumin, and has a t ½ of approximately 2 hours. Ketoprofen is primarily metabolised to the glucuronide conjugate to form an unstable acyl-glucuronide, which is excreted in the urine.

Several studies have been carried out looking at the topical usage of ketoprofen. One study compared the plasma concentration of ketoprofen after intra-muscular injection and topical application using microdialysis (Tegeder *et al*, 2001). After topical dosing most of the dialysate sample concentrations were below the limit of detection and only reached a plateau of 7-40 ng/ml after 10-12 hours. After an intra-muscular injection of an identical dose to the topical application the plasma concentration was approximately 10 times higher. Cyclooxygenase inhibiting concentrations of ketoprofen were only achieved with intra-muscular injections and not topical dosing which questions the reported clinical efficacy of topical ketoprofen.

Another study examined the effect of four terpene enhancers and ethanol concentration on the percutaneous permeation of ketoprofen through hairless mouse skin *in vitro*. Increasing the ethanol concentration of the vehicle was found to result in increased permeation of ketoprofen. All four terpenes were found to increase permeation of ketoprofen compared to terpene free controls with the terpene limonene having the greatest effect (El-Kattan *et al*, 2000).

Topical and peroral ketoprofen was studied by Steen *et al* (2001) for their clinical efficacy to reduce muscle pain. Muscle pain was induced by low pH intra-muscular infusions followed by topical, oral or peroral dosing of ketoprofen. Peroral ketoprofen resulted in the largest pain reduction followed by oral ketoprofen. Topical ketoprofen did not give pain reduction of the same magnitude but was faster to develop. Overall topical gel based ketoprofen was not found to provide long lasting analgesia compared to perorally dosed ketoprofen.

1.5.3 Fenbufen and Biphenylacetic acid

Fenbufen is the pro-drug form of the drug biphenylacetic acid both are used as an analgesic and for their anti-pyretic action, see fig 1.12. Fenbufen is pharmacologically inactive and requires metabolism via β-oxidation to biphenylacetic acid to gain activity (Kohler *et al*, 1980; Dollery, 1999). Fenbufen is used orally instead of biphenylacetic acid as it has a lower ulcerogenic potential and is better absorbed from the gastro-intestinal tract (Kerwar, 1983). However topically only biphenylacetic acid is used as it is better absorbed and there is no evidence that the skin is capable of metabolising fenbufen.

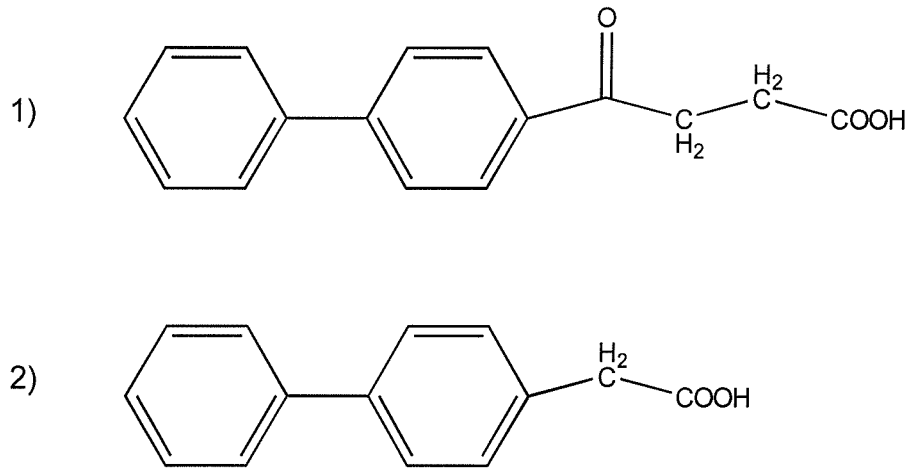

Figure 1.12: Chemical structures of 1) fenbufen and 2) biphenylacetic acid.

Both fenbufen and biphenylacetic acid are highly protein bound ≈99% and are both highly lipophillic, fenbufen and biphenylacetic acid have a P_{ow} value of 3.5 and 3 respectively (Martindale, 1996). Fenbufen and biphenylacetic acid have molecular weights of 254 and 212 respectively, the pK_a of both compounds is between 5 and 6. Fenbufen is practically insoluble in water and sparingly soluble in ethanol, biphenylacetic acid is slightly more soluble in water and readily soluble in ethanol

Topically biphenylacetic acid is applied as a 3% gel or foam (30 mg/ml) for the symptomatic treatment of soft tissue injuries and is available under the proprietary name of Traxam.

Orally fenbufen is prescribed at 900mg daily for the relief of pain and inflammation associated with musculoskeletal and joint disorders and is available under the proprietary name of Lederfen. Fenbufen is rapidly absorbed from the gastrointestinal tract and approximately 80% is absorbed in total (Chiccarelli *et al*, 1980). The T_{max} for fenbufen and biphenylacetic acid are approximately 1.2 and 7.5 hours respectively. The synovial fluid can reach approximately 2/3 of the corresponding plasma levels (Dawson *et al*, 1988).

The active metabolite biphenylacetic acid inhibits cyclooxygenase activity with a reduction in the tissue production of prostaglandins such as $PGF_{2\alpha}$ and PGE_2 (Kerwar, 1983).

A number of studies have been carried out looking at the topical application of biphenylacetic acid however the majority of them are purely clinical only studying and measuring the effectiveness of the drug at treating the symptoms of various conditions. All of these studies found that biphenylacetic acid compared to a placebo control was effective at reducing inflammation and alleviating pain (Leeb, 1994; Lee *et al*, 1991).

One study has looked at the disposition of biphenylacetic acid after topical application. Biphenylacetic acid gel (3%) was applied to one knee of patients suffering arthritis. It was found that the peak concentration in the synovial fluid of the applied knee reached almost 2/3 of the peak plasma concentration. However the synovial fluid from the ipsilateral knees had concentrations that were not significantly different suggesting that there was no direct absorption into the synovial space but was initially into the systemic circulation and subsequently into the synovium (Dawson *et al*, 1988).

1.6 Aims and Objectives

Cutaneous microdialysis is a technique that has been mostly used to qualitatively study physiological processes and detect endogenous compounds. Only relatively recently has any attempt been made to use it as a quantitative tool for the measurement of topically applied drugs. To support the quantitative use of cutaneous microdialysis this thesis has the following aims-

- To investigate the potential of microdialysis to follow the absorption profile of topically applied compounds and to determine an experimental protocol which will maximise tissue concentration and prolong penetration.
- To investigate the potential of microdialysis to detect and measure the cutaneous metabolism of topically applied compounds without interference from hepatic/systemic metabolism.
- To attempt to determine the contribution of distinct skin layers to cutaneous metabolism.
- To study the effect of vehicle formulation on topical absorption
- To study how differences in physico-chemical properties affect the topical absorption of structurally similar compounds.
- To detect and measure the level of protein binding in the skin and determine its effect topical absorption and cutaneous tissue concentration.

The drugs selected for studying cutaneous microdialysis were all non-steroidal anti-inflammatories (NSAID's) for the following reasons-

- There are a large number of topically used NSAID's available for study.
- They are generally well tolerated topically and are unlikely to cause adverse affects.

- A wide range of physico-chemical properties are covered by the different NSAID's available.
- There are published methods of analysis for all commercially available NSAID's reducing the time required for method development.
- There are many NSAID's available that are structurally similar.

The results of these studies should provide an indication of the strengths and weaknesses of cutaneous microdialysis.

2 Materials and Methods

2.1 Materials

2.1.1 HPLC System:

Grant SUB14 Water Bath (Grant Instruments Ltd, Herts, UK)

Waters Millipore 490 Programmable Multiwavelength Detector

Waters Millipore 717 plus Autosampler

Waters Millipore Automated Gradient Controller

Waters Millipore Pump model 510

Waters Millipore 745 Data Module (Millipore (UK), Waters Chromatography Division, Herts,UK)

2.1.2 Solvents:

Acetic Acid 100% (BDH, Poole, UK)

Acetonitrile (BDH, Poole, UK)

Diethyl Ether 100% (BDH, Poole, UK)

Double Distilled Water (Produced on site)

Ethanol 100% (BDH, Poole, UK)

Formic Acid 99% (Sigma-Aldrich, Poole, UK)

Methanol (BDH, Poole, UK)

Perchloric Acid 66% (BDH, Poole, UK)

Propylene Glycol (Sigma-Aldrich, Poole, UK)

2.1.3 Compounds:

Ammonium Acetate (Sigma-Aldrich, Poole, UK)

Biphenylacetic acid (Sigma-Aldrich, Poole, UK)

Carboxy-methylcellulose (Sigma-Aldrich, Poole, UK)

Fenbufen (Sigma-Aldrich, Poole, UK)

Ketoprofen (Sigma-Aldrich, Poole, UK)

Methyl Salicylate >99% pure (Sigma-Aldrich, Poole, UK)

Methyl-2-methoxybenzoate (Sigma-Aldrich, Poole, UK)

Salicylic Acid (Sigma-Aldrich, Poole, UK)

2.1.4 Equipment used in *in vitro / in vivo* studies:

0.6ml Eppendorf vials (Life Science (UK) International, UK)

21G x 1 ¼ inch Sterile Disposable Needles (Terumo Europe NV, Leuven, Belgium)

23G x 1 ¼ inch Sterile Disposable Needles (Terumo Europe NV, Leuven, Belgium)

2kDa Renal Dialysis Cartridge Fibres (Gambro Gmbh, Germany)

3MDa Plasmoflo Asahi Plasma Separator Fibres, OP-02 (Asahi Medical Co Ltd, Tokyo, Japan)

Autoclavable Nylon Tubing (Portex Ltd, Kent, UK)

Comfeel Plus Ulcer Dressing (Coloplast Ltd, Cambs, UK)

Disposable Petri Dishes 9cm (Bibby Sterilin Ltd, Staffs., UK)

EMLA Cream 5% (Astra Pharmaceuticals Ltd, Herts, UK)

Human Albumin 4.5% Solution Ph. Eur. (Bio Products Lab, Herts, UK)

Hyperfix Medical Tape (Smith & Nephew Ltd, Hull, UK)

Loctite Superglue (Henkel Home Improvement & Adhesive Products, Cheshire, UK)

MAB 20 Peristaltic Pump (MAB, Sweden)

Noradrenaline Base 1mg/ml, 4ml ampoules (Abbot Laboratories Ltd, Kent, UK)

Oruvail Gel, 2.5% Ketoprofen (May & Baker, Dagenham, UK)

IVAC P3000 Syringe Drivers (Alaris Medical UK Ltd, Hants, UK)

Ringer's solution (Fresenius)

Silicon Sealant (Unibond, Henkel Home Improvement & Adhesive Products, Cheshire, UK)

Sterile 10ml Syringes (Terumo Europe NV, Leuven, Belgium)

IVAC Tubing: Syringe Extension Tubing (Alaris Medical UK Ltd, Hants, UK)

Tygon Tubing (Cole Palmer, Illinois, USA)

Dermascan 3-D ultrasound scanner (Dermascan, Denmark)

Coomassie protein assay kit (Coomassie, USA)

2.2 Methods

2.2.1 Solutions

2.2.1.1 Perfusion Solution

The perfusion solution used throughout all experiments was Ringer's solution (155 mM sodium chloride). This was used neat in all *in vitro* experiments and either neat or with noradrenaline present at a concentration of 5μg/ml for *in vivo* experiments. As sterility is the overriding concern in any invasive *in vivo* technique great care was taken when preparing the solutions. Sterile Ringer's solution was taken directly from a sterile drip bag. The perfusate containing noradrenaline was prepared by making a serial dilution from a 1mg/ml stock solution of noradrenaline taken from a sterile 4ml ampoule using sterilised needles and syringes.

2.2.1.2 Standard Solutions used in standard curves for sample analysis

Ten mg/ml stock solutions of methyl salicylate and salicylic acid were prepared in methanol. A 10mg/ml stock solution of ketoprofen, fenbufen and biphenyl acetic acid was prepared in acetonitrile. The working concentration of all the compounds was 5μg/ml, which was achieved by diluting the stock using Ringer's solution. The standard curves were produced by diluting the working solutions with Ringer's solution to give 5, 4, 3, 2, 1, 0.5, 0.25 μg/ml.

2.2.1.3 Topical Preparations

The methyl salicylate formulation used in the *in vivo* studies had a base consisting of 50% propylene glycol and 50% double distilled water. To obtain the final preparation 1ml of methyl salicylate was added to 8mls of the base and agitated for 24 hours at room temperature to allow it to reach saturation. The resulting mixture was centrifuged to separate any undissolved methyl salicylate, which was removed. The final solution contained between 7-9% methyl salicylate and was stored at 4°C until required.

A formulation with a base of 50% ethanol and 50% double distilled water was also used in some studies, and this was thickened using carboxymethyl-cellulose (medium viscosity), which was added to give a final concentration of 1.5%. The carboxymethyl-cellulose was premixed with water and allowed to

form a gel over night, this was then mixed with ethanol which contained 2%$^{w}/_{v}$ methyl salicylate, (giving a final concentration of 1%).

A prescription only formulation of ketoprofen was used throughout the *in vivo* studies; it contained 2.5 % w/v ketoprofen.

The fenbufen and BPAA studies used the same ethanol/water formulation used in some of the methyl salicylate studies. Due to solubility problems achieving a 1% solution of both drugs proved possible only when the pH of the final formulation was kept at 7 by addition of sodium hydroxide.

For all topical preparations the compounds were found to be stable at room temperature for at least a period equal to the duration of the studies performed.

2.2.2 Microdialysis Probe Preparation

2.2.2.1 2kDa Probes and 5kDa probes

The microdialysis probes consist of a semi-permeable fibre attached to a short length of nylon tubing; the probes were made rather than purchased, due to the high cost of the commercial brands. The 2kDa fibres were made from Cuprophan® having an internal diameter of 200 μm, a wall thickness of 8μm and a 2000 daltons molecular weight cut-off. The 5kDa fibres had the same dimensions but were made from a material called Hemophan® and had a molecular weight cut-off of 5000 daltons. The fibres were acquired from a renal dialysis cartridge; both ends of the cartridge were sawn off with a hacksaw and the fibres carefully removed. Once the fibres were removed they were kept in a clean, dust free environment. Before probe manufacture, the tips of the fibres were removed using a scalpel because the ends of the fibres were usually closed or blocked as a result of removing them from the cartridge. The fibres were inserted for a distance of approximately 2-3cm into a 10 cm length of nylon tubing (inner diameter 0.58mm, outer diameter 1.02mm) and glued in-place using a commercial brand of glue that contained methylacrylate. Once the glue had set the probes were placed in autoclavable bags in batches of eight and sent for sterilisation at Cardiff General hospital. As the fibres were unlikely to survive high temperatures they were sterilised using ethylene oxide which has been shown not to alter the probe properties (Hegeman *et al*, 1995).

The fibres themselves are manufactured from individual threads of the relevant trademarked polymer (Cuprophan or Hemophan). Thousands of these individual threads are spun together so that they interlock and form a hollow fibre, in sense they form a hollow piece of rope. The pores through which dialysis occurs are merely the gaps between the individual threads. The nature of the production means that pore size cannot be regulated and a whole range of pore sizes are produced. However by controlling the production parameters the fibres can be limited to a maximum pore size i.e. the manufacturer will guarantee that all pores will exclude molecules above a certain molecular weight. The 2kDa and 5kDa fibres will only allow molecules below 2000 and 5000 daltons respectively to freely diffuse through the membrane. The pore size distribution is also heavily influenced by its method

of manufacture. Due to the essentially random nature of the pore size, distribution follows an exponential curve with the number of pores decreasing as pore size increases. Therefore at the maximum pore size or cut-off point the number of pores is extremely small. This means that the ability of the fibre to dialyse molecules is reduced as the molecular weight increases.

2.2.2.2 3MDa Probe

The fibres used for the 3MDa probes were taken from a plasma phoresis cartridge; these cartridges are used to manufacture pure plasma from whole blood by filtering out blood cells and large protein complexes. The fibres have an inner diameter of 400μm, a wall thickness of 10μm and a 3 million dalton molecular weight cut-off. The fibres were removed as for the 2 KDA fibres and also had to have their tips trimmed. The fibres were fragile and became easily kinked; to prevent this they were strengthened by the insertion of a stainless steel wire along their entire length. Because of the increased external diameter of the 3KDa fibres the nylon tubing could not be used. The strengthened fibre was inserted into a short length of flexible tubing (tygon tubing, I.D= 380μm, O.D.= 2200μm), which gripped the fibre tight enough to remove the need for glue. To prevent leakage, the fibre-tubing junction was sealed using a proprietary silicon based sealant. The finished probes were sterilised using ethylene oxide, as described above. The method of manufacture is not known but the pore size distribution follows the same trend as the 2kDa and 5kDa fibres, as pore size increases the number of pores decreases.

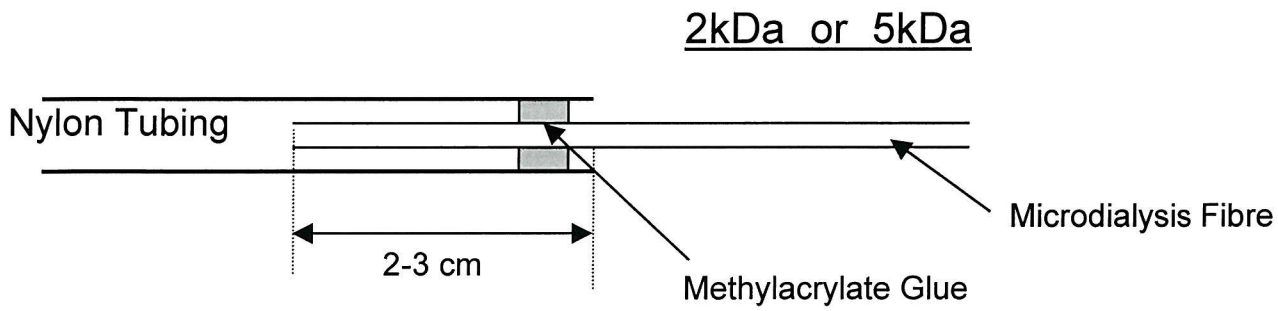

Fig 2.1 Construction of 2kDa and 5kDa fibres

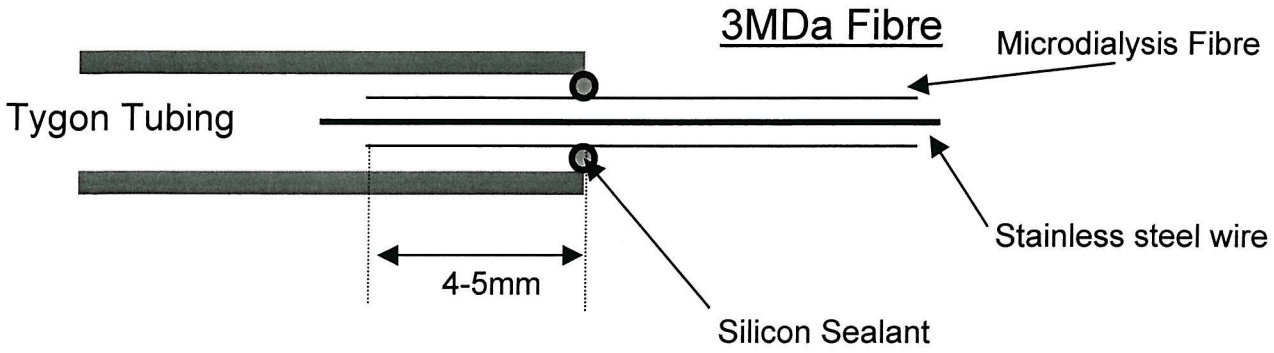

Fig 2.1a Construction of 3MDa fibres

2.2.3 *In vitro* Studies

2.2.3.1 Experiments with 2kDa and 5kDa fibres

The dialysis probes were mounted in standard 9 cm disposable petri dishes, one per dish by making small holes in the side of the dish, and connected via a length of IVAC tubing to 10 ml disposable syringes. The probes were positioned so that only a 3 cm length of fibre was exposed inside the petri dish and the fibre ends were allowed to feed into 600 μl eppendorf vials to collect the dialysate samples. Syringes were mounted in IVAC syringe drivers, which are designed to depress the plungers at a constant rate, which can be adjusted to give a flow rate between 0-500 μl/min in increments of 1.67 μl/min.

The fibres were perfused for one hour to give a blank dialysate sample prior to filling the petri dishes with 40mls of bath solution. After addition of the bath solution the pumps were run for a further 4 hours with collections taken hourly. All the *in vitro* experiments carried out were for the determination of recovery from the medium, therefore it was always the bath solution that contained the compound of interest and not the dialysate. The bath solution consisted of Ringer's solution plus the drug under study, occasionally protein was added to observe any effect on recovery The recovery was calculated as the ratio between the concentration in the dialysate and the concentration of the solution added to the petri dish. Samples of the bath solution were taken at the beginning and end of the experiments.

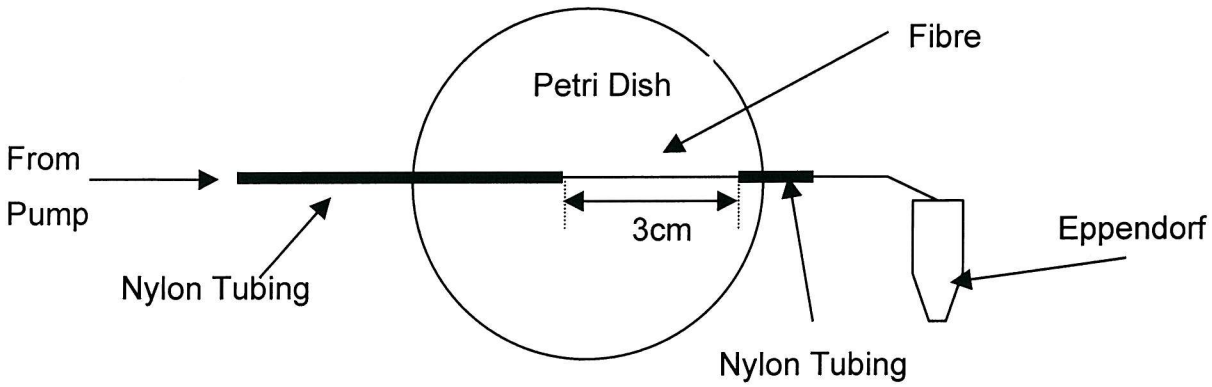

Fig 2.2 Construction of *in vitro* probe used to study relative recovery for both 2 and 5 KDa fibres

The parameters studied were

Concentration:

The fibres were perfused with Ringer's solution and the bath solutions contained differing concentrations of drug.

Flow Rate:

The fibres were perfused with Ringer's solution at differing flow rates while the bath solutions all contained the same concentration.

Temperature:

Most studies were performed at room temperature. Experiments were also carried out in a high temperature room kept at a constant 32°C.

Protein:

The effect of adding human albumin in the bath solution and/or in the perfusate was studied.

Fibre:

Two fibre types were compared, the original manufactured by Gambro from a material called Cupraphan with a 2kDa molecular weight cut off, 200 μm internal diameter and a 8μm wall thickness against a similar fibre made by Fresenius from Hemophan which has identical dimensions but a higher molecular weight cut-off of 5 kDa.

Wires:

In later experiments, thin wires were inserted down the lumen of the fibres before the probes were made and the results compared to data derived using the non-wired fibres.

2.2.3.2-Experiments using single 3MDa probes

The 3MDa probes used in *in vitro* experiments were constructed in a slightly different fashion to those used *in vivo*. Both ends of the 3MDa fibre were inserted into lengths of flexible tubing so that a 3cm span was left exposed then both joins were sealed using silicon sealant. The probe was then mounted in a 9cm disposable petri dish prior to use. The difference in construction was necessary because of the need to use a peristaltic pump to create the perfusate flow. Initial attempts used an *in vivo* fibre (see figure 2.1)

connected to a syringe mounted on a syringe driver similar to that for the 2kDa experiments. However the volumes collected in the hourly samples varied considerably and could be less or greater than the volume of perfusate delivered. The variation was a result of siphoning because the large pore size and diameter of the 3MDa fibre posed little barrier to the free movement of water. If the collecting vial was slightly below the level of the petri dish the volume collected was higher than could be accounted for by the flow rate, if however the vial was slightly raised then the volume collected was reduced markedly. This difficulty was overcome by using a peristaltic pump, the pump was used to both push perfusate through the fibre and pull it through simultaneously at the same flow rate. The push/pull system generated no net pressure within the fibre so that there would be no net flux of water either into or out of the bath solution. This method gave consistent and realistic sample volumes. Once the 3MDa probe was attached to the push/pull pump the procedure followed was the same as that used with the 2kDa probes. The fibres were perfused for an hour prior to the bath solution being added, following which samples were collected hourly.

2.2.3.3 Dual Probe Experiments

Experiments were also carried out where both a 2kDa and a 3MDa probe were mounted in the same petri dish and both perfused using the push/pull technique. This required that the 2kDa probes be modified; existing probes were glued to a second length of nylon tubing so that only 3cm was exposed before being mounted in a petri dish. Apart from this, the protocol was the same as with the single 3MDa probe.

2.2.3.4 Relative Recovery

Complete equilibrium between the dialysate and the extracellular fluid is almost never reached, this is mainly due to the residence time of the perfusate within the fibre, which is normally between 6 and 60 seconds depending on the flow rate. Relative recovery as described in the introduction is the ratio of the sample or dialysate concentration in relation to the tissue concentration. Throughout all the studies the relative recoveries of each drug used were calculated *in vitro* under different microdialysis conditions.

Initially the average bath concentration was calculated using the initial and final bath sample concentrations. The relative recovery is simply the sample concentration as a percentage of the average bath concentration. The relative recovery (RR) was calculated for each time point of the *in vitro* experiments, the RR's for all the time points for an individual fibre were then averaged. To get a final RR for a particular set of experimental conditions, the RR of 4 or more different lines were averaged.

The RR calculated in this way represents an ideal recovery i.e. diffusion of drug is not impeded, bath concentration remains essentially unchanged and there is little in the bath solution to cause pore blockage. *In vivo* the situation is not ideal, the tissue in which the fibre resides is viscous and the drug is not free to diffuse as in an aqueous solution (Fettweis & Borlak, 1996) and there is a high concentration of protein, which may adhere to the fibre surface and cause pore blockage. Therefore *in vivo* the RR will always be less than the measured RR *in vitro*. If *in vivo* data is corrected using *in vitro* RR to calculate actual tissue concentration, the value calculated will be a minimum tissue concentration.

2.2.4 *In vivo*

The *in vivo* experiments were all performed on healthy adult human volunteers. The experiments were approved by Southampton and South West Hampshire Health Authority Joint Research Ethics Committee and legally covered by University insurance. All volunteers were questioned as to any medication they were taking, had the study fully explained to them and gave written consent before the study took place. The volunteers were requested to refrain from drinking alcohol or beverages containing caffeine the evening before and on the morning of the study, because of concerns that this may affect skin blood flow.

A minimum of one hour before the start of the study the volunteers applied EMLA cream, a local anaesthetic cream, to six areas of skin along their forearms see figures 2.3 and 2.4 below. The areas were at least 3cm across, were equally spaced out along the entire length of the forearm and were occluded; the volunteers carried out this procedure before arriving at the hospital. Once in the investigation room the volunteers were asked to lie on a standard hospital bed where they remained for the duration of the study.

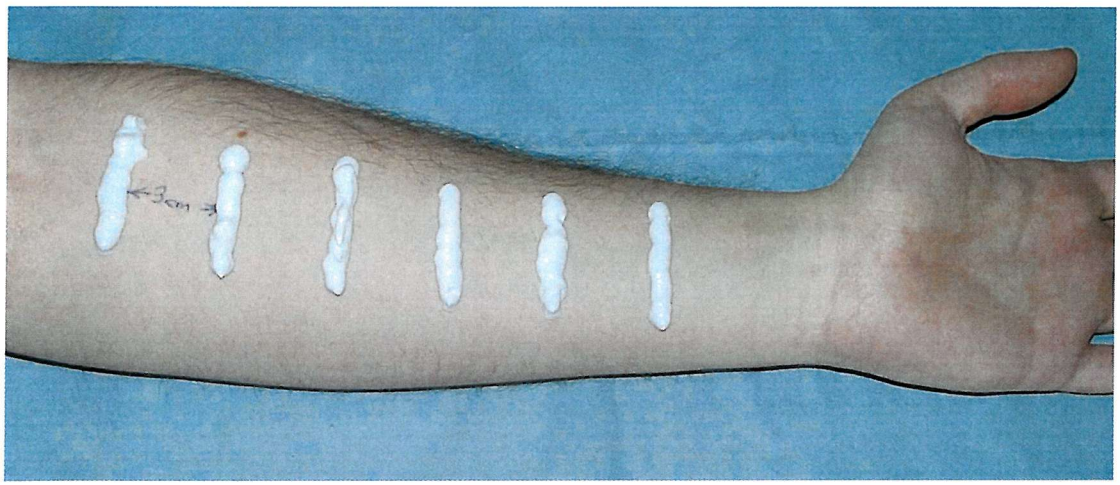

Fig. 2.3: Forearm of volunteer with EMLA applied in six equally placed strips. Distance between strips was 3cms.

The occlusive dressing and the EMLA were carefully removed from one of the patches. The skin was lightly pricked using a sterile needle to check the effect of the anaesthetic, and if there was no pain sensation needle insertion would

proceed. Any sensation of pain meant that further EMLA was applied and left for 30 minutes before being rechecked, see figure 2.5 below.

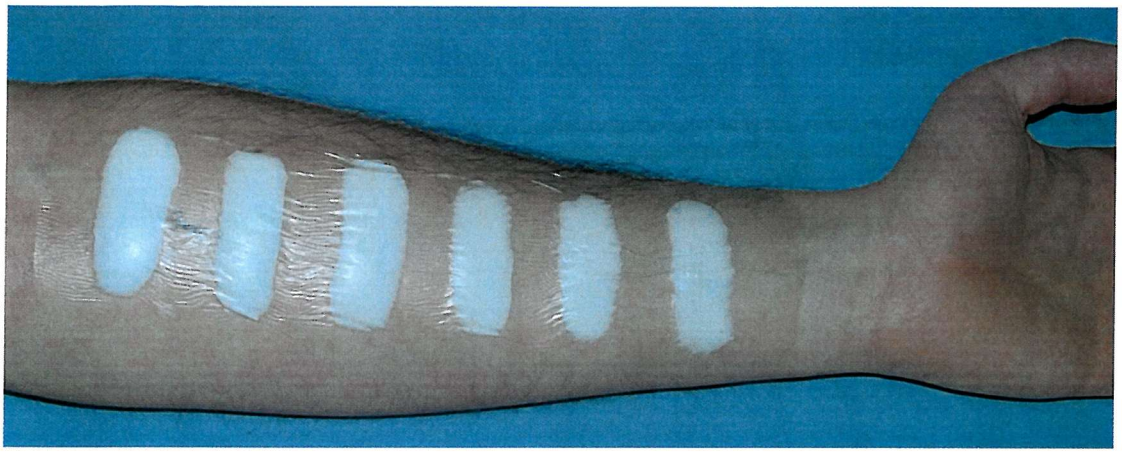

Fig. 2.4: Volunteers forearm with equally spaced strips of EMLA covered with Tagaderm patches.

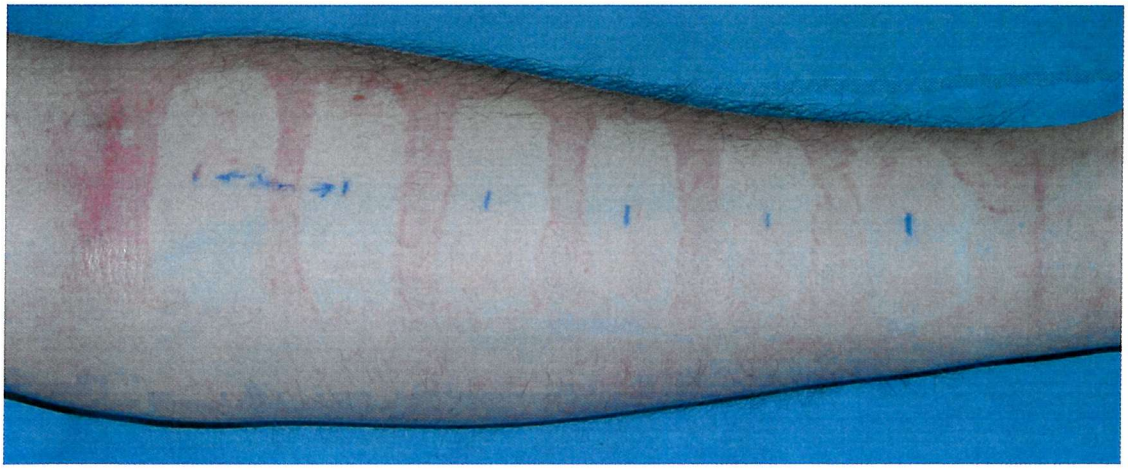

Fig 2.5: Volunteers arm after removal of EMLA. EMLA had been applied for 90 minutes, areas of EMLA application are clearly marked by vasoconstriction of the cutaneous blood flow.

2.2.4.1 2kDa and 5kDa Probe Insertion

A 23 gauge sterile needle was inserted through the surface of the skin into the dermis and then pushed intra-dermally parallel to the skin surface for a distance of 30 mm at which point the needle tip was allowed to emerge. If the needle tip broke the skin surface before the 30mm point the needle was removed and with the volunteers permission a new needle inserted in an area

adjacent to the original insertion. Under sterile conditions the fibre end of a 2kDa or 5kDa probe was inserted through the bevel end of the needle so that it protruded from the syringe fitting. The needle was then removed and the probe was taped to the skin and connected via IVAC tubing to a syringe filled with Ringer's solution mounted on a syringe driver. The probe was purged with Ringer's solution and if no leaks were detected the needle removed. The procedure was repeated until six probes had been inserted along the forearm, see figure 2.6.

For the majority of the studies the perfusate contained 5μg/ml noradrenaline which causes vasoconstriction in the peripheral circulation. When noradrenaline is pumped through the fibres it constantly diffuses into the surrounding tissue. The constant supply of noradrenaline causes vasoconstriction up to 5mm either side of the implanted fibre which lasts for the duration of perfusion. There are 2 reasons for the use of noradrenaline in the perfusate.

1. Reduce variability: Level of blood flow in skin varies between different subjects. By reducing the blood flow to a minimum in all volunteers variability will be kept to a minimum.
2. Increase tissue concentration: Blood vessels will continuously remove absorbed drug from the skin tissue therefore the measured tissue concentration may not necessarily reflect the total flux of drug across the stratum corneum. By reducing the blood flow to a minimum, tissue concentration will be more reflective the of the total drug flux across the skin.

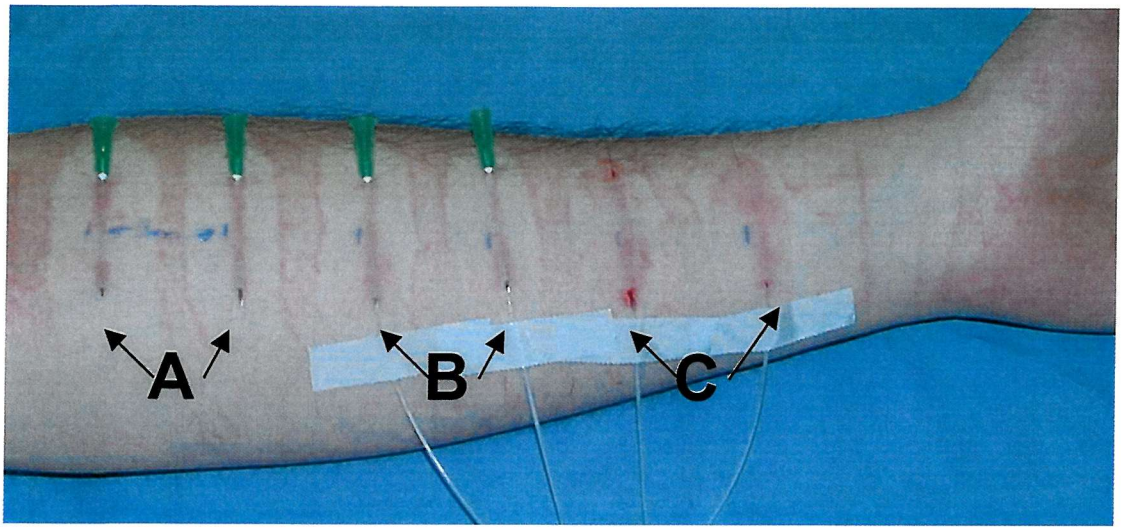

Fig.2.6: Volunteers arm showing stages of insertion. A) Needle insertion , intradermally at a depth of approximately 0.5mm for distance of 3cm. B) Fibre insertion, threaded through needle, taped to skin and perfused to check for leaks. C) Needle removal, microdialysis fibre left in situ.

2.2.4.2 3MDa Probe Insertion

3MDa probe insertion is essentially the same as described for the 2kDa probes, however because of increased diameter of the probe a larger needle had to be used, in this case a 21 gauge needle. The larger needle resulted in a small increase in bleeding at the entry and exit points, however as for the 23 gauge needles all bleeding stopped within 10 minutes.

The syringe drivers were set at the desired flow rate and then allowed to run continuously for the duration of the study. The protruding ends of the probes were placed into 600μl Eppendorf vials, which were taped onto the side of the forearm. The vials were changed at set intervals and immediately frozen at -20°C.

Probe insertion requires a syringe needle to be inserted through dermis parallel to the epidermis to act as a guide cannula for the microdialysis fibre. Once the fibre has been threaded through the needle it is removed leaving the probe in the dermal space. Inevitably needle insertion causes trauma to the tissue which is dependant on the needle type and possibly insertion depth (Clapp-Lilly *et al*, 1999), as microdialysis is normally used for the study of normal tissue a sufficient time must be left before the commencement of the study to allow the trauma to subside and for the cutaneous blood flow to

stabilize (Groth *et al*, 1998; Groth & Serrup, 1998). For human studies 60 minutes has been found to be long enough for the skin hyperaemia to return to baseline values (Groth & Serup, 1998).

Drug wells were fixed over the inserted fibres at least half an hour prior to drug application. Previous researchers made drug wells from adhesive pads used to affix ECG monitors. However they had noted that due to the stiffness of the plastic foam they were made off, there was a tendency for the drug well not to follow the curve of the forearm. Practically this meant that quite regularly the drug wells lifted away from the skin at the edges allowing the applied compound to contaminate the samples. Several alternative materials were found, the most promising of which was a product called Comfeel Plus. This is an adhesive backed sheet of methyl cellulose used with patients suffering from bed sores or who have colostomy bags to prevent rubbing. The sheets were far more flexible than the ECG pads and appeared to have a stronger adhesive. To test the Comfeel Plus sheets, several sets of drug wells were made from both materials. The two sets of material were cut into squares 3 x 2.5 cm with an opening 2 x 0.5 cm in the middle see figure 2.7 below. Several sets were positioned onto the researcher's forearm and into the central hole a 0.1ml solution of 50% propylene glycol and 50% water was pipetted. The solution was spiked with a non-toxic dye that showed up well on skin. The drug wells were then covered with an occlusive cover made from the same material as the drug well and left for 5 hours. When the wells were removed it was clear that very little of the dye solution had crept under the Comfeel Plus drug wells. However around the ECG monitor wells there was a large area of dye on the surrounding skin and several of the wells had lifted up allowing dye solution to run freely across the skin. It was decided to use Comfeel Plus drug wells for all studies.

After the pre-dose collection, 0.1ml of the drug preparation was syringed into the drug wells so that it evenly coated the exposed skin. In most studies a patch of methyl cellulose sheet was applied immediately to the drug well in order to occlude the application site. The study continued until at least 5 hours worth of samples had been collected. At the end of the study each line was scanned using ultrasound to measure the probe depths.

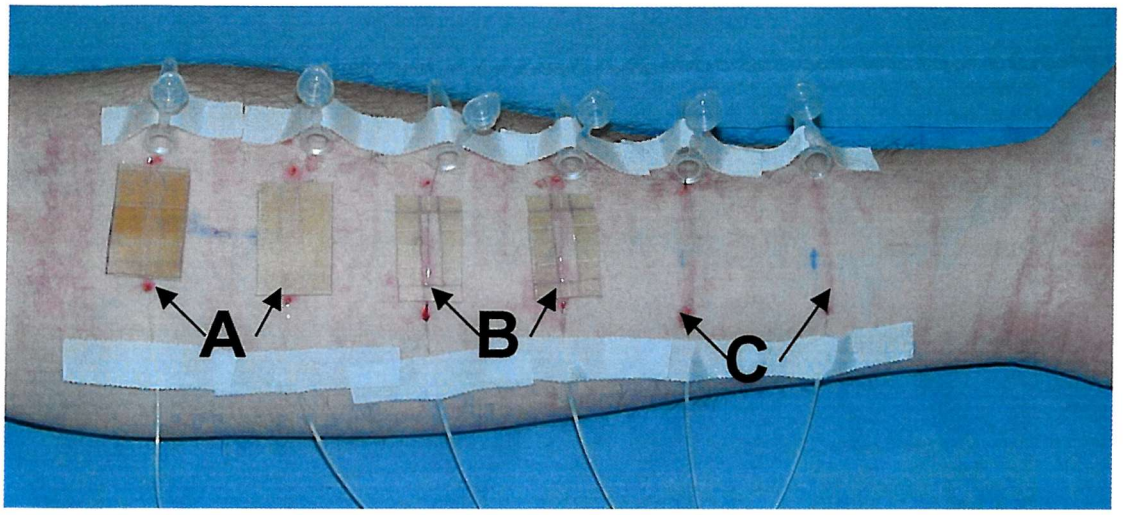

Fig. 2.7: Volunteers forearm, six microdialysis lines inserted and fed into collecting vials, drug wells in various stages of being applied. A) Drug wells with occlusive cover. B) Drug wells open. C) No drug wells.

2.2.4.3 Dual Probe

Several studies were performed in which the drug under examination was introduced directly to the dermis via a microdialysis fibre so that movement through and metabolism by the dermis could be studied.

This required two parallel probes to be inserted under the skin with very limited separation, one probe to introduce the drug to the dermis and the second to remove the drug plus any metabolite. The probes used were 2kDa and the insertion technique was similar, the only difference being that the needles were inserted in pairs with the maximum separation between them approximately 2-3mm. Once the probes were in place only the receiving probe was perfused for the initial hour in order to give a control sample.

2.2.4.4 Fibre Depth Measurement

At the conclusion of all studies each *in situ* fibre was scanned using a high frequency 3-D ultrasound scanner (Dermascan) in order to measure the depth of the probe. The scanner used was specially designed for working with skin as it works at a frequency of 20,000kHz far higher than that used for foetal

monitoring. The increased frequency results in a greatly reduced penetration of the ultrasound, the maximum depth measurable is approximately 3cm, however this is compensated by an increase in resolution allowing the detection of objects as small as 0.1mm.

Once microdialysis had finished the probes were disconnected from the pumps, the drug wells were removed and the forearm cleaned to remove all traces of the drug preparation. Prior to scanning the probes were briefly attached to an empty syringe via a length of tubing and the residual perfusate left in the fibres was expelled so that the fibres contained air. This was necessary to enhance detection of the fibres. Ultrasound works on the principle that sound is reflected at the barrier between two areas of differing density. If the fibres were left fluid filled there would be little difference in density between the fibres and the surrounding tissue, by filling the fibres with air the density difference is increased and therefore allowing the fibre to be detected more easily. This was not necessary in the later studies which had a fibre that contained a strengthening wire.

The areas of skin above the air filled fibres were covered in an aqueous gel. The Dermascan probe head was positioned so that it rested on the skin above the fibre, the gel was necessary to ensure an air free junction between the skin and probe. Any air present would prevent the ultrasound from reaching the skin. The skin was scanned along the entire length of the fibre and the probe depth measured three times per fibre at 5mm intervals along the fibre.

2.2.4.5 Skin Hydration Measurement

Skin hydration is an important factor in topical drug absorption; the degree that drug vehicles hydrated the skin was investigated using a trans-epidermal water loss (TEWL) meter. This consists of a hollow chamber that rests on the surface of the skin and measures the water content of the air close to the skins surface. The more the skin is hydrated the more water is lost through evaporation and therefore the water content of the air is higher.

The hydrating effect of several drug vehicles was tested using the TEWL meter. A set area of skin was liberally smeared with a particular base formulation and covered with an occlusive dressing. After a pre-determined period of time the dressing was removed and all traces of the vehicle carefully

wiped off. The skin hydration of the area was measured immediately and then by repeat measurements every 10 minutes for one hour. This procedure was repeated with various formulations and application times.

Measurements were taken in a closed room with precautions taken to prevent air movement as even a slight air movement could cause incorrect measurements. The volunteers were also kept immobile for the duration of the study, any exertion might have caused a degree of sweating which would artificially raise the level of hydration recorded.

2.2.5 HPLC Analysis

2.2.5.1 Methyl Salicylate / Salicylic Acid

All the samples generated from both the *in vitro* and *in vivo* experiments were analysed using HPLC with the following method.

- 10 μl of a 20 μg/ml solution of internal standard (methyl-2-methoxybenzoate) was added to a 200μl aliquot of each sample.
- The samples were vortexed and loaded into the autosampler.
- The UV detector was operated at 277nm.
- The mobile phase was 70% double distilled water, 30% methanol with the pH adjusted to 2.5 using formic acid.
- The flow rate was set at 0.8 ml/min.
- The column was a Phenomenex Prodigy ODS3 4μ column (75 x 2 mm) kept at a constant temperature of 50°C.
- The run time per injection was 12 minutes.
- All injections were 100μl, which gave a lower limit of detection of 10ng on column or 100 ng/ml. The retention times were:- methyl salicylate = 9 min, salicylic acid = 3.3 and methyl-2-methoxy benzoate = 4.5 min.
- Absorption data was integrated and plotted using a Waters 745 data module, which was set to give peak areas rather than peak heights.

Sample concentrations were calculated by comparison to standard curves for methyl salicylate and salicylic acid. Triplicate standards were prepared as detailed in the solutions section and then analysed by HPLC in the method described above for the samples. A standard curve was prepared for every batch of samples and HPLC run. The peak areas from both the samples and the standards from a run were entered into an Excel spreadsheet. The standard curve was calculated by plotting the ratio of the drug (methyl salicylate or salicylic acid) over the internal standard against the drug concentration. The slope of the standard curves was used to calculate the concentration of both compounds in all samples in the run. Figures 2.3 and 2.4 show typical standard curves for both methyl salicylate and salicylic acid. The average CoV for the methyl salicylate and salicylic acid assays were respectively: 8.1 and 2.1.

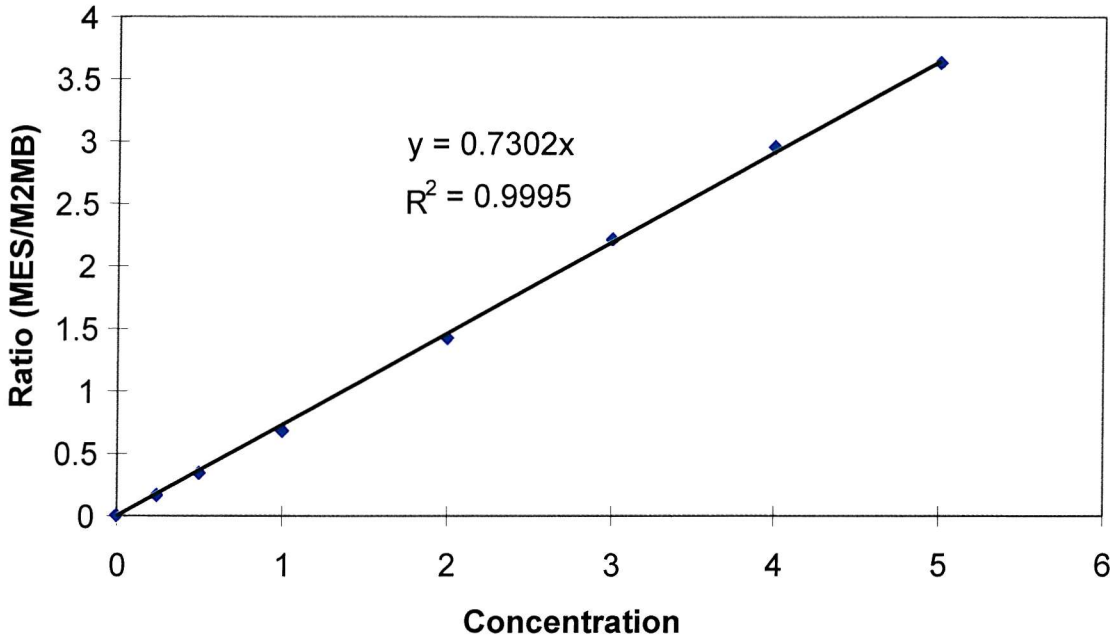

Figure 2.8: Typical standard curve for methyl salicylate (MES), each point is the average of triplicate standards, (M2MB = methyl-2-methoxy benzoate, internal standard).

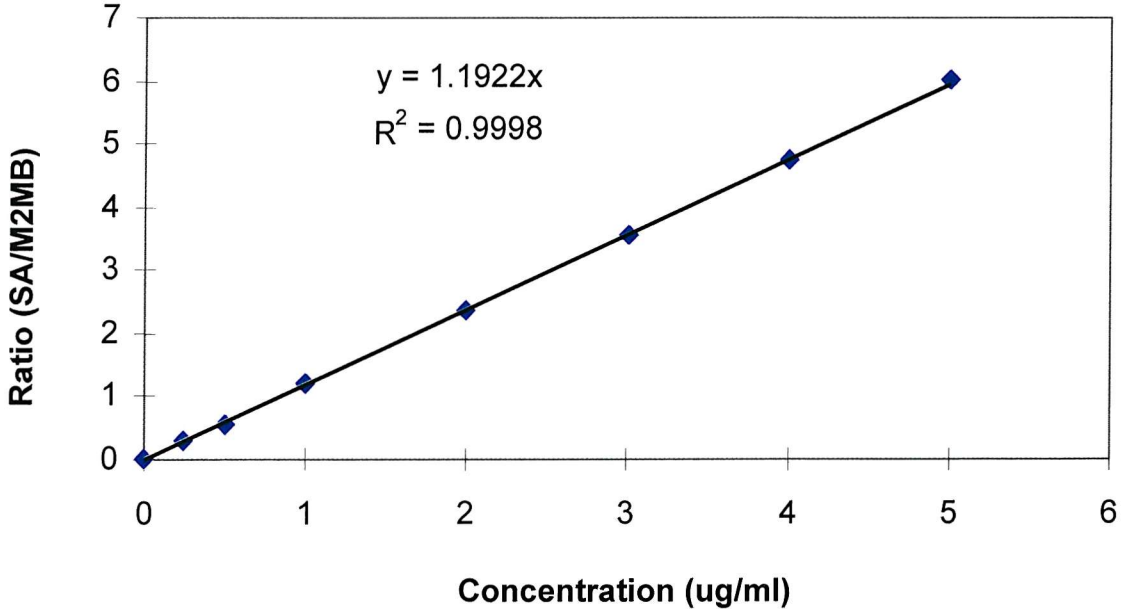

Figure 2.9: Typical standard curve for salicylic acid (SA) each point is the average of triplicate standards, (M2MB = methyl-2-methoxy benzoate, internal standard).

The average and standard deviations for the R^2 values for all standard curves produced in this thesis for methyl salicylate and salicylic acid were:

Methyl salicylate: 0.983 ± 0.039 Salicylic acid: 0.999 ± 0.000

2.2.5.2 Ketoprofen

All samples produced using the 3MDa probes went through an initial extraction procedure to remove proteins prior to the general procedure that was used for samples from 2kDa fibres.

- 100 μl of sample was added to 200ul of 2M perchloric acid, vortexed and allowed to stand for one hour.
- 5 ml of diethyl ether was added, and the tube vortexed and shaken for one hour.
- All samples were centrifuged for 10 minutes at 4000 rpm.
- The organic solvent layer was removed and retained and the aqueous layer discarded
- The organic solvent layer was evaporated to dryness under a flow of nitrogen at a temperature of 40°C.
- 100μl of mobile phase was added to the residue and agitated overnight.

All samples (the extracted 3MDa samples and the neat 2kDa samples) were then analysed using the following HPLC method.

- 100μl of the samples were added to a HPLC vial and 10 μl 30μg/ml biphenyl acetic acid (BPAA) added as internal standard and loaded into the auto-sampler.
- The detector was operated at 350nm.
- The mobile phase was 70% 250mM ammonium acetate, 30% acetonitrile with the pH adjusted to 5.0 using acetic acid.
- The flow rate was set at 2 ml/min.
- The column was a Phenomenex Prodigy ODS3 5μ column (100 x 4.6 mm) maintained at room temperature.
- All injections were 90μl, which gave a lower limit of detection of less than 1ng on column or 11 ng/ml.
- The run time per injection was 10 minutes.

- The retention time for ketoprofen and BPAA was 6 and 8 minutes respectively.

Sample concentrations were calculated by comparison to standard curves for ketoprofen. Two sets of duplicate standards were prepared as detailed in the solutions section one duplicate also contained 2.25% human albumin. The set containing protein was put through the extraction procedure above and then both sets analysed by HPLC in the method detailed above. A standard curve was prepared for every HPLC run. The peak areas from both the samples and the standards from a run were entered into an Excel spreadsheet. The standard curve was calculated by plotting the peak area of ketoprofen against the drug concentration. The slope of the standard curves was used to calculate the concentration of ketoprofen in all samples in the run. Figures 2.5 and 2.6 below show typical standard curves for both the extracted and unextracted ketoprofen standards. The average CoV for both extracted and unextracted ketoprofen standard curves were respectively 10.5 and 5.6.

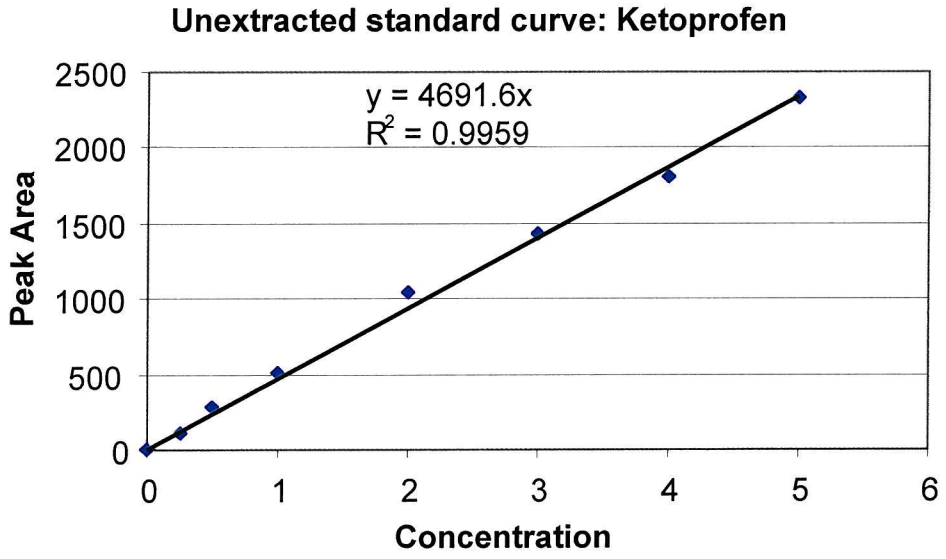

Figure 2.10: Typical standard curve for unextracted ketoprofen standards

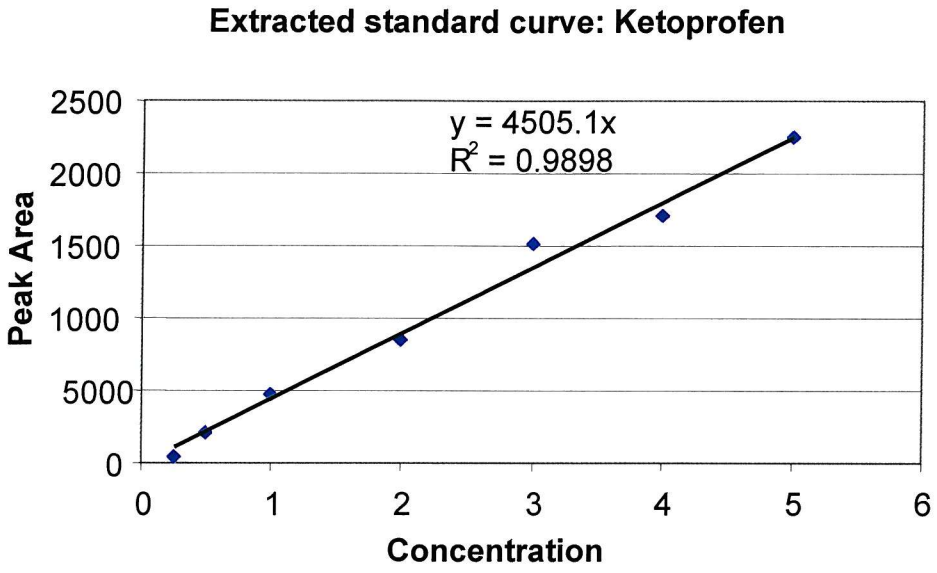

Figure 2.11: Typical standard curve for extracted ketoprofen standards

The average and standard deviations of the R^2 value for all standard curves produced for extracted and unextracted ketoprofen samples were:

Unextracted : 0.990 ± 0.007 Extracted : 0.978 ± 0.010

2.2.5.3 Fenbufen and BPAA

- 100μl of the samples were added to a HPLC vial and 10 μl 30μg/ml ketoprofen added as internal standard and loaded into the auto-sampler.
- The detector was operated at 260nm.
- The mobile phase was 70% 250mM ammonium acetate, 30% acetonitrile with the pH adjusted to 5.0 using acetic acid.
- The flow rate was set at 2 ml/min.
- The column was a Phenomenex Prodigy ODS3 5μ column (100 x 4.6 mm) maintained at room temperature.
- All injections were 90μl, which gave a lower limit of detection of less than 1ng on column or 11 ng/ml.
- The run time per injection was 15 minutes.
- The retention time for ketoprofen, fenbufen and BPAA was 4, 6 and 8 minutes respectively.

Sample concentrations were calculated by comparison to standard curves for BPAA and fenbufen. A standard curve was prepared for every HPLC run. The peak areas from both the samples and the standards from a run were entered into an Excel spreadsheet. The standard curve was calculated by plotting the peak area of BPAA and fenbufen against the drug concentration. The slope of the standard curves was used to calculate the concentration of ketoprofen in all samples in the run. Figure 2.7 below show a typical standard curve. The average CoV for both fenbufen and biphenylacetic acid standard curves are respectively 2.9 and 2.0.

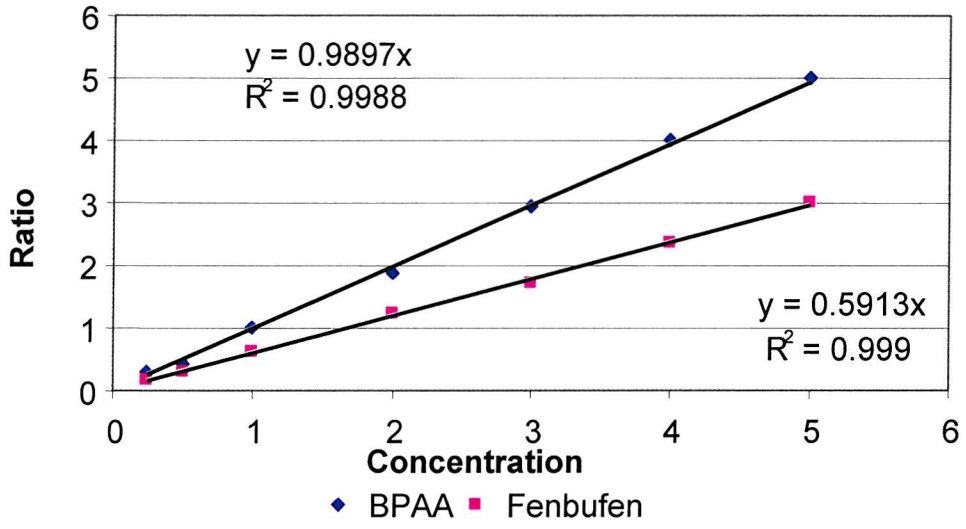

Fig 2.12: Typical standard curve for fenbufen and BPAA.

The average and standard deviations of the R^2 value for all standard curves produced for fenbufen and BPAA samples were:

Fenbufen : 0.989 ± 0.013 BPAA : 0.991 ± 0.010

2.2.5.4 Protein determination

Samples were analysed for protein using a Coomassie protein assay kit:

- Protein standards were prepared using bovine serum albumin and Ringer's solution, final concentrations were 2000, 1500, 1000, 750, 500, 250, 125 and 0 μg/ml.

- 5μl of the standards and all samples were pipetted in duplicate into separate wells on a standard 96 well plate.
- 250μl of Coomassie reagent was added to all wells.
- Plate was agitated for 30 seconds.
- Absorbence at 595 nm was measured for all wells.
- Sample values were calculated using the standard curve.

2.2.6 Statistical Analysis

All statistical analysis was performed using Microsoft Excel.

In vitro results were analysed using either the Student's t-Test (2 sample, equal variance) or analysis of variance (ANOVA).

The in-vivo data was analysed using either paired student t-Tests or ANOVA. In all experiments with duplicate lines, the averages of the lines were used in the analysis to prevent an uneven weighting of the results.

2.2.7 Calculation of C_{max}, T_{max} and AUC

For each study performed the C_{max}, T_{max} and AUC were calculated for the averaged results. C_{max} of an individual line was taken as the maximum observed concentration reached throughout the course of the study period and was simply the concentration at the time point with the highest concentration. The T_{max} was taken as the time at which the C_{max} above was reached and so was always a specific time point.

The AUC or area under the curve is a measure of exposure to the drug and has units of μg.min/ml. As the majority of the studies did not show an elimination phase the AUC could not be calculated to infinity. The AUC was calculated using Excel and the averaged data for each volunteer within a particular study using the trapezoidal method. The sample concentrations of

each pair of consecutive time points were averaged and then multiplied by the time period between them. This gave the AUC between the two time points, by adding the AUC's for all the consecutive time points together, the AUC for a particular fibre type in a particular volunteer was calculated.

3 Results: Methyl Salicylate - *In vitro*

3.1 Introduction

A series of *in vitro* microdialysis experiments were carried out to investigate the relative recovery of methyl salicylate and its metabolite salicylic acid. The purpose was to determine the effect on relative recovery when the experimental conditions were altered. This would give the optimum parameters for recovery for any *in vivo* studies. The experimental conditions studied were: fibre type (2kDa vs. 5kDa), perfusion flow rate, concentration gradient across the dialysis membrane, temperature (room temperature versus skin temperature) and the insertion of wire through the fibre.

3.2 Methods

The method used is given in the materials and methods chapter (Section 2.2.3.1). The only modification to the method described was in the studies investigating the effect of inserting wire through the fibres on the relative recovery. The probes used in these studies were constructed by first threading a fine stainless steel wire through a 2kDa fibre before it was glued to the nylon tubing. The resulting probe was then used in a similar manner to the unmodified probes.

3.3 Results: Methyl salicylate

3.3.1 Concentration

Four concentrations of methyl salicylate in the bath solution were investigated using the 2kDa fibres (see below); 500, 50, 10 and 5 μg/ml, using a perfusion flow rate of 0.2ml/hr (3.33μl/min). Concentrations of 50 and 10 μg/ml both showed an average recovery of approximately 29% with no statistical difference between them (table 3.1 and 3.2). The average recovery at 5 μg/ml was 20% and statistical analysis gave a p value of <0.01 when compared to both 50 and 10 μg/ml (table 3.3). The average recovery at 500 μg/ml was 8% significantly lower than the other three concentrations (table 3.4). When preparing 500μg/ml solutions there was a tendency for the solutions to become turbid indicating that the methyl salicylate was forming a micellar solution which would reduce the concentration of available drug in solution.

The extent of equilibrium which is reached should be dependent on the flow and fibre characteristics and independent of the concentration of the drug in the bath solution so all recoveries measured should be approximately equal. Based on results from other studies (see later) it is possible that an amount of drug may be adhering to the petri dish surface, which would be more noticeable at low concentrations, i.e. 5μg/ml, as it would represent a larger fraction of the total available drug.

Time	0.05mg/ml			
	Fibre 1	Fibre 2	Fibre 3	Fibre 4
0.00	0.00	0.00	0.00	0.00
1.00	29.84	23.54	32.08	25.37
2.00	29.80	26.01	30.50	33.01
3.00	22.97	26.89	34.81	33.01
4.00	25.07	27.01	29.74	28.80
Average	26.92	25.86	31.78	30.05
St Dev	3.46	1.61	2.24	3.70
	Group Average		28.65	
	Group St Dev		2.74	

Table 3.1: Percentage Relative Recovery of Methyl Salicylate (%) from a 50 μg/ml bath solution using a 2kDa fibre perfused at 0.2ml/hr with Ringer's solution.

Time	0.01 mg/ml			
	Fibre 1	Fibre 2	Fibre 3	Fibre 4
0.00	0.00	0.00	0.00	0.00
1.00	34.44	27.90	30.40	30.60
2.00	29.02	31.50	26.40	29.79
3.00	28.16	24.00	32.07	25.92
4.00	23.86	30.10	30.10	23.07
Average	28.87	28.38	29.74	27.35
St Dev	4.34	3.27	2.39	3.50
	Group Average		28.58	
	Group St Dev		1.00	

Table 3.2: Percentage Relative Recovery of Methyl Salicylate (%) from a 10 μg/ml bath solution using a 2kDa fibre perfused at 0.2ml/hr with Ringer's solution.

Time	0.005 mg/ml			
	Fibre 1	Fibre 2	Fibre 3	Fibre 4
0.00	0.00	0.00	0.00	0.00
1.00	23.20	18.00	18.60	22.63
2.00	21.84	24.00	17.20	19.69
3.00	19.91	25.30	23.70	17.01
4.00	20.28	13.80	21.90	18.34
Average	21.31	20.28	20.35	19.42
St Dev	1.51	5.36	2.98	2.41
	Group Average		20.34	
	Group St Dev		0.77	

Table 3.3: Percentage Relative Recovery of Methyl Salicylate (%) from a 5 μg/ml bath solution using a 2kDa fibre perfused at 0.2ml/hr with Ringer's solution.

Time	0.5 mg/ml			
	Fibre 1	Fibre 2	Fibre 3	Fibre 4
0	0.00	0.00	0.00	0.00
1	7.19	4.64	1.86	3.67
2	6.66	4.40	4.17	6.13
3	10.09	3.77	6.68	4.77
4	7.98	4.27	4.24	5.80
Mean	7.98	4.27	4.24	5.09
St Dev	1.51	0.37	1.97	1.11
	Group average		5.39	
	Total StDev		2.00	

Table 3.4: Percentage Relative Recovery of Methyl Salicylate (%) from a 500 μg/ml bath solution using a 2kDa fibre perfused at 0.2ml/hr with Ringer's solution.

3.3.2 Flow Rate

The effect of perfusate flow rate on the relative recovery of methyl salicylate was investigated using 2kDa fibres by setting the syringe pumps at 0.4ml/hour and 0.2ml/hour (6.7 and 3.3 μl/min) and running a series of *in vitro* experiments with the bath concentration MeS fixed at 5 μg/ml. The average recovery for 0.2ml/hour was 22% (Table 3.6) and for 0.4ml/hour it was 12% (Table 3.5), which was statistically significant ($p<0.01$), this agrees with the hypothesis that recovery is inversely proportional to the flow rate.

Time(hr)	Fibre 1	Fibre 2	Fibre 3	Fibre 4	Fibre 5	Fibre 6
0	0.00	0.00	0.00	0.00	0.00	0.00
1	14.92	15.09	9.75	11.73	12.94	16.99
2	16.64	17.65	10.48	10.20	12.70	10.37
3	16.30	15.83	8.97	9.99	10.74	13.19
4	9.48	12.77	6.17	5.95	8.46	5.01
Average	14.33	15.34	8.84	9.47	11.21	11.39
St Dev	3.32	2.02	1.89	2.47	2.08	5.04
			Group Average	11.76		
			Group StDev	3.60		

Table 3.5: Percentage Relative Recovery of Methyl Salicylate (%) from a 5 μg/ml bath solution using a 2kDa fibre perfused at 0.4ml/hr with Ringer's solution.

Time(hr)	Fibre 1	Fibre 2	Fibre 3	Fibre 4	Fibre 5	Fibre 6
0	0.00	0.00	0.00	0.00	0.00	0.00
1	23.20	18.00	18.60	24.20	17.14	26.44
2	21.84	24.00	17.20	17.11	28.00	28.80
3	19.91	25.30	23.70	14.74	17.81	22.59
4	20.28	13.80	21.90	22.33	19.86	29.20
Average	21.31	20.28	20.35	19.59	20.70	26.76
St Dev	1.51	5.36	2.98	4.41	5.00	3.03
			Group Average	21.50		
			Group St Dev	2.63		

Table 3.6: Percentage Relative Recovery of Methyl Salicylate (%) from a 5 μg/ml bath solution using a 2kDa fibre perfused at 0.2ml/hr with Ringer's solution.

3.3.3 Temperature

Most *in vitro* experiments were performed at room temperature, however *in vivo* experiments would by definition occur at body temperature. The effect of temperature was studied in an experiment run at an elevated temperature in a specially heated room using the 2kDa fibres. Body temperature is on average 37°C however at the skins surface the temperature is on average 32°C. The average bath temperature for the studies was 32°C, the flow rate was 0.4ml/hr (6.67μl/min) and the bath concentration was 5μg/ml. The temperature caused the average recovery to rise from 12%, table 3.6 (room temperature, 21°C), to 18% (table 3.7). The difference between the two values was statistically significant ($p<0.05$).

Time (hrs)	Fibre 1	Fibre 2	Fibre 3	Fibre 4	Fibre 5	Fibre 6
0	0.00	0.00	0.00	0.00	0.00	0.00
1	23.36	16.26	17.68	19.81	15.60	21.36
2	17.70	16.78	21.54	17.24	19.69	16.54
3	16.19	9.94	16.41	13.06	16.50	15.74
4	26.21	15.21	19.70	20.71	22.41	9.87
Average	20.87	14.55	18.83	17.71	18.55	15.88
St Dev	4.71	3.14	2.26	3.43	3.11	4.71
Group Average				17.73		
Group St Dev				2.25		

Table 3.7: Percentage Relative Recovery of Methyl Salicylate (%) from a 5 μg/ml bath solution kept at 32°C using a 2kDa fibre perfused at 0.4ml/hr with Ringer's solution

3.3.4 Fibre Type

All fibres used were taken from kidney dialysis cartridges and fibres from two different types of cartridge were found to be robust enough to be used in probe production. A 2kDa fibre made by Gambro from a material called Cuprophan and a 5kDa fibre made by Fresenius using a material called Hemophan. The 2kDa fibre gave an average recovery of 19% using a flow rate of 0.2 ml/hr and 5μg/ml in the bath at room temperature (Table 3.8). Under similar experimental conditions the 5kDa fibre showed a recovery of 20% (Table 3.9). There was no statistically significant difference between the two values.

Time	Fibre 1	Fibre 2	Fibre 3	
0	0.0	0.0	0.0	
1	25.3	22.5	14.0	Total Mean
2	19.6	24.6	13.9	19.1
3	21.0	17.3	15.3	Total St Dev
4	18.1	23.5	13.9	4.3
Mean	21.0	22.0	14.3	
St Dev	3.1	3.2	0.7	

Table 3.8: Percentage Relative Recovery of Methyl Salicylate (%) from a 5 μg/ml bath solution using a 2kDa fibre perfused at 0.2ml/hr with Ringer's solution..

Time	Fibre 1	Fibre 2	Fibre 3	
0	0.0	0.0	0.0	
1	25.2	21.2	10.8	Total Mean
2	17.8	25.3	14.1	20.3
3	23.5	26.8	17.2	Total St Dev
4	23.6	20.6	17.3	4.9
Mean	22.5	23.5	14.9	
St Dev	3.2	3.0	3.1	

Table 3.9: Percentage Relative Recovery of Methyl Salicylate (%) from a 5 μg/ml bath solution using a 5kDa fibre perfused at 0.2ml/hr with Ringer's solution.

3.3.5 Luminal Wire

It was discovered that if wire was threaded along the length of the fibres the resulting probes were more robust and less likely to kink and block during *in vivo* studies. Probes with and without wires were investigated to determine the effect on the relative recovery. Probes without wires had an average recovery of 9.2% (table 3.10) and with wires a recovery of 8.7% (table 3.11).

Time	Wire	Wire	Wire
0	0.0	0.0	0.0
1	9.8	7.3	7.4
2	9.6	8.5	8.2
3	7.2	8.2	8.4
4	11.7	7.5	9.1
Average	9.6	7.9	8.3
St Dev	1.9	0.6	0.7
	Total Mean	8.6	
	Total St Dev	0.9	

Table 3.10: Percentage Relative Recovery of Methyl Salicylate (%) from a 5 μg/ml bath solution using a 2kDa fibre containing a luminal steel wire perfused at 0.4ml/hr with Ringer's solution.

Time	No Wire	No Wire	No Wire
0	0.0	0.0	0.0
1	8.1	11.8	9.9
2	10.0	10.2	9.7
3	9.7	7.5	9.1
4	4.5	9.4	10.2
Average	8.1	9.8	9.7
St Dev	2.6	1.8	0.5
	Total Mean	9.2	
	Total St Dev	1.1	

Table 3.11: Percentage Relative Recovery of Methyl Salicylate (%) from a 5 μg/ml bath solution using a 2kDa fibre not containing a luminal steel wire perfused at 0.4ml/hr with Ringer's solution.

3.4 Results: Salicylic Acid

3.4.1 Concentration

The effect of concentration on the relative recovery of salicylic acid was investigated in a series of *in vitro* experiments. Three different bath concentrations were tested: 5, 10 and 20 ug/ml. Recoveries for 10 and 20 μg/ml were approximately 60% (tables 3.12 and 3.13), however when the bath solution was 5μg/ml the recovery was slightly lower (52%, table 3.14). The results for 10 and 20 μg/ml were not statistically significant with respect to each other but 5μg/ml was significantly different with respect to both 10 and 20 μg/ml ($p<0.05$). This result mirrors that seen when using methyl salicylate and may have arisen for the same reasons, i.e. due to a fixed amount of drug adhering to the bath surface, which would have a correspondingly higher effect at lower concentrations.

Time (hr)	5ug/ml			
0	0.00	0.00	0.00	0.00
1	55.15	49.81	46.74	56.15
2	54.12	47.73	47.03	55.76
3	56.11	48.76	48.72	55.50
4	54.32	52.96	49.60	58.44
Mean	54.93	49.81	48.02	56.46
St Dev	0.91	2.26	1.37	1.35
		Group average	52.31	
		Group St Dev	4.03	

Table 3.12: Percentage Relative Recovery of Salicylic acid (%) from a 5 μg/ml bath solution using a 2kDa fibre with no wire perfused at 0.2ml/hr with Ringer's solution at room temperature.

Time (hr)	10ug/ml			
0	0.00	0.00	0.00	0.00
1	58.51	57.54	59.20	58.54
2	64.49	60.01	57.25	59.01
3	60.04	59.65	57.96	59.26
4	60.84	58.74	60.12	64.84
Mean	60.97	58.99	58.63	60.41
St Dev	2.54	1.10	1.28	2.97
		Group average	59.75	
		Group St Dev	1.12	

Table 3.13: Percentage Relative Recovery of Salicylic acid (%) from a 10 μg/ml bath solution using a 2kDa fibre with no wire perfused at 0.2ml/hr with Ringer's solution at room temperature.

Time (hr)	20ug/ml			
0	0.00	0.00	0.00	0.00
1	66.59	61.25	62.79	57.80
2	61.16	62.54	62.41	61.26
3	54.21	59.14	58.14	61.51
4	60.65	59.38	57.18	57.79
Mean	60.65	60.58	60.13	59.59
St Dev	5.07	1.61	2.88	2.08
		Group average	60.24	
		Group St Dev	0.49	

Table 3.14: Percentage Relative Recovery of Salicylic acid (%) from a 20 μg/ml bath solution using a 2kDa fibre with no wire perfused at 0.2ml/hr with Ringer's solution at room temperature.

3.4.2 Flow Rate

The effect of flow rate on recovery of salicylic acid was investigated using three flow rates (0.4, 0.3 and 0.2ml/hour). The average recovery for 0.4ml/hr was 36 ± 2%, for 0.3ml/hr it was 43 ± 2% and for 0.2ml/hr the recovery was 52 ± 4% (Tables 3.15-3.17). The values were all significant with respect to each other ($p<0.01$).The results show an inverse relationship between flow rate and recovery.

Time (hrs)	0.4ml/hr			
0	0.00	0.00	0.00	0.00
1	33.37	35.78	32.05	35.42
2	36.14	31.36	33.36	37.08
3	39.35	37.54	31.47	40.39
4	37.76	38.51	34.97	39.36
Average	36.65	35.80	32.96	38.06
St Dev	2.55	3.17	1.55	2.24
		Group Average	35.87	
		Total St Dev	2.15	

Table 3.15: Percentage Relative Recovery of Salicylic acid (%) from a 5 μg/ml bath solution using a 2kDa fibre with no wire perfused at 0.4ml/hr with Ringer's solution at room temperature.

Time (hrs)	0.3 ml/hr			
0	0.00	0.00	0.00	0.00
1	40.07	45.21	39.87	44.51
2	42.61	41.10	40.15	43.57
3	46.01	42.03	45.35	40.79
4	47.04	40.80	42.07	44.47
Average	43.93	42.29	41.86	43.34
St Dev	3.19	2.02	2.52	1.75
		Group Average	42.85	
		Total St Dev	2.34	

Table 3.16: Percentage Relative Recovery of Salicylic acid (%) from a 5 μg/ml bath solution using a 2kDa fibre with no wire perfused at 0.3ml/hr with Ringer's solution at room temperature.

Time (hrs)	0.2 ml/hr			
0	0.00	0.00	0.00	0.00
1	49.81	55.15	56.15	46.74
2	47.73	54.12	55.76	47.03
3	48.76	56.11	55.50	48.72
4	52.96	54.32	58.44	49.60
Average	49.81	54.93	56.46	48.02
St Dev	2.26	0.91	1.35	1.37
		Group Average	52.31	
		Total St Dev	4.03	

Table 3.17: Percentage Relative Recovery of Salicylic acid (%) from a 5 μg/ml bath solution using a 2kDa fibre with no wire perfused at 0.2ml/hr with Ringer's solution at room temperature.

3.4.3 Fibre

The recoveries of the two different fibre types were compared using salicylic acid in the bath solution (table 3.18). The 2kDa and 5kDa fibres gave recoveries of 54% and 48% respectively. The difference was found not to be significant.

Time(hr)	2kDa			5kDa		
	Fibre 1	Fibre 2	Fibre 3	Fibre 1	Fibre 2	Fibre 3
0	0.0	0.0	0.0	0.0	0.0	0.0
1	47.3	55.6	58.4	40.9	55.9	41.8
2	51.7	54.4	59.6	41.6	49.8	52.4
3	49.5	53.7	54.8	45.0	48.6	54.1
4	49.2	54.9	57.6	42.5	47.1	54.4
Mean	49.4	54.6	57.6	42.5	50.4	50.6
St Dev	1.8	0.8	2.1	1.8	3.9	6.0

Total Mean	Total St Dev
53.9	4.2

Total Mean	Total St Dev
47.8	2.1

Table 3.18: Comparison of percentage relative recovery (%) between 2kDa and 5kDa fibres at room temperature using a flow rate of 0.2ml/hour and a bath concentration of Salicylic acid of 5μg/ml.

3.4.4 Luminal Wire

The effect of wire insertion on the recovery of salicylic acid using a 2kDa wire was investigated (table 3.19). With wire gave 61% whereas no wire gave 62% recovery.

Time (hr)	Wire			No wire		
	Fibre 1	Fibre 2	Fibre 3	Fibre 1	Fibre 2	Fibre 3
0	0.0	0.0	0.0	0.0	0.0	0.0
1	63.5	57.9	57.8	61.2	72.0	54.9
2	62.1	62.0	60.7	61.7	69.5	55.8
3	57.8	58.9	68.5	57.5	70.9	56.4
4	54.1	59.9	63.9	59.6	66.9	59.2
Mean	59.4	59.7	62.7	60.0	69.9	56.6
St Dev	4.3	1.8	4.6	1.9	2.2	1.9

Total Mean	Total St Dev
60.6	1.9

Total Mean	Total St Dev
62.1	6.9

Table 3.19: Comparison of percentage relative recovery (%) between 2kDa fibres with and without wires at room temperature using a flow rate of 0.2ml/hour and a bath concentration of Salicylic acid of 5μg/ml.

The difference between the relative recovery in fibres with and without wire was not statistically significant.

3.5 Discussion

Theoretically the percentage recovery from a microdialysis probe should be independent of concentration, however the *in vitro* experiments showed a lower recovery at the lowest concentration tested especially for methyl salicylate. Methyl salicylate is a hydrophobic compound that can be adsorbed to non-polar surfaces (the petri dish), and this was shown in later *in vitro* experiments looking at loss or reverse recovery (see chapter 5). In these experiments the methyl salicylate was contained in the perfusate and not the bath solution, so that the movement of drug is now from the fibre to the bath solution. When samples were analysed no methyl salicylate could be detected at all showing the methyl salicylate had been adsorbed onto the inside of the tubing used to connect the probe to the syringe. This was confirmed when samples were taken directly from the end of the tubing and analysed and again no methyl salicylate could be detected. In comparison to the tubing the petri dish has a much lower surface area to volume ratio and therefore it is to be expected that less drug will be adsorbed. However it is likely that enough is adsorbed that it measurably affects the bath concentration and therefore the calculated recovery at the lowest concentration. At this stage in the research the bath solutions were not analysed and the concentration assumed to be that added so there are no data to show that the bath solution concentration was lower than that originally prepared.

A reduction in the relative recovery was also shown at the highest concentration. Attempts to achieve concentrations higher than 500μg/ml in an aqueous solution had resulted in turbid suspensions and occasionally a 500μg/ml solution also resulted in a suspension. It is likely that at the highest concentration methyl salicylate may not have been freely in solution and may have formed a micellar solution. If this is true the reduction in recovery at the highest concentration is merely a reflection of the reduction of available methyl salicylate in the bath solution.

Previous microdialysis studies have shown that tissue concentrations of topically applied drugs in general tend to be low (Muller *et al* 1997, Benfeldt & Groth 1998, Cross *et al* 1998). *In vitro* work should as far as possible reflect *in vivo* conditions so it was important to determine the recovery of a low concentration of methyl salicylate *in vitro,* that was large enough to give

clearly measurable concentrations in the dialysate samples for analysis. At a flow rate of 0.4ml/hour, a bath concentration of 5μg/ml resulted in recovered concentrations roughly ten times the limit of detection. This concentration was used for further experiments, as lower concentrations would have given unreliable data.

The flow rate of the perfusate has a direct correlation to the percentage recovery, the lower the flow rate the longer the perfusate is in contact with the fibre membrane, therefore the concentration of the drug in the perfusate gets closer to equilibrium with the external concentration. Theoretically at zero flow rate there would be complete equilibration. However a flow rate must be found that gives a large enough sample to analyse but yet still allows the perfusate concentration to reach a significant percentage of the tissue or bath concentration. The initial flow rate tried was 0.4 ml/hour, which gave a recovery of ≈ 12% for methyl salicylate and a sample size of approximately 350 μl, (the samples are smaller than the theoretical 400μl probably because of evaporation from the collection tube). Halving the flow rate gave a recovery of ≈22% and a sample size of approximately 175μl. Initially 0.4ml/hour was chosen as the flow rate for any *in vivo* studies purely because it gave more sample to analyse so if any problems arose there would enough to re-analyse. Once the entire *in vivo* procedure had been established it was envisioned that the flow rate would be reduced to 0.2ml/hour.

Increasing the temperature of an *in vitro* study to 32°C caused an increase in recovery from 12% to 18% at 0.4ml/hr. The increase in temperature would increase the rate of diffusion across the membrane which allowed the perfusate and bath solution to get closer to equilibrium. Ideally all the *in vitro* experiments would be carried out at this temperature, as it is a closer reflection of the *in vivo* conditions however practical considerations made this difficult. However when calculating the absolute quantities of drug absorbed in the *in vivo* studies it would be better to use the recovery at 32°C.

A problem arose during the study concerning the fibres used because the renal dialysis cartridges used initially were no longer manufactured and once the existing stock is depleted there would be no chance of replacing them.

Even though enough fibres were available to continue experiments for several years it was thought best to investigate alternatives as soon as possible (especially if the aim is to produce a validated method available to other researchers). One of the more likely substitutes is a fibre made by Fresenius. The *in vitro* results showed that the recoveries from both fibres are almost identical. The Fresenius fibre has a molecular weight cut-off of 5kDa whereas the fibre made by Gambro is only 2kDa. The cut off limit refers to the maximum pore size of the fibre i.e. a 2kDa fibre will have pores with a range of sizes but the largest will only allow molecules equal to or smaller than 2000 Da to pass through. Both methyl salicylate and salicylic acid have molecular weights about ten fold less than the cut of limit and it is not expected that the maximum pore size should make any difference. A more important element is the pore size distribution, since the higher the density of pores large enough to allow the free movement of drug the higher the expected relative recovery. Due to the method of production the manufacturer's were unable to give any information on the distribution apart from that it followed an exponential curve with the number of pores decreasing as pore size increased. The similarity of the relative recovery would tend to indicate that the density of pores able to allow the free passage of drug was similar in both fibres. Another factor which might affect the relative recovery is the charge characteristics of the different fibre materials, however no information has yet obtained from the manufacturers concerning the specific characteristics of the fibres.

A recent development in microdialysis probe construction is the insertion of fine wires along the inside of the fibres, which makes the fibre easier to handle, less prone to blockage and readily detectable using ultrasound. The results showed a slight reduction in recovery but statistically it was not significant and the results were well within the ranges found in studies with normal fibres. The reduction was expected because the introduction of wire to the fibre results in a considerable reduction in the cross-sectional area and therefore a reduction to the overall volume of the probe. The cross sectional area of the dialysis tubing is approximately $0.004cm^2$ and that of the wire $0.0009\ cm^2$ .The flow rate is solely controlled by the syringe pump and remains constant no matter which probe is used therefore the reduction in cross-sectional area results in an increased perfusate speed within the fibre. If

the speed of the perfusate increases then the time the perfusate resides within the fibre decreases. In consequence the relative recovery should be reduced by the insertion of wire as the perfusate would have less time to reach equilibrium with the surrounding fluid. The similarity of the results suggests that the conditions within the fibre are more complicated and that other factors such as turbulence and the presence or absence of a laminar flow may be occurring.

It was possible to use ultrasound in some of the later *in vivo* studies and therefore the use of the wire probes as standard would prove to be beneficial.

3.6 Conclusion

The *in vitro* results indicated that the optimum conditions for methyl salicylate recovery are: A flow rate of 0.2 ml/hour, this gives the best balance of recovery and sample size and running the experiment at skin temperature, approximately 32°C.

All the other parameters tested had little or no effect on the overall recovery.

4 Results: Methyl Salicylate - *In vivo*

4.1 Introduction

Methyl salicylate was chosen as the model drug for the first set of studies for four reasons:

1. Methyl salicylate is tolerated well topically and is present in a variety of over the counter topical preparations
2. Its physico-chemical properties suggests a high degree of transdermal penetration.
3. There is a sensitive and robust analytical method present in the literature.
4. Microdialysis has the potential to study local metabolism in the dermis.

The aims of the studies were first to determine whether microdialysis could be used to detect and measure the transdermal absorption of methyl salicylate and its metabolism to salicylic acid, and secondly to assess the influence of dermal blood flow and occlusion on accumulated tissue concentration.

4.2 Method: Propylene glycol formulation

The 8 volunteers for the *in vivo* studies had an average age of 21 years (max.=24, min.= 19) and were 4 women and 4 men. All were students studying at Southampton University. Due to problems with the fibres (which did not contain a luminal wire) remaining patent during the studies not all of the volunteers had the same numbers of functioning lines (Table 4.1).

Volunteer	Age	Sex	No. Control	No. NA	No. NA
1	19	F	2	2	2
2	20	F	1	1	2
3	20	M	1	1	2
4	21	F	2	2	2
5	22	F	1	1	2
6	21	M	2	2	2
7	24	M	2	2	2
8	21	M	2	2	2

Table 4.1: Volunteer information for methyl salicylate in a propylene glycol based formulation study: Age, sex and the number of patent lines for each experimental condition.

All studies used the 2kDa probes without wire inserts, the manufacture and insertion of which are described in detail in the materials and methods chapter. All inserted fibres were perfused at a flow rate of 0.4ml/hour.

In all volunteers the six lines were divided into three sets of two lines. The first set was unoccluded and was a control, which had Ringer's solution as the perfusate. The second set had a low concentration of noradrenaline present in the Ringer's solution perfusate (5μg/ml). The third set had the same concentration of noradrenaline and the drug well had an occlusive patch made from the same material as the drug well itself. The drug wells (see chapter 2) contained approximately 0.1ml of a saturated solution of methyl salicylate; the vehicle was 50/50 mixture of water and propylene glycol.

The positioning of the lines was controlled in that the three probes nearest the wrist and the three nearest the elbow would always contain one of each of the experimental sets i.e. Ringer's perfusate unoccluded, Ringer's plus noradrenaline unoccluded and Ringer's plus noradrenaline occluded. The arrangement of the three sets in each of the two groups of fibres (nearest the wrist vs. nearest the elbow) was random.

All other parameters remained constant.

4.3 Methyl Salicylate Absorption and Recovery

Subject	Fibre Type	Pre dose		0->1		1->2		2->3		3->4		4->5	
		Fibre 1	Fibre 2	Fibre 1	Fibre 2	Fibre 1	Fibre 2	Fibre 1	Fibre 2	Fibre 1	Fibre 2	Fibre 1	Fibre 2
1	Ringer's	0	0	0.82	1.13	0.22	0.44	0.00	0.00	0.00	0.00	0.00	0.00
	Noradrenaline	0	0	1.54	1.04	0.85	0.53	0.47	0.30	0.30	0.17	0.21	0.16
	Noradrenaline & Occlusion	0	0	2.01	1.55	2.19	1.33	1.27	0.96	0.89	0.83	0.80	0.58
2	Ringer's	0	0	0.63	NA	0.49	NA	0.45	NA	0.45	NA	0.52	NA
	Noradrenaline	0	0	0.21	NA	0.17	NA	0.25	NA	1.24	NA	0.32	NA
	Noradrenaline & Occlusion	0	0	0.30	0.97	0.29	0.72	0.29	0.78	0.66	0.90	0.13	0.73
3	Ringer's	0	0	0.22	NA	0.21	NA	0.00	NA	0.00	NA	0.00	NA
	Noradrenaline	0	0	0.36	NA	0.19	NA	0.00	NA	0.00	NA	0.00	NA
	Noradrenaline & Occlusion	0	0	0.17	0.13	0.50	0.31	0.38	0.34	0.25	0.36	0.25	0.33
4	Ringer's	0	0	0.63	0.54	0.47	0.12	0.15	0.00	0.00	0.00	0.00	0.00
	Noradrenaline	0	0	0.96	1.27	0.50	0.50	0.17	0.16	0.11	0.11	0.10	0.00
	Noradrenaline & Occlusion	0	0	0.91	0.38	0.96	0.55	0.68	0.29	0.57	0.21	0.46	0.25
5	Ringer's	0	0	0.63	NA	0.00	NA	0.00	NA	0.00	NA	0.00	NA
	Noradrenaline	0	0	0.97	NA	0.22	NA	0.16	NA	0.00	NA	0.00	NA
	Noradrenaline & Occlusion	0	0	1.94	1.00	0.91	0.73	0.50	0.49	0.61	0.33	0.00	0.00
6	Ringer's	0	0	0.39	1.04	0.35	0.41	0.00	0.00	0.17	0.00	0.00	0.00
	Noradrenaline	0	0	0.50	2.95	0.48	0.87	0.28	0.23	0.20	0.23	0.00	0.00
	Noradrenaline & Occlusion	0	0	1.13	1.12	1.15	1.40	0.50	0.69	0.48	0.47	0.00	0.42
7	Ringer's	0	0	0.30	0.30	0.10	0.09	0.00	0.00	0.00	0.00	0.00	0.00
	Noradrenaline	0	0	0.50	1.60	0.20	0.30	0.08	0.10	0.00	0.07	0.00	0.00
	Noradrenaline & Occlusion	0	0	1.00	0.30	0.60	0.30	0.40	0.20	0.20	0.10	0.10	0.08
8	Ringer's	0	0	1.70	1.93	0.27	0.43	0.14	0.24	0.17	0.13	0.12	0.00
	Noradrenaline	0	0	1.65	0.47	0.53	0.18	0.27	0.05	0.17	0.00	0.00	0.00
	Noradrenaline & Occlusion	0	0	0.65	0.48	0.64	0.34	0.34	0.21	0.21	0.16	0.09	0.05

Table 4.2: Summary of methyl salicylate concentrations from all volunteers and all lines following topical methyl salicylate application. The values are the concentration (μg/ml) of methyl salicylate in the dialysate sample for each line and at each time point (NA = not available due to analytical problems).

Collection	RINGERS		NA		NA OCC	
Time	MEAN	SD	MEAN	SD	MEAN	SD
Pre-dose	0.00	0.00	0.00	0.00	0.00	0.00
0-1	0.77	0.51	1.17	0.28	0.92	0.57
1-2	0.25	0.14	0.47	0.18	0.86	0.50
2-3	0.05	0.07	0.20	0.09	0.52	0.29
3-4	0.03	0.06	0.11	0.09	0.40	0.25
4-5	0.01	0.02	0.03	0.07	0.31	0.22

Table 4.3: Table showing the effect of noradrenaline and occlusion on methyl salicylate concentration in the dialysate following topical methyl salicylate application . The results are the mean sample concentration (μg/ml) ± the standard deviation for 8 subjects (using the average value where data from 2 similar fibres were available).

Figure 4.1 below and table 4.2 and 4.3 above show the data from all microdialysis probes from all volunteers for the three sets of experimental conditions. Figure 4.1 shows the corrected concentrations, which are calculated by dividing by the relative recovery, measured *in vitro*, this gives the minimum tissue concentration (Song *et al*, 1999). The concentrations for

all three sets peaked after one hour with maximum concentration for the noradrenaline and noradrenaline occluded line peaking at ≈9.2μg/ml and 7.8μg/ml respectively and the Ringer's solution line peaking at ≈ 4.8μg/ml.

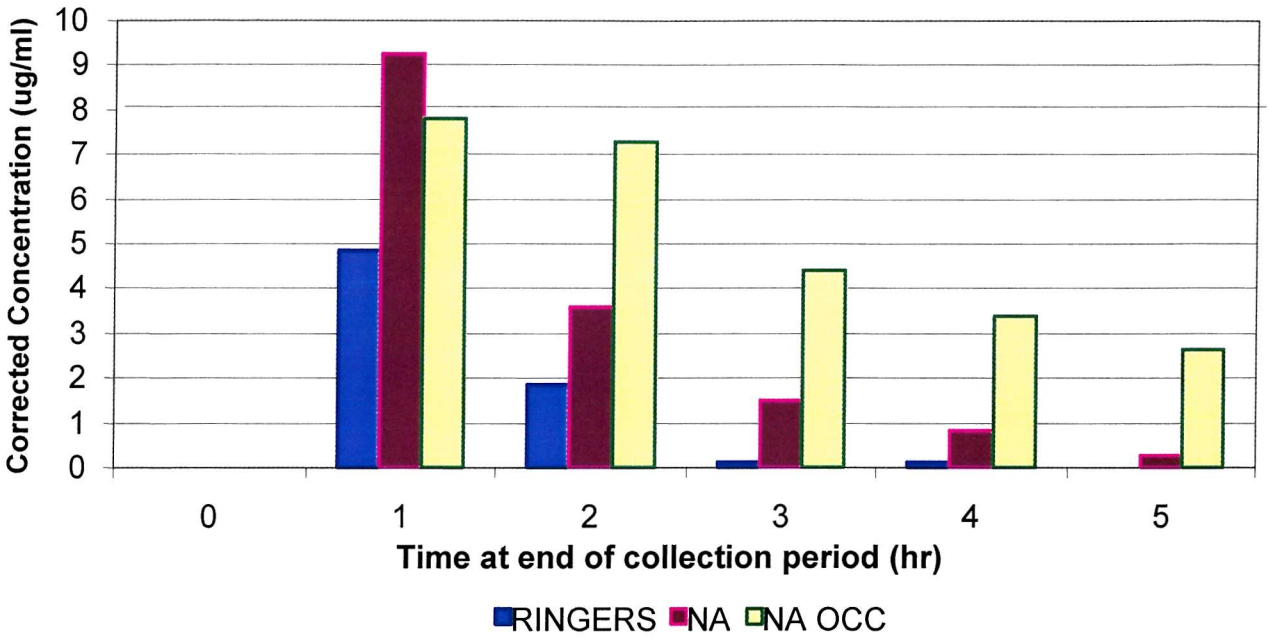

Figure 4.1: Graph showing the effect of noradrenaline (NA) and occlusion on methyl salicylate tissue concentration following topical methyl salicylate application . n = 8.

After the first hour the concentrations in both the Ringer's solution lines and the noradrenaline lines decreased, the Ringer's solution line reached the limit of detection at about 4 hours and the noradrenaline line decreased to 0.3μg/ml after 5 hours. The noradrenaline occluded line decreased far more slowly and appeared to plateau between hours 1 and 2; it decreased to 2.6 μg/ml by five hours.

For all lines the inter-subject variability was high (table 4.3) as indicated by the large standard deviations. Analysis of the AUC's using paired Student's t-tests showed that all three lines were significantly different to each other.

Parameter	Control		NA		NA Occ	
	Mean	St Dev	Mean	St Dev	Mean	St Dev
C_{max} (ug/ml)	0.58[a]	0.22	1.04[a]	0.42	0.95[b]	0.52
T_{max} (hr)	1	0	1	0	1.5	0.63
AUC (ug.min/ml)	47.4[a]	21.8	110.9[b]	48.0	180.4[c]	90.5

Table 4.4: Table of pharmacokinetic values for the three experimental parameters following topical methyl salicylate application using uncorrected data. Values with different superscripts were significantly different ($p<0.05$). n = 8.

Three pharmacokinetic properties were calculated, C_{max}, T_{max} and AUC (Table 4.4), using the uncorrected data. The C_{max} for the control line was approximately 0.6 μg/ml whereas both the noradrenaline and noradrenaline occluded lines gave a value of approximately 1μg/ml. The control line was significantly different to the two noradrenaline lines however there was no significant difference between the noradrenaline lines.

The T_{max} for both the control and noradrenaline lines was 1 hour, the noradrenaline occluded line gave a T_{max} of 1.5 hours.

The average AUC values for the control, noradrenaline and noradrenaline occluded lines were 48, 111 and 180 μg.min/ml respectively. The results were all significantly different from each other

Figure 4.1a shows the relationship of the AUC's from each of the three experimental conditions for all 8 volunteers. The general trend is for the AUC to increase when noradrenaline is present in the perfusate and increase further when the drug wells are occluded in addition to the presence of noradrenaline.

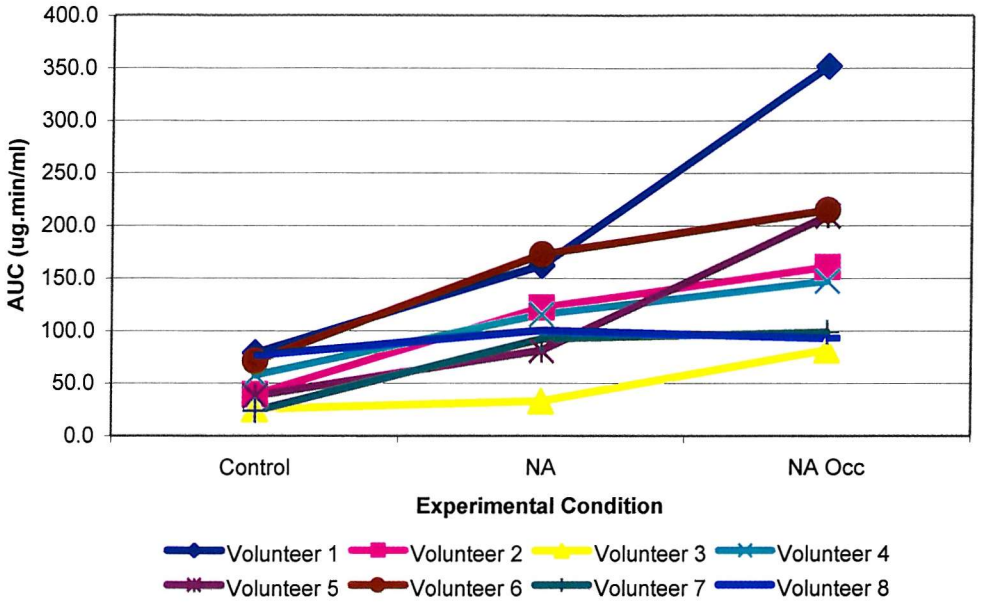

Figure 4.1a: Relationship of AUC between the three experimental conditions following the topical application of methyl salicylate.

4.4 Salicylic Acid Recovery From the Dermis After Topical Methyl Salicylate Application

The dialysate samples were analysed for salicylic acid which was detected in all samples (table 4.5) indicating cutaneous hydrolysis.

Subject	Fibre Type	Pre dose		0->1		1->2		2->3		3->4		4->5	
		Fibre 1	Fibre 2	Fibre 1	Fibre 2	Fibre 1	Fibre 2	Fibre 1	Fibre 2	Fibre 1	Fibre 2	Fibre 1	Fibre 2
1	Ringer's	0.12	0.13	0.28	0.32	0.54	0.60	0.39	0.51	0.29	0.36	0.18	0.13
	Noradrenaline	0.14	0.11	0.25	0.04	0.41	0.36	0.50	0.43	0.72	0.37	0.48	0.37
	Noradrenaline & Occlusion	0.12	0.13	0.37	0.32	0.83	0.53	1.02	0.84	1.23	0.70	1.31	0.94
2	Ringer's	0.04		0.15		0.48		0.84		0.93		0.61	
	Noradrenaline	0.10		0.20		0.39		0.48		0.50		0.47	
	Noradrenaline & Occlusion	0.09	0.06	0.21	0.14	0.47	0.22	0.44	0.27	1.17	0.46	0.99	0.47
3	Ringer's	0.08		0.07		0.15		0.15		0.08		0.09	
	Noradrenaline	0.00		0.52		1.01		1.05		1.16		0.97	
	Noradrenaline & Occlusion	0.03	0.04	0.13	0.12	0.47	0.65	0.73	1.29	0.91	1.46	1.11	1.55
4	Ringer's	0.00	0.00	0.11	0.05	0.45	0.16	0.47	0.17	0.31	0.09	0.21	0.07
	Noradrenaline	0.07	0.04	0.36	0.44	1.15	1.24	1.22	1.11	1.23	0.84	0.69	0.59
	Noradrenaline & Occlusion	0.05	0.04	0.25	0.20	0.98	0.70	1.46	1.06	1.59	1.14	1.59	1.07
5	Ringer's	0.00		0.37		0.54		0.38		0.21		0.00	
	Noradrenaline	0.10		0.65		1.41		1.35		0.90		0.00	
	Noradrenaline & Occlusion	0.05	0.00	0.44	0.44	1.49	1.74	1.33	2.88	2.26	2.32		
6	Ringer's	0.00	0.04	0.07	0.30	0.20	0.28	0.11	0.13	0.09	0.14	0.06	0.07
	Noradrenaline	0.04	0.10	0.34	0.10	0.38	0.24	0.40	0.25	0.36	0.24	0.29	0.21
	Noradrenaline & Occlusion	0.12	0.04	0.12	0.30.	0.51	0.60	0.63	0.62	0.62	0.85	0.69	0.96
7	Ringer's	0.00	0.00	0.19	0.20	0.23	0.24	0.14	0.13	0.06	0.03	0.00	0.06
	Noradrenaline	0.00	0.00	0.61	1.31	1.46	2.08	1.28	1.37	0.82	0.99	0.00	0.64
	Noradrenaline & Occlusion	0.03	0.04	0.76	0.86	1.97	2.40	2.46	2.54	2.05	2.03	1.70	1.84
8	Ringer's	0.00	0.00	0.36	0.62	0.45	1.02	0.53	0.82	0.19	0.55	0.13	0.33
	Noradrenaline	0.00	0.02	0.54	0.80	1.29	1.77	1.17	1.71	0.81	1.36	0.48	0.86
	Noradrenaline & Occlusion	0.00	0.00	0.52	0.54	1.44	1.26	1.82	1.61	1.83	1.52	1.66	1.17

Table 4.5: Summary of data from all volunteers and all lines. The values are the concentration (μg/ml) of salicylic acid in the dialysate sample for each line and at each time point following topical methyl salicylate application (space = not available due to analytical problems).

Collection	Ringers		NA		NA & Occ	
Period	Mean	SD	Mean	SD	Mean	SD
Pre-dose	0.05	0.08	0.06	0.05	0.05	0.04
0-1	0.24	0.15	0.48	0.29	0.38	0.24
1-2	0.43	0.19	1.07	0.55	1.08	0.66
2-3	0.42	0.20	1.01	0.41	1.41	0.74
3-4	0.30	0.21	0.84	0.23	1.48	0.55
4-5	0.22	0.17	0.55	0.17	1.28	0.34

Table 4.6: Table of mean value (in μg/ml) of salicylic acid concentration following topical methyl salicylate application, standard deviation for each time point for the three parameters. n = 8.

Figure 4.2 below and table 4.5 and 4.6 above show the results for the analysis for salicylic acid, the hydrolysis product/metabolite of methyl salicylate. Figure 4.2 shows the corrected average concentrations for all volunteers, the figures are derived by dividing by the relative recovery measured *in vitro* to give a

minimum tissue concentration. The concentrations of salicylic acid for all three lines were not zero in the pre-dose sample indicating either interference from an endogenous compound or a background concentration of salicylic acid. The averaged concentration for all three parameters increased more slowly than the methyl salicylate data. The Ringer's solution and noradrenaline lines (without occlusion) reached a peak between hours two and three. The Ringer's solution and noradrenaline lines reached a maximum concentration of 1.2μg/ml and 3.2μg/ml respectively before decreasing to 0.6 and 1.6μg/ml at 5 hours.

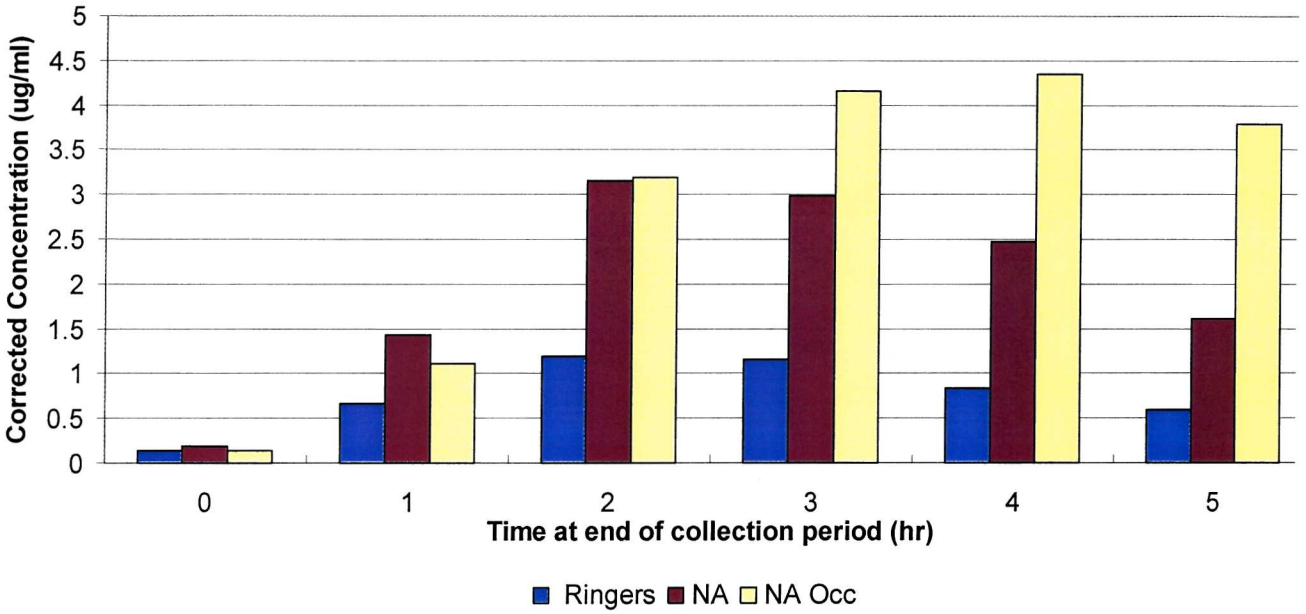

Fig. 4.2: Graph showing the effect of noradrenaline and occlusion on salicylic acid recovery following topical methyl salicylate application. n = 8.

The noradrenaline occluded line peaked later at hour 4 after dose application and reached a maximum concentration of 4.4μg/ml. This decreased to 3.8μg/ml by the fifth time point. Analysis by ANOVA using the AUC's showed that all three lines were statistically different from one another ($p<0.05$) (table 4.7).

Parameters	Control		NA		NA Occ	
	Mean	St Dev	Mean	St Dev	Mean	St Dev
Cmax (ug/ml)	0.46^{a}	0.21	1.01^{b}	0.52	1.49^{c}	0.63
Tmax (hr)	2.5	0.76	3.13	0.99	3.88	0.83
AUC (ug.min/ml)	84.3^{a}	39.8	202.7^{b}	91.9	277.7^{c}	129.6

Table 4.7 Table of pharmacokinetic values for the three experimental parameters for salicylic acid following topical methyl salicylate application. Values with different superscripts were significantly different ($p<0.05$) n = 8.

The C_{max}, T_{max} and AUC were calculated for all three experimental conditions (table 4.7) using the uncorrected data. The C_{max} showed significant differences between all three parameters with an increase from control to noradrenaline and finally noradrenaline occluded.

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The same trend exists with the AUC with the control having the lowest value and noradrenaline occluded giving the highest, the values were all significantly different with respect to each other.

The T_{max} appeared to be later for each successive experimental condition

4.5 Comparison Of Methyl salicylate and Salicylic acid Recovery

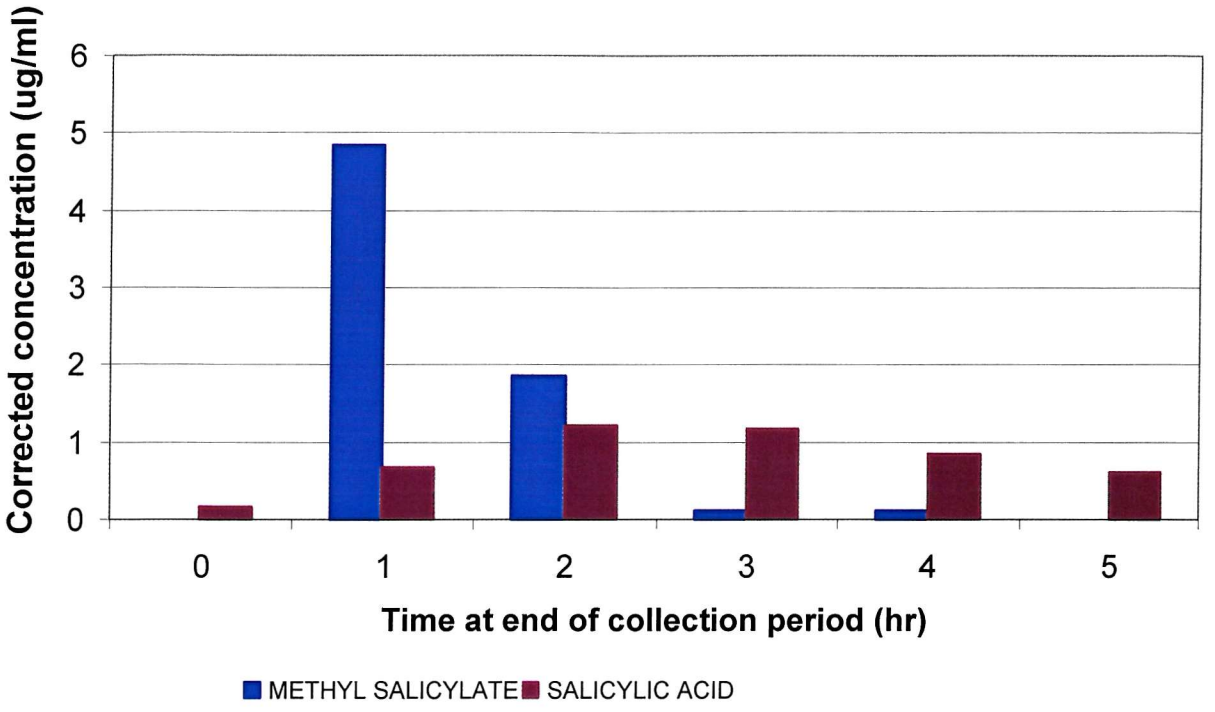

Fig. 4.3 Comparison of methyl salicylate and salicylic acid recovery from Ringer's solution lines following topical methyl salicylate application. n = 8.

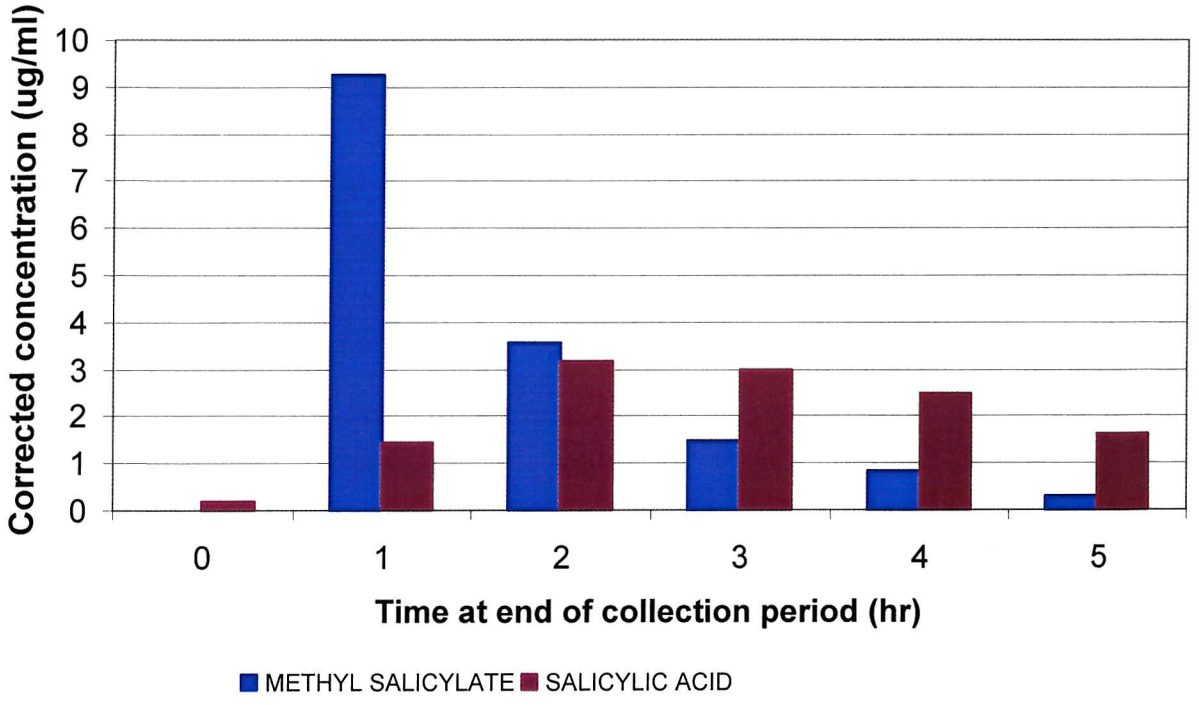

Fig. 4.4 Comparison of methyl salicylate and salicylic acid recovery from noradrenaline lines following topical methyl salicylate application. n = 8.

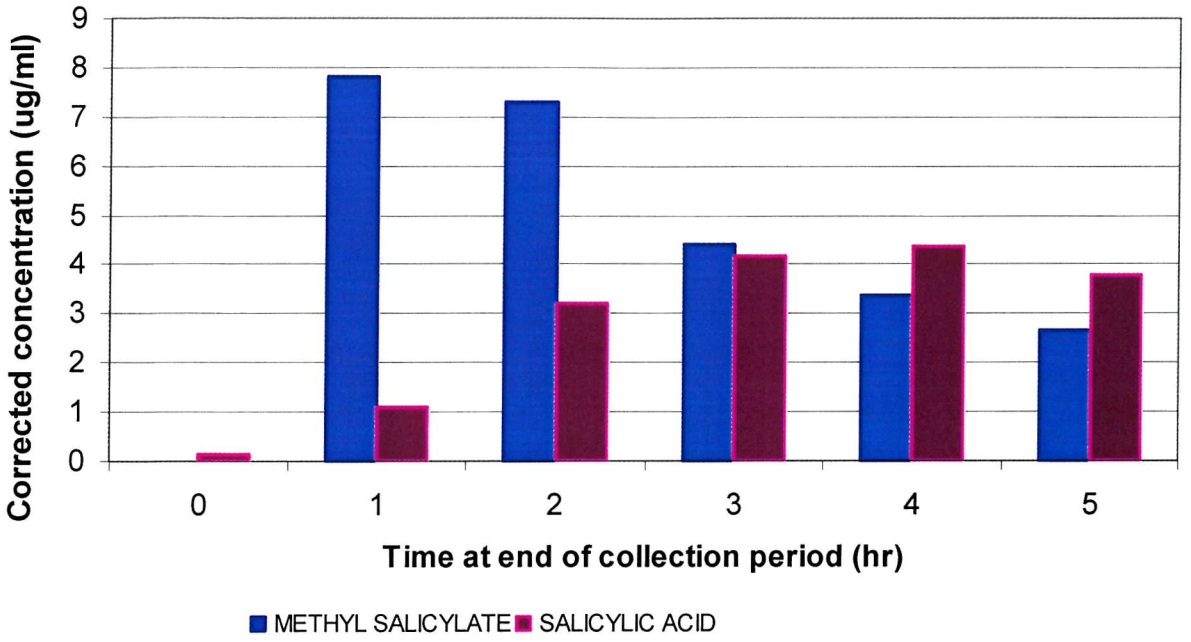

Fig. 4.5 Comparison of methyl salicylate and salicylic acid recovery from noradrenaline occluded lines following topical methyl salicylate application . n = 8.

Figures 4.3, 4.4 and 4.5 above all show a similar pattern, since the methyl salicylate peak recovery was always in the first hour followed by a continuous decrease, either sharply in the Ringer's solution and noradrenaline lines or slowly in case of the noradrenaline occluded lines. Salicylic acid showed a slow increase in concentration and peaked later in hours 3-4.

Figure 4.5a, shows the relationship of the methyl salicylate and salicylic acid AUC's for the three different experimental conditions. It clearly shows the inter-individual variability in both methyl salicylate absorption and cutaneous hydrolysis.

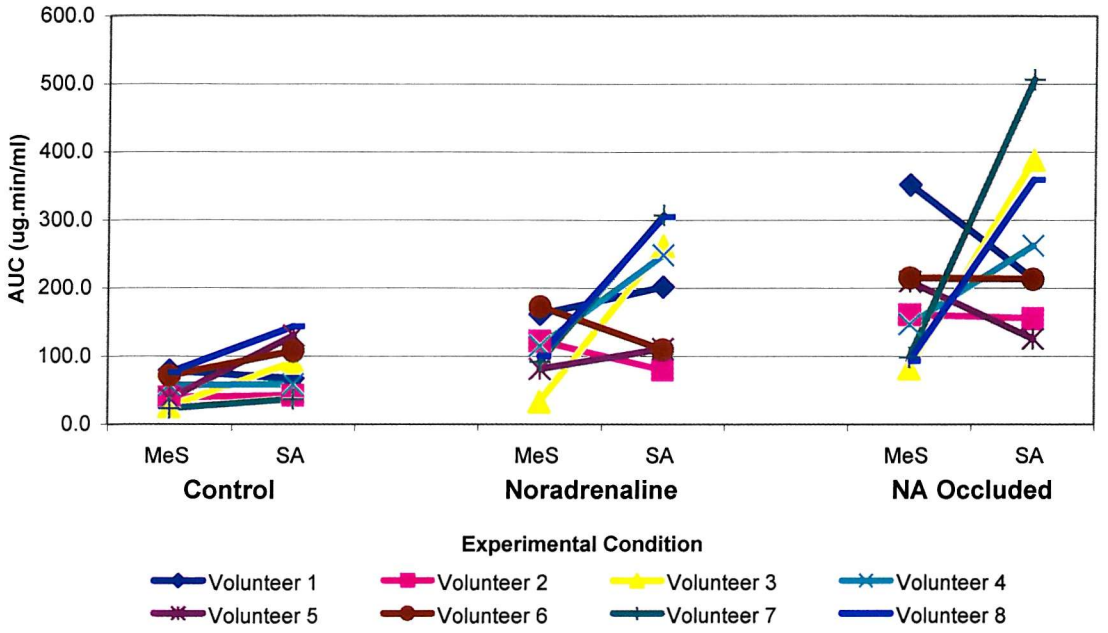

Figure 4.5a: Relationship of methyl salicylate and salicylic acid AUC's following topical application of methyl salicylate.

4.6 Probe Depth

At the end of each individual study the depth of the probes in the dermis was measured using an ultrasound scanner as detailed in the materials and methods section. None of the fibres contained the luminal wire inserts however filling the fibres with air prior to measurement made the fibres visible to ultrasound. Figures 4.6, 4.7 and 4.8 below show the relationship between the average measured probe depth and the AUC for each set of conditions and for each volunteer.

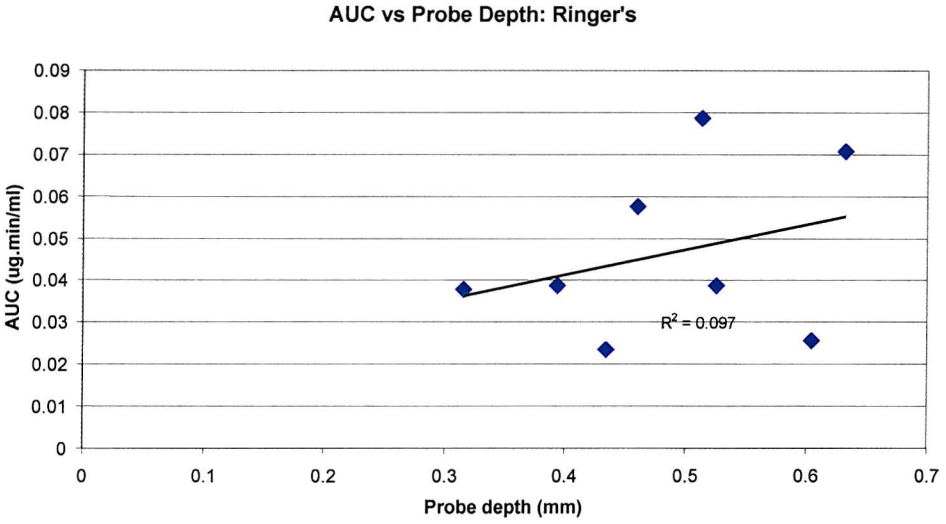

Figure 4.6: AUC (Ringer's line) vs probe depth for 8 volunteers. $R^2 = 0.097$.

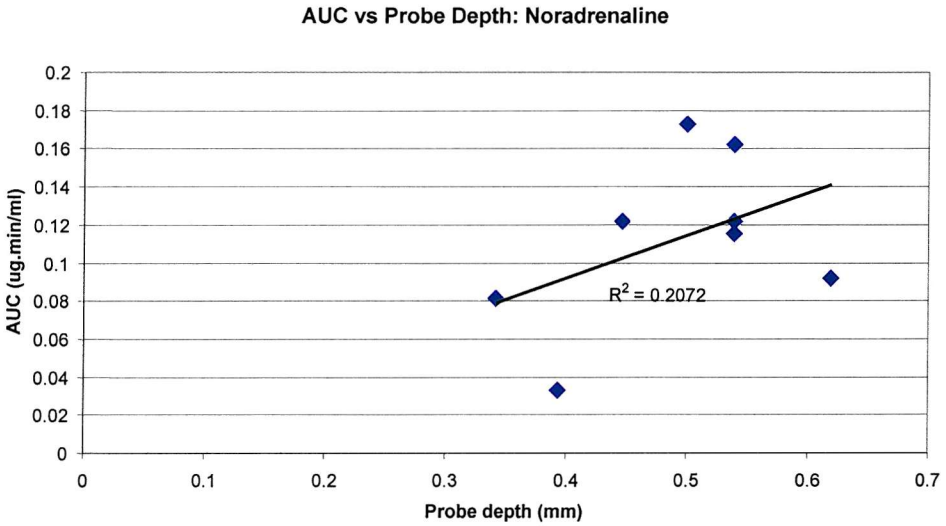

Figure 4.7: AUC (Noradrenaline line) vs probe depth for 8 volunteers. $R^2 = 0.2072$..

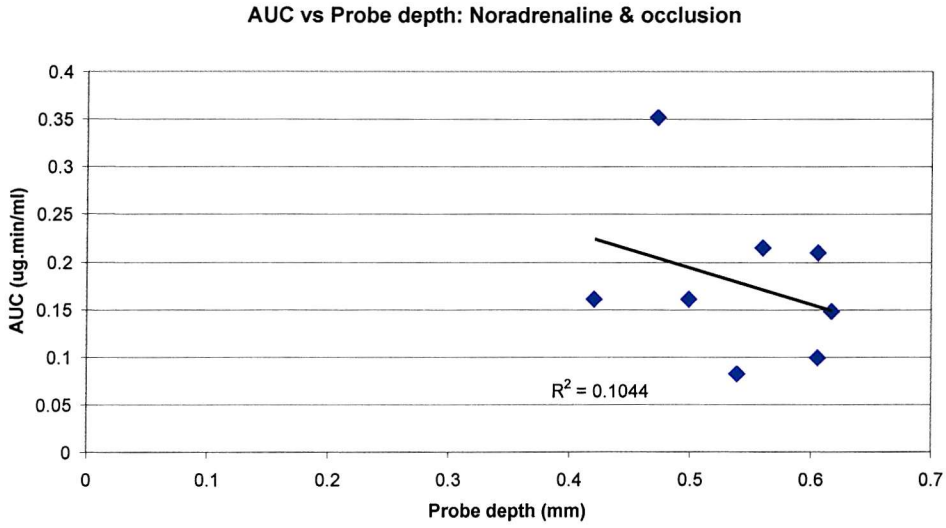

Figure 4.8: AUC (Noradrenaline & occlusion line) vs. probe depth for 8 volunteers. R^2 = 0.1044

The R^2 values show no positive correlation between probe depth and AUC in any of the three conditions studied. However the probes were inserted to a consistent depth of approximately 0.4-0.6mm, so that this conclusion only applies to this restricted range of depth.

4.7 Method: Ethanol Formulation

Two similar smaller studies were completed shortly after the study above. The protocol used was identical to the primary study apart from two exceptions; the formulation, and the drug used in the second of the smaller studies. The formulation base was changed to 50% water, 50% ethanol mix, to this base was added 3% carboxy-methylcellulose. This was required to thicken the formulation enough to prevent it from leaking from the sides of the drug well. In the first study methyl salicylate was added to the thickened base to make a 1% solution and in the second study the active metabolite salicylic acid was added to make a final concentration of 1%. There were 4 volunteers in each study, the average age for both studies was 20.6 (max. =24, min.=18), and all volunteers were students at the University of Southampton.

Due to problems with fibres remaining patent during the studies not all volunteers had the same number of functioning lines, (see table 4.8).

Volunteer	Study	Age	No. of Ringers Lines	No. of Noradrenaline Lines	No. NA Occluded Lines
1	Methyl salicylate	24	2	2	2
2	Methyl salicylate	20	1	2	2
3	Methyl salicylate	21	2	2	2
4	Methyl salicylate	20	2	2	2
5	Salicylic acid	23	1	2	2
6	Salicylic acid	18	1	2	2
7	Salicylic acid	20	2	1	2
8	Salicylic acid	19	2	2	2

Table 4.8: Volunteer Information: Study, age and number of patent lines per experimental parameter.

Subject	Line Type	Pre-Dose		0-0.5 hr		0.5-1 hr		1-1.5 hr		1.5-2 hr		2-2.5 hr		2.5-3 hr		3-3.5 hr		3.5-4 hr		4-4.5 hr		4.5-5 hr	
		1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
1	Ringers	0.00	0.00	2.94	2.43	2.26	4.26	0.83	1.80	0.25	0.55	0.16	0.22	0.00	0.15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Noradrenaline	0.00	0.00	2.95	1.99	5.89	3.97	4.60	3.75	3.31	3.53	2.02	2.25	0.74	0.97	0.45	0.76	0.17	0.55	0.09	0.34	0.00	0.13
	NA & Occlusion	0.00	0.00	2.27	1.97	4.68	3.93	2.20	3.39	2.28	2.86	3.12	2.67	3.14	2.49	1.77	2.52	1.79	2.56	0.76	2.60	2.76	2.63
2	Ringers	0.00		1.52		1.05		0.12		0.00		0.00		0.00		0.00		0.00		0.00		0.00	
	Noradrenaline	0.00	0.00	4.53	9.92	12.66	23.39	6.88	10.01	3.37	5.50	1.48	2.57	0.84	0.69	0.52	1.42	0.21	0.12	0.43	0.18	0.00	0.11
	NA & Occlusion	0.00	0.00	7.41	5.03	15.51	8.75	13.29	8.65	15.35	8.90	14.64	7.85	12.71	7.65	12.76	7.37	10.90	5.84	9.50	5.48	8.15	3.39
3	Ringers	0.00	0.00	2.31	1.72	1.67	2.04	0.77	0.57	0.00	0.19	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		
	Noradrenaline	0.00	0.00	8.02	4.02	7.77	5.89	7.10	1.60	2.28	1.16	1.03	0.55	0.51	0.34	0.20	0.16	0.15	0.11	0.14	0.08		
	NA & Occlusion	0.00	0.00	1.43	1.61	2.43	2.15	1.01	2.14	1.57	1.54	1.97	1.17	1.46	1.39	1.21	1.08	0.90	0.80	1.18	0.71		
4	Ringers	0.00	0.00	1.89	2.62	2.45	1.96	0.83	0.80	0.24	0.13	0.07	0.08	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Noradrenaline	0.00	0.00	6.96	3.51	12.35	7.51	7.24	4.08	3.70	2.69	1.88	1.42	0.64	0.72	0.69	0.48	0.15	0.29	0.14	0.28	0.06	0.06
	NA & Occlusion	0.00	0.00	3.66	2.91	7.20	5.29	6.27	3.95	6.58	4.25	6.16	4.31	5.53	4.08	5.45	3.45	4.75	2.84	4.27	2.47	3.89	2.07

Table 4.9 Summary of data from all volunteers and all lines. The values are the concentration (μg/ml) of methyl salicylate in the dialysate sample following topical methyl salicylate application. Spaces indicate sample was lost.

Subject	Line Type	Pre-Dose		0-0.5 hr		0.5-1 hr		1-1.5 hr		1.5-2 hr		2-2.5 hr		2.5-3 hr		3-3.5 hr		3.5-4 hr		4-4.5 hr		4.5-5 hr	
		1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
1	Ringers	0.00	0.00	0.00	0.14	0.14	0.20	0.16	0.28	0.23	0.33	0.22	0.31	0.19	0.31	0.20	0.28	0.12	0.21	0.19	0.20	0.11	0.15
	Noradrenaline	0.00	0.00	0.52	0.28	1.04	0.56	1.68	1.29	2.32	2.03	2.97	2.24	3.61	2.45	3.17	2.45	2.73	2.45	2.65	2.45	2.57	2.45
	NA & Occlusion	0.00	0.00	0.53	0.41	1.06	1.90	1.55	1.41	2.04	2.58	2.70	4.63	3.36	6.18	3.75	5.32	4.13	6.95	4.51	2.05	4.90	5.75
2	Ringers	0.00		0.80		1.10		0.82		0.44		0.25		0.25		0.15		0.14		0.05		0.05	
	Noradrenaline	0.00	0.00	0.78	0.83	3.63	2.86	3.02	3.67	4.51	3.79	4.50	4.53	4.70	4.14	5.22	4.32	3.50	3.94	3.11	3.92	2.64	2.21
	NA & Occlusion	0.00	0.00	0.33	1.06	1.81	3.34	2.27	4.23	3.09	4.67	3.49	4.92	3.06	4.79	3.32	5.32	3.13	4.44	3.18	5.18	3.15	4.76
3	Ringers	0.00	0.00	0.41	0.84	0.73	1.29	0.93	0.67	0.71	0.95	0.48	0.70	0.34	0.00	0.21	0.30	0.20	0.27	0.11	0.41		
	Noradrenaline	0.00	0.00	1.26	0.78	2.22	2.37	2.78	2.09	2.61	2.55	2.93	2.73	3.03	3.09	2.89	2.74	2.86	2.42	2.32	2.09		
	NA & Occlusion	0.00	0.00	0.60	0.77	1.84	1.24	3.54	1.92	3.27	2.50	2.61	2.52	3.56	3.00	4.15	2.73	3.43	2.96	3.78	2.68		
4	Ringers	0.00	0.00	0.59	0.21	0.86	0.43	0.59	0.55	0.57	0.47	0.42	0.35	0.19	0.26	0.24	0.21	0.21	0.16	0.22	0.15	0.10	0.09
	Noradrenaline	0.00	0.00	0.83	0.61	2.33	2.19	2.62	2.14	2.99	3.03	4.03	3.16	4.45	3.41	4.18	3.47	4.58	2.79	2.76	2.55	3.98	2.55
	NA & Occlusion	0.00	0.00	0.54	0.73	1.36	2.08	1.96	3.11	2.64	3.32	2.99	3.41	3.22	3.90	3.07	4.41	2.94	4.00	2.84	4.49	1.36	3.83

Table 4.10 Summary of data from all volunteers and all lines. The values are the concentration (μg/ml) of salicylic acid in the dialysate sample following topical methyl salicylate application. Spaces indicate sample was lost.

4.8 Methyl Salicylate Absorption and Recovery: Ethanol formulation

Tables 4.9, 4.10, 4.11 and 4.12 show the methyl salicylate and salicylic acid data from all microdialysis probes from volunteers 1-4 from the methyl salicylate study. The concentration profile for both methyl salicylate and salicylic acid follows the same shape seen in the larger propylene glycol study. The major difference between the studies is the level of concentrations measured. At all time points in this study the concentration of methyl salicylate and salicylic acid measured was statistically higher than that seen in their respective propylene glycol study samples ($p<0.05$). For statistical purposes each time point from the propylene glycol study was compared to the average of the two respective half hour collection points from the ethanol study. Again like the previous study the inter-subject variability was high (table 4.11, 4.12).

MeS	Ringers		NA		NA Occ	
TIME	Mean	SD	Mean	SD	Mean	SD
Pre-Dose	0.00	0.00	0.00	0.00	0.00	0.00
0-0.5 hr	2.07	0.59	5.24	2.48	3.28	2.56
0.5-1 hr	2.06	1.12	9.93	7.08	6.24	5.20
1-1.5 hr	0.70	0.60	5.66	2.42	5.11	5.11
1.5-2 hr	0.16	0.21	3.19	1.37	5.42	5.83
2-2.5 hr	0.06	0.11	1.65	0.75	5.24	5.25
2.5-3 hr	0.02	0.04	0.68	0.23	4.81	4.71
3-3.5 hr	0.00	0.00	0.58	0.40	4.45	4.89
3.5-4 hr	0.00	0.00	0.22	0.12	3.80	4.01
4-4.5 hr	0.00	0.00	0.21	0.10	3.37	3.59
4.5-5 hr	0.00	0.00	0.06	0.01	4.23	2.18

Table 4.11: Table showing the effect of noradrenaline and occlusion on methyl salicylate concentration (μg/ml) following topical methyl salicylate application. The results are the mean sample concentration ± the standard deviation for 4 subjects (using the average value where data from two similar fibres were available).

SA	Ringers		NA		NA Occ	
TIME	Mean	SD	Mean	SD	Mean	SD
Pre-Dose	0.00	0.00	0.00	0.00	0.00	0.00
0-0.5 hr	0.50	0.38	0.91	0.57	0.73	0.28
0.5-1 hr	0.76	0.52	2.11	1.23	2.12	0.57
1-1.5 hr	0.61	0.34	2.14	1.05	2.39	0.89
1.5-2 hr	0.52	0.28	2.63	1.36	2.92	0.84
2-2.5 hr	0.37	0.19	2.90	1.50	3.57	0.69
2.5-3 hr	0.22	0.05	2.96	1.50	3.92	0.86
3-3.5 hr	0.21	0.06	2.91	1.81	3.89	0.94
3.5-4 hr	0.18	0.05	2.39	1.44	3.99	1.46
4-4.5 hr	0.17	0.11	2.34	1.30	3.22	0.99
4.5-5 hr	0.10	0.07	2.47	0.06	4.64	0.97

Table 4.12: Table showing the effect of noradrenaline and occlusion on salicylic acid concentration (μg/ml) following topical methyl salicylate application. The results are the mean sample concentration ± the standard deviation for 4 subjects (using the average value where data from two similar fibres were available).

Figures 4.9 and 4.10 below show the corrected tissue concentrations for the methyl salicylate and salicylic acid data taking into account the relative recovery measured *in vitro*. The concentration calculated this way was a minimum tissue concentration; the level of disparity to the actual tissue concentration depends on how close the *in vitro* and *in vivo* relative recoveries were.

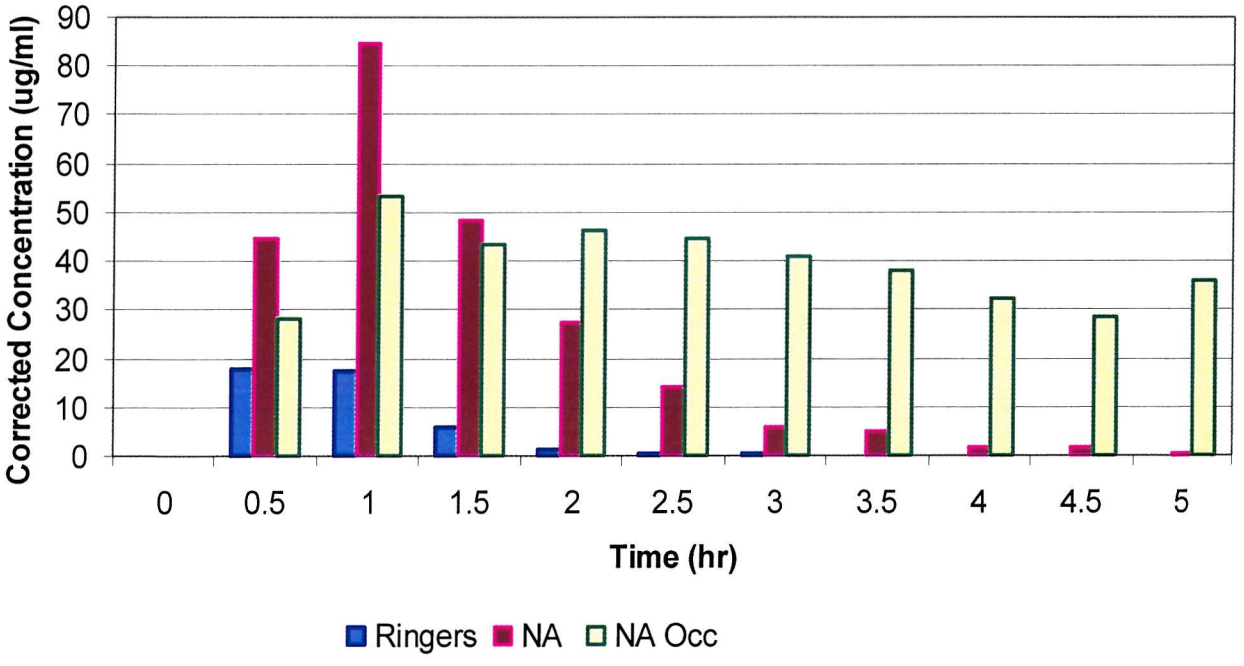

Fig. 4.9: Corrected methyl salicylate concentration using *in vitro* relative recovery following topical methyl salicylate application. Average value for 4 volunteers.

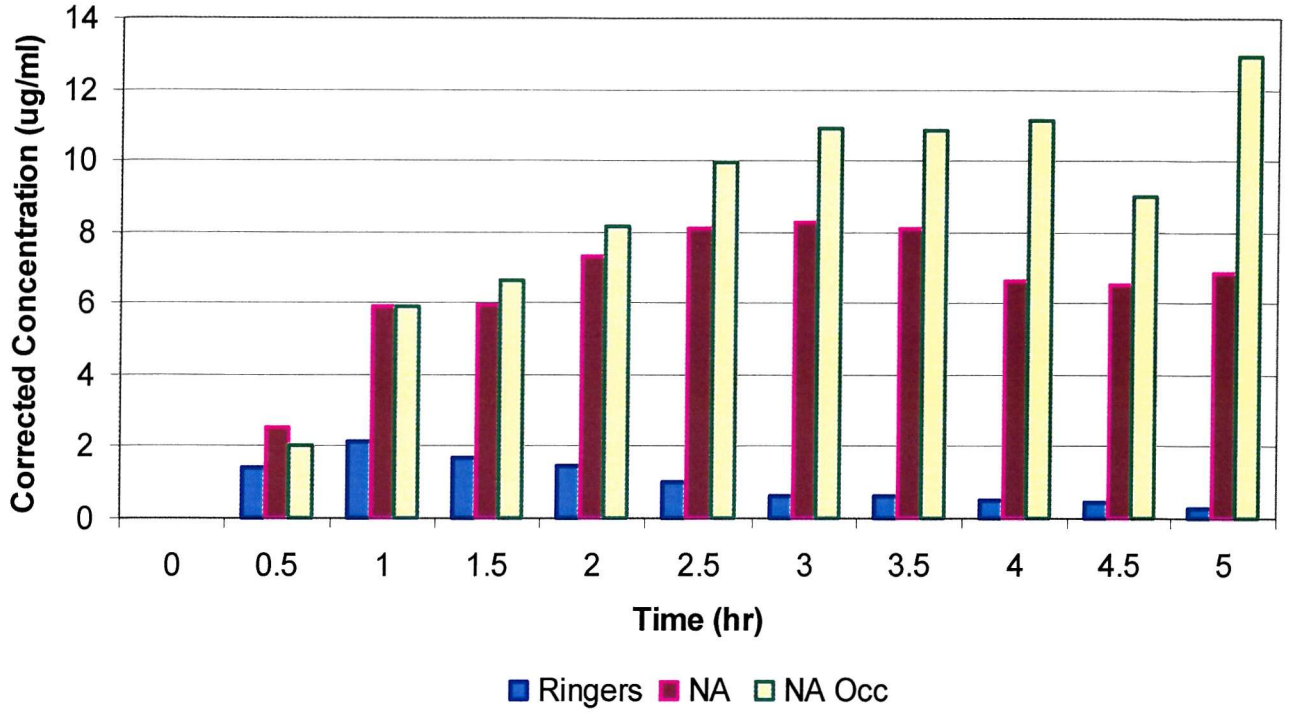

Fig. 4.10: Corrected salicylic acid concentration using in vitro relative recovery following topical methyl salicylate application. Average value for 4 volunteers.

Tables 4.13 and 4.14 show three pharmacokinetic properties for the methyl salicylate and salicylic acid data, C_{max}, T_{max} and AUC. For all measured properties for both compounds the control line was found to be significantly different to the noradrenaline and noradrenaline occluded lines. There was no significant difference between the two noradrenaline lines in any of the properties measured in either compound.

Parameter	Control		Noradrenaline		Noradrenaline Occluded	
	Mean	SD	Mean	SD	Mean	SD
C_{max} (μg/ml)	2.26^a	0.73	9.93^b	5.78	6.23^b	3.26
T_{max} (hr)	0.63	0.25	1.00	0.00	1.00	0.00
AUC (μg.min/ml)	156.02^a	65.28	821.49^b	318.94	1294.54^b	1040.25

Table 4.13: Table of pharmacokinetic values for parameters derived from the methyl salicylate data following topical methyl salicylate application. Values with different superscripts were significantly different ($p<0.05$) n = 4.

Parameter	Control		Noradrenaline		Noradrenaline Occluded	
	Mean	SD	Mean	SD	Mean	SD
C_{max} (μg/ml)	0.76^{a}	0.37	3.6975^{b}	0.83	4.0675^{b}	0.59
T_{max} (hr)	1.25	0.50	3.125	0.25	3.375	0.25
AUC (μg.min/ml)	105.65^{a}	36.48	772.33^{b}	188.68	849.98^{b}	120.74

Table 4.14: Table of pharmacokinetic values for parameters derived from the salicylic acid data following topical methyl salicylate application. Values with different superscripts were significantly different ($p<0.05$) n = 4.

When the ethanol formulation AUC's were compared to their respective propylene glycol formulation AUC's, they were found to be all significantly larger ($p<0.05$) except for the control line salicylic acid AUC ($p>0.05$).

4.9 Salicylic acid Absorption and Recovery: Ethanol formulation

Tables 4.15 and 4.16 and figure 4.11 show all the data from volunteers 4-8 from the salicylic acid study. The two tables show the actual sample concentration measured, figure 4.11 shows the corrected tissue concentration taking into account the *in vitro* relative recovery.

Collection Point	Control		NA		NA Occ	
	Mean	SD	Mean	SD	Mean	SD
Pre-Dose	0.00	0.00	0.06	0.08	0.02	0.03
0-0.5 hr	2.83	2.61	4.80	3.43	2.69	1.98
0.5-1 hr	8.60	6.66	28.35	20.85	17.38	15.07
1-1.5 hr	11.84	9.03	37.80	27.06	43.46	37.87
1.5-2 hr	12.10	10.73	40.82	28.36	61.11	43.50
2-2.5 hr	10.40	10.77	36.83	26.05	68.79	43.07
2.5-3 hr	11.30	10.15	39.64	23.15	69.33	41.56
3-3.5 hr	8.78	5.59	42.83	28.80	62.09	38.02
3.5-4 hr	12.49	7.73	40.58	27.44	73.38	54.37
4-4.5 hr	12.47	7.99	38.79	27.43	66.24	45.97
4.5-5 hr	12.96	8.24	36.54	27.64	68.46	53.85

Table 4.15: Table showing the effect of noradrenaline and occlusion on salicylic acid concentration (μg/ml) following topical salicylic acid application. The results are the mean sample concentration ± the standard deviation for 4 subjects (using the average value where data from two similar fibres were available).

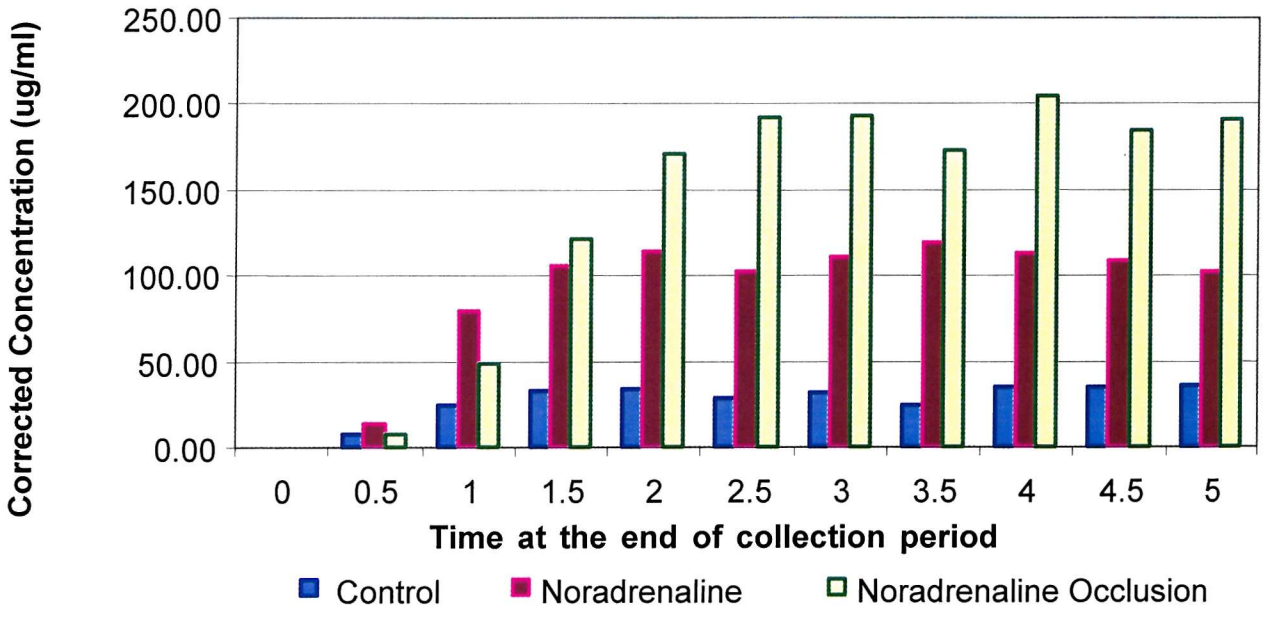

Fig. 4.11 Corrected salicylic acid concentration following topical salicylic acid application. Average value for 4 volunteers

Subject	Line Type	Pre-Dose		0-0.5 hr		0.5-1 hr		1-1.5 hr		1.5-2 hr		2-2.5 hr		2.5-3 hr		3-3.5 hr		3.5-4 hr	
		Fibre 1	Fibre 2	Fibre 1	Fibre 2	Fibre 1	Fibre 2	Fibre 1	Fibre 2	Fibre 1	Fibre 2	Fibre 1	Fibre 2	Fibre 1	Fibre 2	Fibre 1	Fibre 2	Fibre 1	Fibre 2
1	Ringers	0.00		2.76		14.95		19.87		26.26		25.70		25.21		12.76		21.50	
	Noradrenaline	0.00	0.00	7.91	2.63	64.18	35.29	73.89	54.63	67.82	65.75	81.15	52.85	77.47	54.75	84.53	71.05	83.70	62.52
	Noradrenaline & Occlusion	0.00	0.00	7.68	1.79	69.23	4.36	109.43	80.39	130.62	107.80	146.35	104.58	150.33	99.29	99.36	126.86	152.62	146.57
2	Ringers	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.18	0.26	0.34	0.28	0.67	0.64	1.03	0.63	2.36	1.63	4.47
	Noradrenaline	0.00		0.00		0.00		0.13		0.43		4.39		10.47		8.76		7.06	
	Noradrenaline & Occlusion	0.00	0.00	0.00	0.00	0.13	0.00	6.71	3.02	15.72	13.40	21.87	20.38	24.82	24.74	16.56	27.21	28.40	24.63
3	Ringers		0.00		6.30		12.56		17.90		12.09		7.02		9.95		13.54		14.82
	Noradrenaline	0.21	0.12	8.22	8.04	41.01	16.48	59.95	21.34	66.64	27.16	40.41	23.66	47.08	24.65	42.28	27.95	37.81	31.91
	Noradrenaline & Occlusion	0.12	0.00	5.97	0.69	20.24	10.29	36.14	25.05	72.20	26.92	87.55	32.04	86.04	30.75	74.46	28.08	64.50	23.54
4	Ringers	0.00	0.00	3.15	1.38	6.28	7.47	8.95	10.02	6.17	13.30	3.65	13.18	5.29	13.12	7.08	7.56	8.22	12.99
	Noradrenaline	0.11	0.04	8.06	3.56	52.59	17.26	66.92	25.37	67.23	31.11	60.78	26.97	62.28	29.96	63.41	35.92	60.75	33.83
	Noradrenaline & Occlusion	0.00	0.04	0.83	4.55	4.93	29.82	37.38	49.53	50.15	72.07	52.83	84.76	51.62	87.04	57.16	67.01	66.17	80.58

Table 4.16: Summary of data from all volunteers and all lines. The values are the concentration (μg/ml) of salicylic acid in the dialysate sample following topical salicylic acid application. Spaces indicate sample was lost.

The profile shown by salicylic acid for all three line types was broadly similar, all three line types showed an initial increase which appeared to reach steady state after several hours. The control and noradrenaline lines seemed to reach steady state slightly earlier than the occluded line, 1-1.5 hrs compared to 2-2.5 hrs. The corrected steady-state concentrations for the control, noradrenaline and noradrenaline occluded lines are approximately 35, 110 and 190 μg/ml respectively.

For all lines the inter-subject variability was high (table 4.15) as indicated by the large standard deviations.

Table 4.17 shows the average C_{max}, T_{max}, and AUC for the 4 volunteers in the salicylic acid study. The C_{max} from the ringers line was found to be significantly lower than the two noradrenaline lines but no significant difference was found between the noradrenaline and noradrenaline occluded lines. No significant difference was found between any of the lines regarding the T_{max}. Comparison of the AUC showed a significant difference between the ringers line and the two noradrenaline lines

Parameter	Ringers		Noradrenaline		Noradrenailine Occluded	
	Mean	SD	Mean	SD	Mean	SD
C_{max} (μg/ml)	14.24^{a}	10.06	43.39^{b}	23.71	69.84^{b}	41.73
T_{max} (hr)	2.38	1.11	2.3750	0.48	2.7500	0.29
AUC (μg.min/ml)	2918.48^{a}	2141.00	9862.15^{b}	6701.36	14961.04^{b}	10370.86

Table 4.17: Table of pharmacokinetic values for parameters derived from the salicylic acid data from volunteers 4-8 following topical salicylic acid application. Values with different superscripts were significantly different using a paired student t test ($p<0.05$)

The AUC's from this study were compared to the AUC's from the methyl salicylate study calculated for methyl salicylate. The AUC for salicylic acid absorption was significantly higher than methyl salicylate absorption for all three line types ($p<0.05$).

4.10 Discussion

4.10.1 Propylene Glycol Formulation

For all time points Ringer's solution gave consistently lower recoveries than the noradrenaline lines. The presence of noradrenaline in the perfusate had the visible effect of blanching, due to shutting down cutaneous blood flow for up to 0.5cm around the implanted fibre. This reduction in blood flow would decrease removal from the dermis and allow a higher concentration of methyl salicylate to be retained in the tissue surrounding the microdialysis probes. Both the noradrenaline and noradrenaline/occluded lines gave higher initial peaks in comparison to the Ringer's solution lines. The implanted fibres would have been just below or within the papiliary plexus that lies just beneath the dermo-epidermal junction. These capillaries would be able to dialyse drug in the same way as the implanted fibres. Noradrenaline reduced the capability of the capillary network to remove compounds.

All three lines gave their highest concentrations of methyl salicylate at the first or second time point followed by a reduction in the concentration. The unoccluded lines gave an almost identical profile, a sharp initial peak followed by a rapid decrease. The occluded line on the other hand had almost a plateau region after the first hour followed by a gradual decrease. Several theories may be proposed to explain the rapid decrease in concentration over time despite the high concentration remaining in the drug well on the surface. One possibility is that the layer of the formulation adjacent to the skin becomes depleted of drug rapidly and that diffusion of drug from higher layers in the formulation is slow, resulting in much lower concentrations of drug on the skin's surface after the first hour. However this theory would only be a major factor for viscous formulations or those made into creams or gels where diffusion would be limited. The formulation used in these studies had a very fluid consistency, which would offer little resistance to free diffusion of the drug. Also if this was the reason it would not be expected to see such a marked difference between the occluded and unoccluded noradrenaline lines as occlusion should not have any direct effect on the diffusional characteristics within the formulation. Another possibility is that protein

becomes deposited on the outside of the fibre within the dermis as part of the inflammation process triggered by the insertion of the needles and with time the pores in the fibre slowly become blocked reducing recovery. Again if this were a major factor in these studies the two noradrenaline lines (with and without occlusion) should be similar especially at later time points as both should have equal levels of protein present in the skin. The most likely factor accounting for the decrease in the unoccluded lines is that the formulation dried up and no longer remained in solution in contact with the skin. This is supported by observational data, since the formulation in the unoccluded wells became viscous after approximately 3 hours and by the end of the studies resembled a very thick gel. By comparison when the occluded drug wells were removed at the end of the studies the remaining formulation was far less viscous. The drying out of the unoccluded wells allows the viscosity of the formulation to rise to a point where free diffusion of the drug would be hindered, which would make the idea of a drug depleted layer of formulation adjacent to the skin much more feasible. Evaporation of the applied formulation would also result in a mixture that has a much higher concentration of propylene glycol. Methyl salicylate has a higher solubility in propylene glycol than in water and therefore increasing the ratio of propylene glycol to water will have large affect on the partition coefficient between the formulation and the stratum corneum. In effect the point of equilibrium would move towards the formulation as it begins to dry and its composition changes. By occluding the drug well the formulation remains more fluid and retains an advantageous partition coefficient with regards to the stratum corneum.

Analysing for salicylic acid, the major metabolite of methyl salicylate, gave a different profile but a similar correlation between the different conditions. Rather than the concentrations peaking early, a slow gradual rise was seen with the concentrations reaching a maximum between two and four hours. The noradrenaline lines showed consistently higher concentrations than the Ringer's lines. The Ringer's and unoccluded noradrenaline lines both peaked after approximately two hours and like the methyl salicylate profiles were almost identical in shape to each other. In contrast the concentrations in the noradrenaline occluded lines increased for four hours. As the salicylic acid concentration is a reflection of the methyl salicylate absorption, the same

explanations are true for the differences between the three parameters. Reduced removal due to skin blood flow would result in the noradrenaline lines showing higher levels of salicylic acid than the Ringer's lines. The thickening of the unoccluded formulations through evaporation/absorption would reduce the amount of the drug absorbed towards the end of the studies causing the concentrations of salicylic acid to decrease quicker than in the noradrenaline occluded lines.

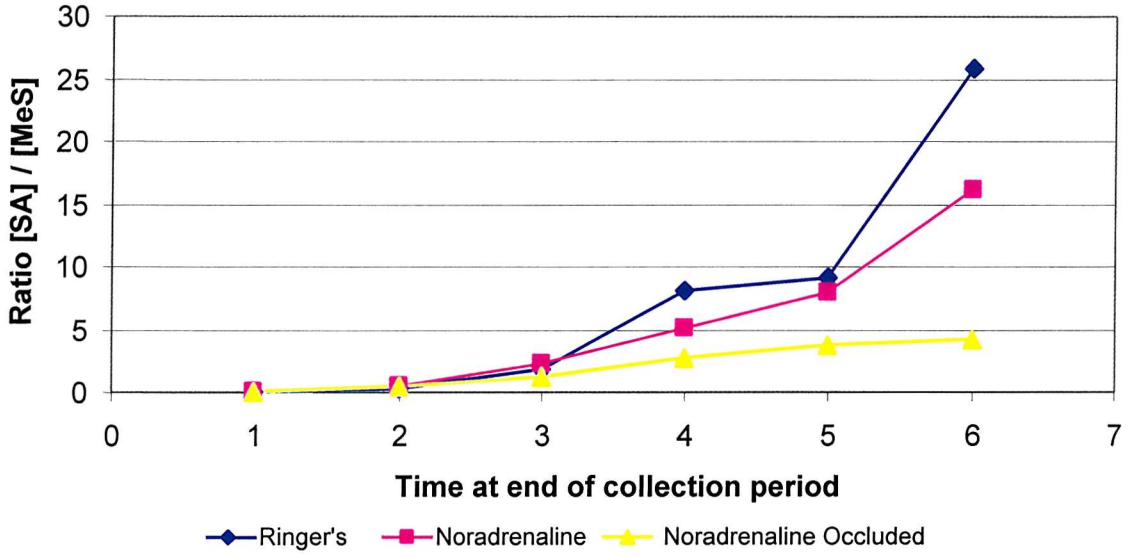

Figure 4.12: Ratio of average salicylic acid and methyl salicylate concentration for the three parameters studied following topical methyl salicylate application. n = 8.

Figure 4.12 above shows how the ratio of salicylic acid to methyl salicylate varies over course of the averaged studies. In all three conditions the ratio increased over time which is consistent with the hypothesis that methyl salicylate is being metabolised to salicylic acid. As methyl salicylate is metabolised, salicylic acid builds up in the tissue space increasing the ratio between the two compounds. The ratios increased at different rates in the order: Ringer's > noradrenaline > noradrenaline & occlusion. This difference is a result of two factors, the first is the delay in the appearance of salicylic acid, and the second is the difference in penetration of methyl salicylate in the three lines over time.

The major difference between the data for salicylic acid and methyl salicylate is the delay in the appearance of the salicylic acid and the much slower fall in

4.10.2 Ethanol Formulation

Both the methyl salicylate and salicylic acid studies show the same effect of altering the cutaneous blood flow as the larger methyl salicylate/propylene glycol study. Reducing the cutaneous blood flow by the use of noradrenaline results in a higher C_{max} and a higher AUC, as absorbed drug is no longer being constantly removed by the systemic circulation.

The effects of occlusion are less clearly shown, both the methyl salicylate and salicylic acid profiles appear to show that occlusion either allows the concentration to reduce more slowly over time or reach a higher steady state concentration. However neither the C_{max} nor the AUC showed any significant difference between the noradrenaline and noradrenaline-occluded lines even though paired t tests were used to minimise the impact of the large inter-individual differences. The lack of significance is most likely a result of the small group sizes in the two studies. It might also be a direct consequence of using ethanol as the base of the formulation. With the propylene glycol formulation the most volatile solvent was the water component, as the water evaporated from the unoccluded drug wells the remaining formulation contained an increasing percentage of propylene glycol. This increased the partition coefficient of the formulation with respect to the drug and so moved the equilibrium between the formulation and the stratum corneum to favour the formulation more. Therefore throughout the course of the study the amount of drug capable of diffusing into the skin from the applied formulation is steadily decreasing. However when using an ethanol based formulation, the most volatile component is the ethanol which evaporates throughout the course of the study. Both salicylic acid and methyl salicylate are more soluble in ethanol than water, therefore the two lipophilic drugs becomes less soluble in the applied formulation over time. The partition coefficient between the stratum corneum and formulation changes to favour the stratum corneum and thereby increasing the flux of drug through the skin. This would tend to offset the effect of the formulation thickening over the course of the study. The formulations used contained 3% carboxy-methylcellulose to prevent the formulation from leaking from the drug wells. Due to evaporation over the course of the study the applied formulation became steadily more viscous in the unoccluded wells, the ability of the drug to diffuse through the formulation

would be reduced as viscosity increased. It is possible that the formulation adjacent to the skins surface would become depleted of compound i.e. drug would be absorbed by the stratum corneum faster than it could diffuse through the formulation effectively reducing the flux across the skin. So in the unoccluded drug wells there are two competing factors modifying the absorption of salicylic acid and methyl salicylate. Firstly the shifting equilibrium towards the stratum corneum as the ethanol component of the formulation evaporates and the increased viscosity of the formulation reducing the concentration of compound immediately adjacent to the skins surface.

The clearest consequence of using ethanol as the base of the formulation was the significantly increased levels of absorption of methyl salicylate compared to the propylene glycol formulation. Both the C_{max} and the AUC for all lines was significantly higher for the ethanol formulation compared to the propylene glycol formulation. Ethanol is an effective penetration enhancer (Guy *et al*, 1990); it is thought to act by penetrating and disrupting the semi-crystalline structure of the lipid matrix in the intercellular space (Barry 1987). The matrix is a lipid bi-layer and the ethanol being a polar molecule disrupts the close packing of the polar head groups of the fatty acids. This increases the fluidity of the intercellular medium, which in turn allows absorbed compounds to have a higher rate of diffusion through the intercellular space. When the drug wells were removed at the end of the ethanol studies the areas of skin where drug solution had been applied were found to be slightly raised in relation with the surrounding skin. This had not been seen in the propylene glycol studies. Furthermore when the depths of the lines were measured using ultrasound the epidermal tissue and to a lesser degree dermal tissue under the application site were found to be far less dense than the surrounding tissue. Again this had not been seen previously in the propylene glycol study, see figures 4.13 and 4.14. The penetration of the ethanol had caused the epidermal tissue to swell and become less dense and had probably allowed the water in the formulation to hydrate the skin more than was the case with the propylene glycol formulation. Both of which resulted in an increased state of fluidity in the upper layers of the skin, which led to a higher rate of diffusion for the applied compound.

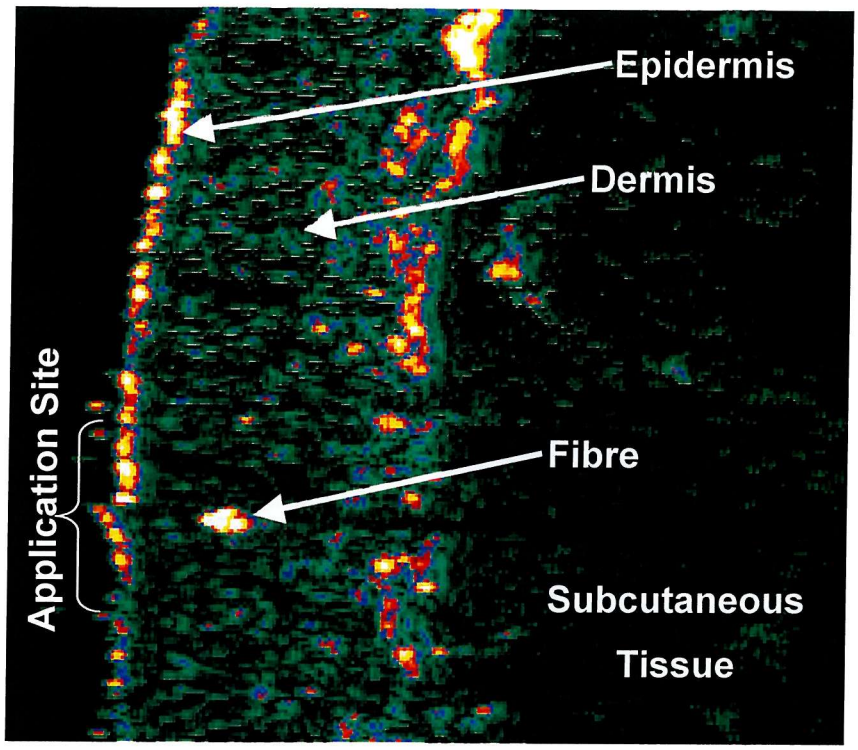

Figure 4.13: Ultrasound image of implanted fibre. Formulation used: methyl salicylate in propylene glycol/water.

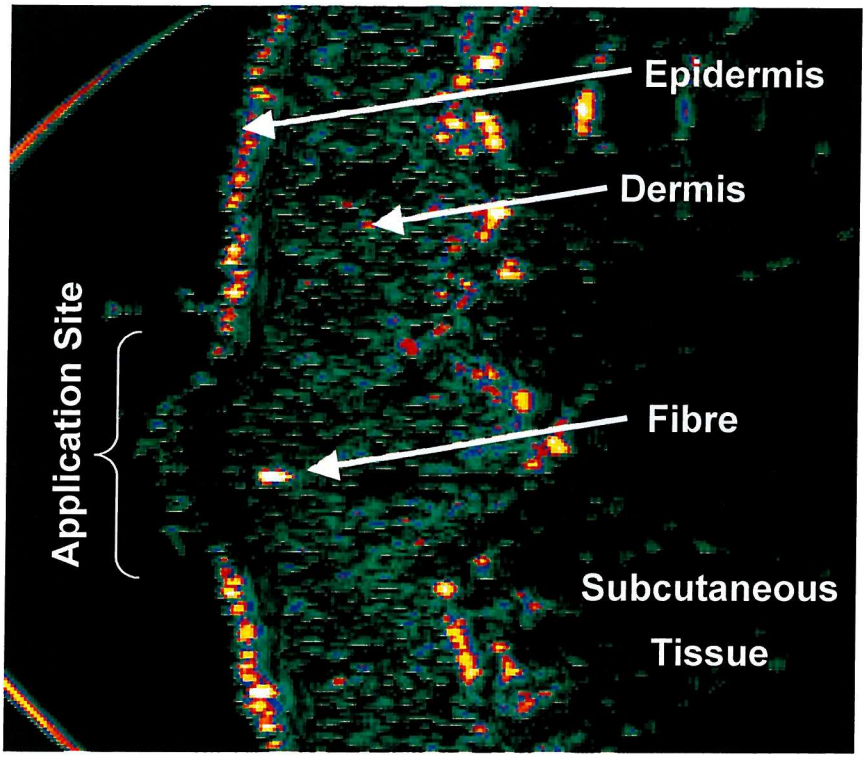

Figure 4.14: Ultrasound image of implanted fibre. Formulation used: methyl salicylate in ethanol/water.

The last observation made was the larger tissue concentrations of salicylic acid compared to methyl salicylate after application of the two different compounds. The AUC and C_{max} were significantly larger for salicylic acid in all three line types compared to methyl salicylate. The applied concentrations of both compounds were identical and both compounds have similar chemical structures. However salicylic acid is more soluble in an ethanol/water solution compared to methyl salicylate and so it should partition more into the formulation more than methyl salicylate would be expected to. This would suggest that salicylic acid ought to give a lower tissue concentration after application, as it would be less likely to partition into the stratum corneum. There are two possible explanations for the extremely high salicylic acid tissue concentrations: the first is the difference in $P_{octanol/water,}$ methyl salicylate has a value of approximately 3 and salicylic acid is approximately 2.5. The values mean that both compounds are very lipophilic and would partition preferentially into a non-aqueous environment. Methyl salicylate however is more lipophilic than salicylic acid and is only sparingly soluble in aqueous solvents unlike salicylic acid, which is more soluble in water. Therefore both compounds will partition into the stratum corneum preferentially to the applied formulation but methyl salicylate has a higher partition coefficient with respect to the stratum corneum and so will have a higher level of flux. However once it has partitioned into the stratum corneum the large $P_{octanol/water}$ value of methyl salicylate means that only a small fraction will partition out of the epidermis and move into the dermis. Only when the compound has partitioned into the dermis can it be detected via the microdialysis fibre. The lower $P_{octanol/water}$ of salicylic acid means that a larger fraction will be able to partition into the dermis leading to a higher measured tissue concentration. In summary there are two partitioning events that control the dermal concentration of both compounds. Firstly the movement of drug from formulation to stratum corneum/epidermis; the higher $P_{octanol/water}$ of methyl salicylate allows it to partition more into the stratum corneum than salicylic acid though both will partition preferentially into the stratum corneum. The second is the movement of drug out of the lipid rich stratum corneum/epidermis into the more aqueous dermal tissue; this time the high $P_{octanol/water}$ value of methyl salicylate will result in a reservoir forming in the upper layers of the skin, however a larger fraction of the absorbed salicylic acid will partition into the dermal tissue due

to its less lipophilic nature. Of the two compounds salicylic acid has a $P_{octanol/water}$ value that best allows penetration to the dermal tissue i.e. lipophilic enough to allow partitioning into the stratum corneum but hydrophilic enough to allow it to partition into the dermal tissue. There is another factor that may allow salicylic acid to be absorbed in greater amounts, which is that salicylic acid is a known de-keratinising agent. Application over the length of the study may result in loss of structural integrity in the keratin structures in the epidermis; this would allow a higher rate of diffusion through the upper layers.

4.11 Conclusion

The *in vivo* studies have shown the importance of skin blood flow and occlusion. Reducing the skin blood flow with noradrenaline prevented methyl salicylate from immediately entering the systemic circulation and therefore allowing higher concentrations to build up in the tissue. Occlusion on the other hand allowed more methyl salicylate to penetrate the skin over the time course of the experiment, giving a more continuous delivery of methyl salicylate rather than an initial peak followed by a decrease. Occlusion is thought to aid penetration of drugs through the hydration of the skin which disrupts the structure of the lipid bi-layer that surrounds the epidermal keratinocytes and increases its fluidity. An increase in fluidity will result in an increased rate of diffusion for topically applied compounds. In these studies the major role of occlusion may not be hydration, though that probably occurs, but preventing the applied formulation from evaporating. As the formulation dries it become more viscous slowing the diffusion of the drug within the applied dose possibly allowing depleted layers to form adjacent to the skin. The drying formulation also contains a higher concentration of propylene glycol, which changes the stratum corneum/formulation partition coefficient to be more in favour of the formulation.

One of the aims of this PhD is to examine the effect of structural and physicochemical differences on the absorption of drugs so it is important to reduce variability caused by factors other than those caused by the drug in question. The probe depth/AUC comparison shows that probe depth need not be a major variable in microdialysis studies. If all the probes are situated within a defined tissue space with minimal variation in depth then inter-individual variation will submerge any variations caused by probe depth. Therefore after this initial study all drug wells should be either occluded to prevent thickening of the formulation, redesigned to accommodate a greater volume of formulation or preferably both. This will hopefully ensure that the absorption profile is affected less by factors resulting from the formulation. The use of noradrenaline will also be continued as this allows a more accurate estimation of the total flux of drug across the skin. By comparing this to results without noradrenaline it should be possible to determine the

optimum chemical characteristics for penetration of the skin and removal from the dermis into the systemic circulation.

The studies also show it is possible to follow and measure cutaneous drug metabolism that is wholly separate from hepatic/first pass metabolism.
The two smaller studies highlighted the important contribution that the chosen vehicle can make to the final tissue concentration. The ethanol based formulation resulted in AUC's 4-9 times greater than that seen from the propylene glycol formulation. The stratum corneum is a very effective barrier to absorption so if a vehicle can be used that reduces the barrier effect in the area of application a potentially more useful tissue concentration can be achieved. The ethanol formulation disrupted the epidermis by swelling the stratum corneum and making the extracellular lipid bi-layer more fluidic aiding diffusion. The studies also showed the impact that the $P_{octanol/water}$ value of the applied compound can have. Methyl salicylate and salicylic acid are structurally similar and have similar molecular weights but salicylic acid gave AUC values $\approx$10 times greater than methyl salicylate. The major physico-chemical difference between the two was the $P_{octanol/water}$ value. Methyl salicylate had a higher value meaning it was more lipophilic allowing it to penetrate the stratum corneum more effectively but it was not able to partition into the dermal tissue particularly well. Salicylic acid's $P_{octanol/water}$ value appeared to give it a better balance of lipo- and hydrophilicity, allowing it to penetrate the stratum corneum but also partition into the dermis.

5 Results: Methyl Salicylate – Double Probe

5.1 Introduction

The previous chapter demonstrated that methyl salicylate is extensively metabolised within the skin to salicylic acid following topical application. However it did not demonstrate whether this activity was present purely in the epidermis or whether the dermis contributed to the formation of salicylic acid. The epidermis is known to be metabolically active and the keratinocytes and the epidermal-origin appendages tend to have the highest baseline activity. The basal cells can be induced by pre treatment to give elevated activities. The activity of the dermis has generally been perceived as being low in comparison. The cell density of the dermis is lower than the epidermis as it consists mainly of connective tissue, which is probably the main reason for its lower metabolic activity.

The aim of the study was to discover whether microdialysis could be used to detect metabolic activity in the dermis alone. The study would also show the extent to which methyl salicylate and salicylic acid migrated through the dermal layer.

5.2 Method

The six volunteers (3 men and 3 women) for the *in vivo* studies had an average age of 21.3 years (max. =22, min.=20). All were students studying at Southampton University.

All studies used 2kDa probe fibres without wire inserts, the production of which is described in the materials and methods chapter. Once inserted the fibres were perfused with Ringer's solution at a flow rate of 0.4ml/hour. The perfusate of all the fibres contained 5μg/ml noradrenaline; previous results had shown that this concentration significantly reduced the amount of drug that was removed by the skin vasculature.

In all volunteers the six inserted fibres were split into three groups:

a. Two fibres (designated 1 and 2) were used as controls, they were inserted as per the method in the materials and methods chapter, section 2.2.4.1. The fibres were then utilised to measure transdermal absorption as described in the previous chapter. The fibres were perfused for one hour before methyl salicylate solution was applied to the surface of the skin (saturated solution contained in a 50/50 mixture of water and propylene glycol). The solution was contained using drug wells to prevent contamination. After application the drug wells were occluded to prevent evaporation. Samples of perfusate were collected every 0.5 hours and frozen at -20° until analysis.
b. Two of the lines inserted were designated as delivery lines. In order to measure dermal metabolic activity and dermal movement independently from epidermal influences, methyl salicylate had to be delivered direct to the dermis. The lines were inserted as normal but were perfused with a modified solution. Methyl salicylate was added to the standard perfusate used in the control lines resulting in a 100μg/ml solution. The two fibres were perfused with the modified solution continuously and samples were collected every 0.5 hours and frozen at -20° until analysis.
c. The other two lines were designated as receptor lines. They were inserted parallel to the delivery lines with an approximate 2mm gap

between them. They were perfused with the standard solution for the duration of the study. Samples were collected every 0.5 hours and frozen at -20° until analysis.

Due to problems with the fibres remaining patent during the studies not all of the volunteers had the same number of functioning lines, see table 5.1.

Volunteer	Age	Sex	No. Delivery Lines	No. Receptor Lines	No. Transdermal Lines
1	20	F	2	2	1
2	21	M	2	2	2
3	22	F	2	2	2
4	22	M	2	2	2
5	21	M	2	2	1
6	22	F	2	2	1

Table 5.1: Volunteer information: Age, sex, and the number of each line type.

5.3 Methyl Salicylate Absorption and Recovery

Tables 5.2, 5.3 and figure 5.1 below show the data from all the control lines for all the volunteers. The results agree with those from chapter 4, methyl salicylate absorption is rapid with a significant concentration present in the first sample. The concentration peaked at the 1-1.5 hr collection point and reduced by approximately three-quarters by the final collection point.

The salicylic acid concentration was initially very low as expected but increased over time until it peaked at the 2.5-3 collection point and then showed little change. This again was consistent with the findings in chapter 4 and indicated that methyl salicylate was being metabolised by endogenous esterases to salicylic acid. The metabolism is easier to see when the ratio of the methyl salicylate and salicylic acid concentrations is plotted, see figure 5.2 below.

Methly Salicylate	Vol 1	Vol 2		Vol 3		Vol 4		Vol 5	
Time	1	1	2	1	2	1	2	1	2
Pre- dose	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0-0.5	0.17	2.08	0.91	0.00	0.00	0.70	0.26	2.31	0.26
0.5-1	0.62	1.30	0.48	0.25	0.13	0.45	0.32	2.52	2.23
1-1.5	0.52	0.70	0.72	0.65	1.39	0.77	0.37	2.29	1.31
1.5-2	0.37	0.94	0.71	0.78	1.24	0.23	0.20	0.17	0.16
2-2.5	0.24	0.50	0.60	0.79	0.80	0.24	0.12	1.33	1.09
2.5-3	0.23	0.80	0.50	0.63	0.64	0.25	0.10	1.15	1.28
3-3.5	0.18	0.50	0.21	0.47	0.65	0.33	0.14	1.01	1.13
3.5-4	0.19	0.22	0.46	0.24	0.61	0.14	0.07	0.51	0.73
4-4.5	0.05	0.20	0.14	0.19	0.51	0.17	0.12	0.65	0.85
4.5-5	0.13			0.13	0.51		0.09		

Table 5.2: Summary of all transdermal data from all volunteers. The values are the concentration (μg/ml) of methyl salicylate in the dialysate for each line and at each collection point following topical methyl salicylate application. The time is expressed in hours.

Salicylic Acid	Vol 2		Vol 3		Vol 4		Vol 5	
Time	1	2	1	2	1	2	1	2
Pre- dose	0.13	0.00	0.00	0.00	0.00	0.00	0.10	0.00
0-0.5	0.36	0.17	0.00	0.00	0.26	0.84	0.14	0.00
0.5-1	0.82	0.51	0.06	0.04	0.87	0.82	0.09	0.46
1-1.5	1.07	0.95	0.69	1.13	1.41	1.15	1.37	0.43
1.5-2	2.72	1.44	1.57	1.77	1.18	2.33	0.21	0.20
2-2.5	1.57	1.65	1.90	1.66	1.69	2.19	2.51	1.42
2.5-3	1.85	2.74	2.08	1.83	1.57	2.59	2.57	1.60
3-3.5	1.74	1.74	2.26	2.57	1.33	2.22	2.15	1.63
3.5-4	1.16	2.05	2.80	2.51	1.04	1.96	1.72	1.51
4-4.5	1.75	1.86	3.15	2.54	1.35	1.95	1.52	1.93
4.5-5			1.02	2.76		1.90		

Table 5.3: Summary of all transdermal data from all volunteers. The values are the concentration (μg/ml) of salicylic acid in the dialysate for each line and at each collection point following topical methyl salicylate application. The time is expressed in hours.

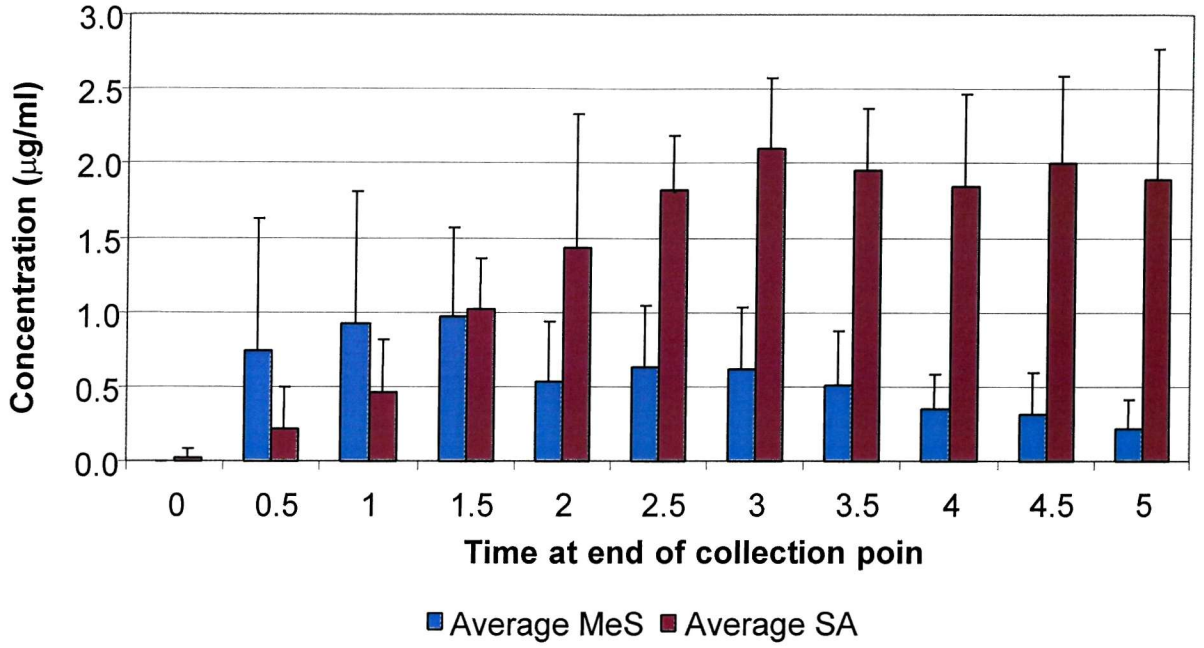

Figure 5.1: Graph showing the average sample concentration ± SD for all 6 volunteers of methyl salicylate and salicylic acid after topical application of methyl salicylate.

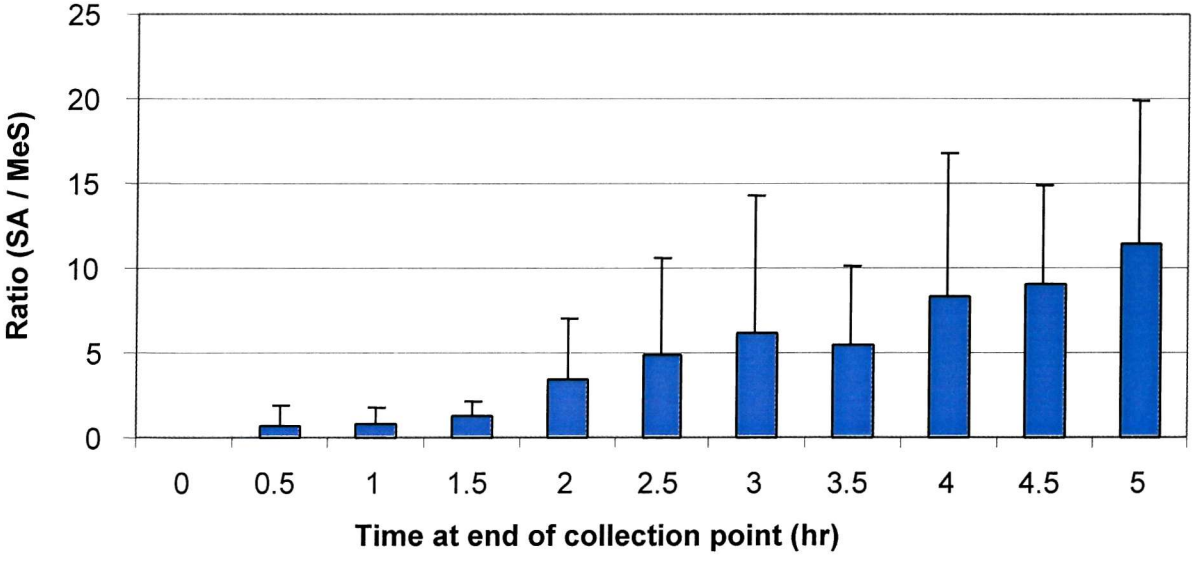

Figure 5.2: Graph showing ratio between methyl salicylate and salicylic acid concentration at each collection point following topical methyl salicylate application. n = 6.

The graph (figure 5.2) shows a steady increase in the ratio over time by the final time point the concentration of salicylic acid is almost nine times greater than that of methyl salicylate. The average AUC for methyl salicylate was 181

± 99 [μg/ml].min and for salicylic acid the AUC was 397 ± 77 [μg/ml].min. The average ratio of the methyl salicylate and salicylic acid AUC's was found to be 3.2 ± 2.8, indicating that the skin tissue contained far more salicylic acid than methyl salicylate.

5.4 Double Probe Results: Initial Studies

The protocol given above was applied to the first 3 subjects only because the measured concentrations of methyl salicylate recovered from the delivery line were extremely low. Tables 5.4 and 5.5 below and figures 5.3 and 5.4 below show all the data collected from all the delivery lines for the first three volunteers. The concentration of methyl salicylate in the perfusate was 100μg/ml however the dialysate or sample concentration was on average only 0.3μg/ml. This suggests that over 99% of the methyl salicylate had diffused into the tissue space through the fibres. The measured concentration of salicylic acid was similar to the measured concentration of methyl salicylate (about 0.1-0.3μg/ml).

Methyl Salicylate	Volunteer 1		Volunteer 2		Volunteer 3			
Time	Fibre 1	Fibre 2	Fibre 1	Fibre 2	Fibre 1	Fibre 2	Average	SD
Pre-dose	0.26	0.05	0.71	0.50	0.75	0.31	0.43	0.27
0-0.5	0.17	0.09	0.59	0.71	0.36	0.45	0.39	0.24
0.5-1	0.09	0.40	0.61	0.25	0.25	0.16	0.29	0.19
1-1.5	0.18	0.09	0.60	0.50	0.26	0.14	0.29	0.21
1.5-2	0.12	0.06	0.45	0.35	0.18	0.33	0.25	0.15
2-2.5	0.10	0.08	0.42	0.32	0.15	0.30	0.23	0.14
2.5-3	0.09	0.00	0.31	0.18	0.19	0.30	0.18	0.12
3-3.5	0.11	0.06	0.80	0.20	0.11	0.10	0.23	0.28
3.5-4	0.09	0.09	0.21	0.21	1.05	0.18	0.31	0.37
4-4.5	0.07	2.52	0.40	0.18	0.15	0.13	0.57	0.96
4.5-5	0.08							

Table 5.4: Summary of all methyl salicylate concentrations from all delivery lines from first three volunteers. The values are the concentration (μg/ml) of methyl salicylate in the dialysate for each line and at each collection point. Methyl salicylate was present only in the perfusate and not applied to the skin. The time is expressed in hours.

Salicylic Acid	Volunteer 1		Volunteer 2		Volunteer 3			
Time	Fibre 1	Fibre 2	Fibre 1	Fibre 2	Fibre 1	Fibre 2	Average	SD
Pre-dose	0.00	0.00	0.27	0.20	0.12	0.25	0.14	0.12
0-0.5	0.00	0.00	0.21	0.22	0.07	0.15	0.11	0.10
0.5-1	0.00	0.00	0.27	0.20	0.09	0.50	0.18	0.19
1-1.5	0.00	0.00	0.23	0.20	0.05	0.08	0.09	0.10
1.5-2	0.00	0.00	0.24	0.18	0.08	0.59	0.18	0.22
2-2.5	0.00	0.00	0.21	0.18	0.05	0.10	0.09	0.09
2.5-3	0.00	0.00	0.23	0.08	0.05	0.08	0.07	0.09
3-3.5	0.00	0.00	0.42	0.08	0.00	0.09	0.10	0.16
3.5-4	0.00	0.00	0.21	0.07	0.44	0.11	0.14	0.17
4-4.5	0.00	0.00	0.23	0.09	0.07	0.06	0.07	0.08
4.5-5	0.00							

Table 5.5: Summary of all salicylic acid concentrations from all delivery lines from first three volunteers. The values are the concentration (μg/ml) of salicylic acid in the dialysate for each line and at each collection point. Methyl salicylate was present only in the perfusate and not applied to the skin. The time is expressed in hours.

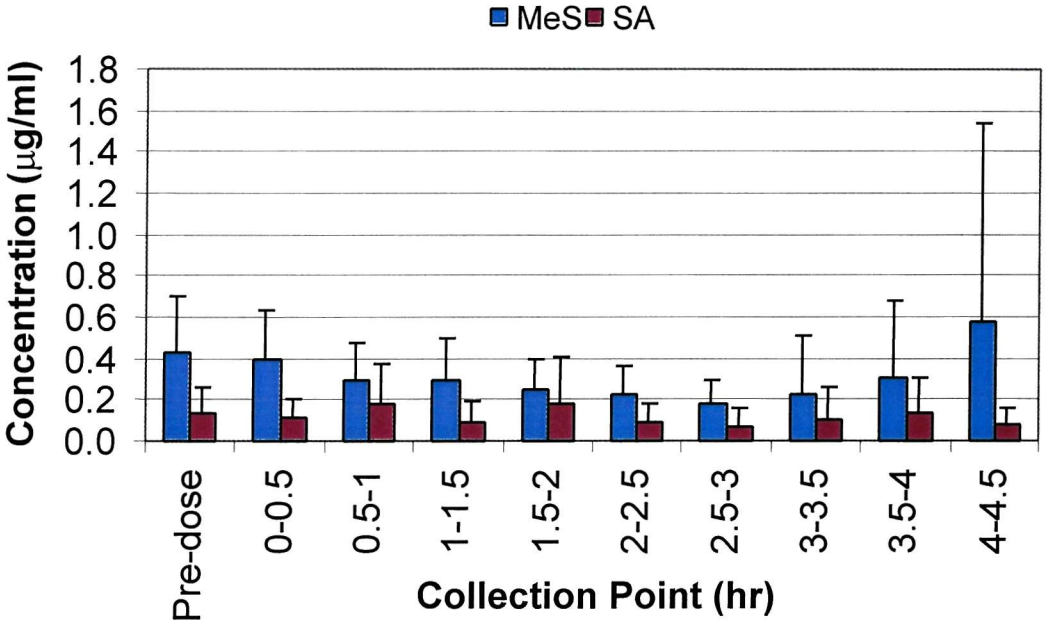

Figure 5.3: Double probe results; Graph showing average methyl salicylate and salicylic acid concentration (μg/ml) in the dialysate from the delivery fibre for all collection points. Methyl salicylate was present only in the perfusate and not applied to the skin. n = 3.

This is shown more clearly in figure 5.4, which shows the ratio between the two concentrations. On average the ratio between the two is 0.4 which is much higher than expected, the ratio of methyl salicylate to salicylic acid in the perfusate solution was approximately 0.01.

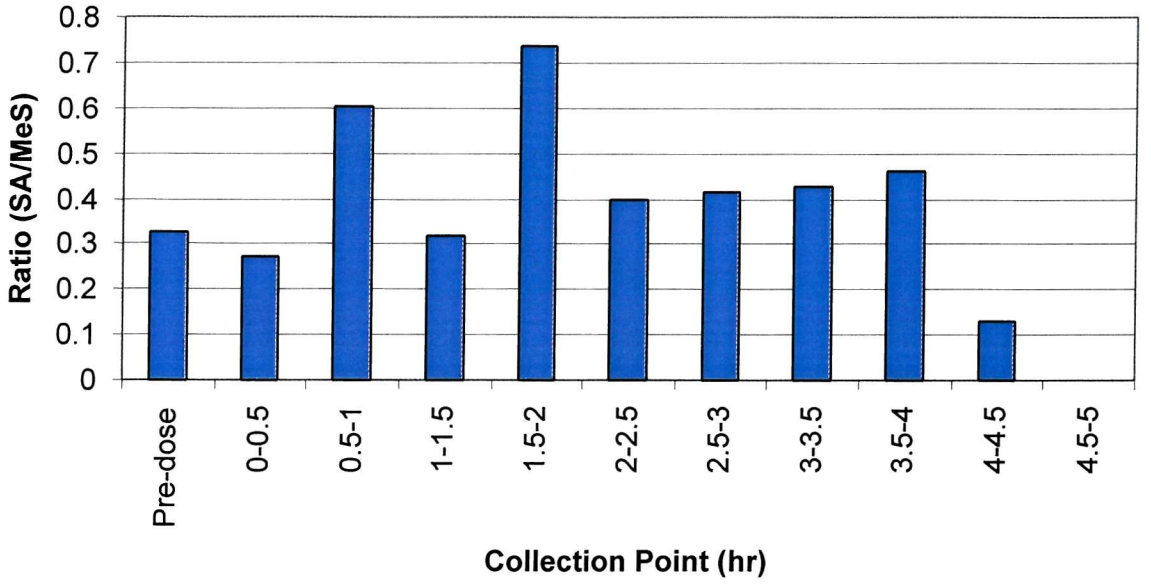

Figure 5.4: Double probe results; Graph showing ratio of methyl salicylate and salicylic acid concentrations for all collection points. Methyl salicylate was present only in the perfusate and not applied to the skin. n = 3.

The methyl salicylate and salicylic acid concentrations in the receptor lines were with a few exceptions, all below the limit of detection of 10 ng/ml, table 5.6 and 5.7.

Methyl Salicylate	Volunteer 1		Volunteer 2		Volunteer 3			
Time	Fibre 1	Fibre 2	Fibre 1	Fibre 2	Fibre 1	Fibre 2	Average	SD
Pre-dose	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0-0.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1-1.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1.5-2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2-2.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2.5-3	0.00	0.76	0.00	0.00	0.00	0.00	0.13	0.31
3-3.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3.5-4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4-4.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4.5-5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 5.6: Summary of all methyl salicylate concentrations from all receptor lines from first three volunteers. The values are the concentration (μg/ml) of methyl salicylate in the dialysate for each line and at each collection point. Methyl salicylate was neither present in the perfusate nor applied to the skin. The time is expressed in hours.

Salicylic Acid	Volunteer 1		Volunteer 2		Volunteer 3			
Time	Fibre 1	Fibre 2	Fibre 1	Fibre 2	Fibre 1	Fibre 2	Average	SD
Pre-dose	0.05	0.00	0.00	0.00	0.00	0.00	0.01	0.02
0-0.5	0.04	0.00	0.00	0.00	0.00	0.00	0.01	0.02
0.5-1	0.06	0.00	0.00	0.00	0.00	0.00	0.01	0.02
1-1.5	0.05	0.00	0.00	0.00	0.00	0.00	0.01	0.02
1.5-2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2-2.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2.5-3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3-3.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3.5-4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4-4.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4.5-5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 5.7: Summary of all salicylic acid concentrations from all receptor lines from first three volunteers. The values are the concentration (μg/ml) of salicylic acid in the dialysate for each line and at each collection point. Methyl salicylate was neither present in the perfusate nor applied to the skin. The time is expressed in hours.

The results would suggest two possible scenarios. The first is that methyl salicylate lost from the delivery lines to the tissue space is over 99% efficient and that its lateral diffusion through the dermal space is essentially zero. *In vitro* studies have shown that at a flow rate of 0.4ml/hr the average recovery is ~12%, and so this would be expected to be the maximum loss both *in vitro* and *in vivo*. The *in vitro* studies are a simplified situation where the only factors to affect the recovery are those related to the fibre. *In vivo* fibre pores may become blocked by proteins, the ionic strength of the tissue space may be different to that of the perfusate both of which would serve to reduce the potential loss of methyl salicylate.

The second scenario is that methyl salicylate was not reaching the fibre but was being adsorbed onto the plastic of the tubing connecting the syringe to the fibre. Methyl salicylate has a high P_{ow} making it very lipophillic, and therefore the likelihood that it could stick to plastic is quite high. This would also explain the high ratio of salicylic acid to methyl salicylate in the recovered/delivery perfusate, salicylic acid has a lower P_{ow} than methyl salicylate and so a greater percentage could be expected to reach the fibre.

The connecting tubes were tested *in vitro*, by passing a 100μg/ml solution of methyl salicylate at 0.4ml/hr through the standard tubing used in all the previous studies. The tubing used was 150 cm long in all cases. The solution was collected at the end of the tubing and not passed through a fibre. When the samples were analysed it was found that less than 1% methyl salicylate

was reaching the end of the tubing. Several alternative sterile tubings were identified and compared to the original tubing, see figure 5.4 below.

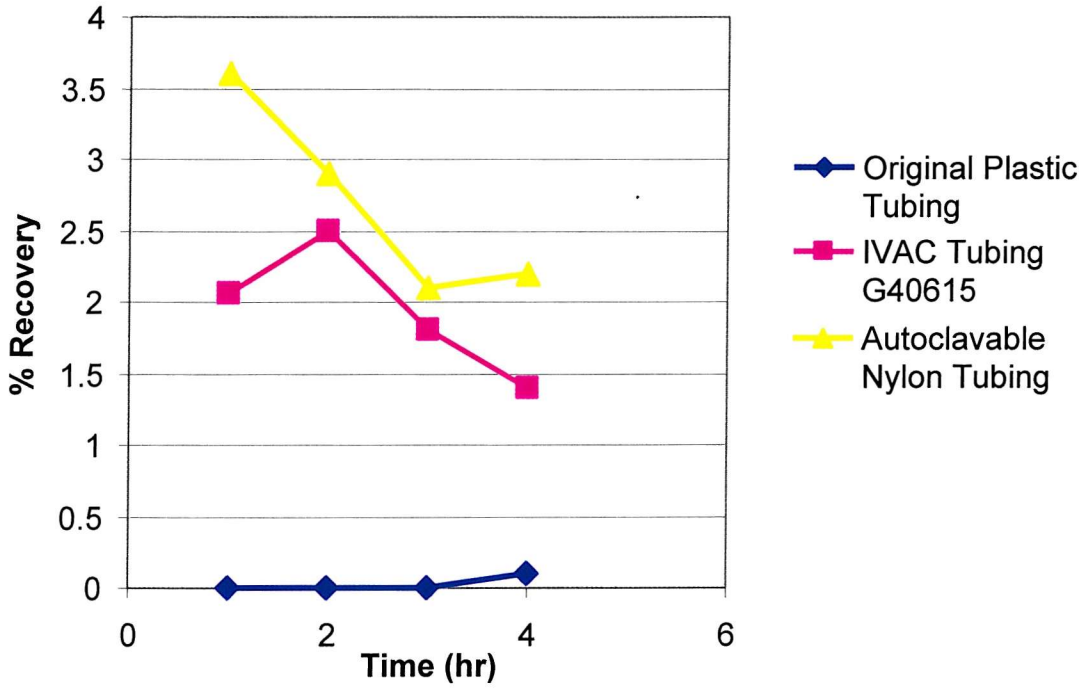

Figure 5.4: Graph showing recovery of methyl salicylate from syringe extension tubing, using a flow rate of 0.4ml/hr and a methyl salicylate concentration of 100μg/ml.

None of the tubing tested prevented methyl salicylate loss due to adsorption, the nylon tubing was used in the next set of studies because the recovery was marginally higher.

5.5 Double Probe Results: Modified Studies

The protocol of the study was changed to maximise the effective concentration of methyl salicylate reaching the microdialysis fibre:

1. The concentration of methyl salicylate in the perfusate was increased to 200μg/ml.
2. The tubing used to connect the syringe to the fibre was changed to the autoclavable nylon tubing and the length was restricted to 30cm.
3. Before being connected to the delivery lines the tubing was purged for 30 minutes with 200μg/ml methyl salicylate in an attempt to saturate the inner surface.
4. After each collection point (every 30 minutes) the tubing was purged for the volume of the tubing (approximately 0.3ml). The reason for this were to measure the concentration of methyl salicylate reaching the fibre and secondly to ensure fresh methyl salicylate solution was reaching the fibre.

Therefore this set of studies generated three sets of concentrations of methyl salicylate and salicylic acid, from the purged samples from the delivery lines, from the normal samples from the delivery lines and the receptor line samples see figure 5.4a.

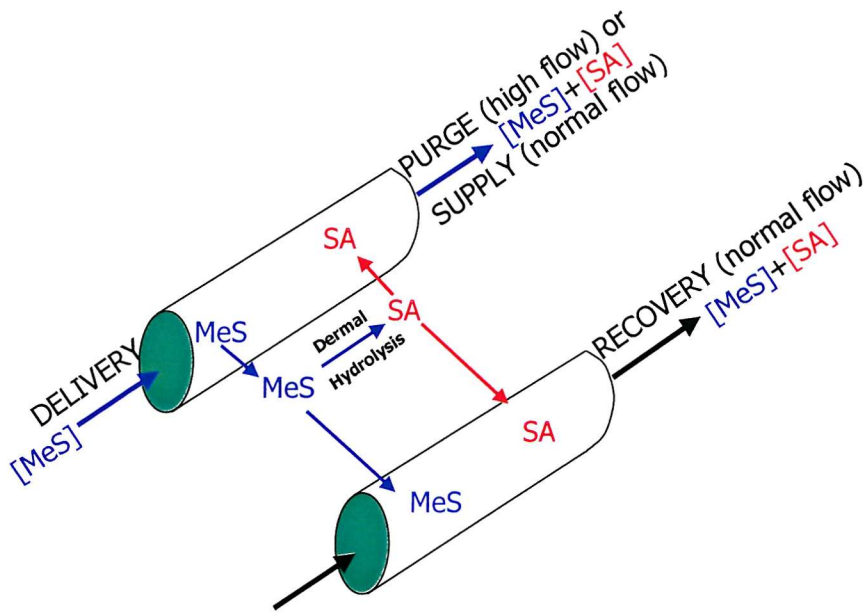

Figure 5.4a: Diagram of the dual probe experiment. Two fibres embedded within the dermis, both are perfused with Ringer's solution but only the delivery fibre is perfused with a 200μg/ml solution of methyl salicylate.

Table 5.8 and figure 5.5 below show the concentration of methyl salicylate in the purged samples which will be similar to the concentration reaching the delivery fibres. The results were far more promising than from the previous studies. The concentration of methyl salicylate was still far lower than the prepared concentration, less than a quarter of the concentration was reaching the fibre. The average methyl salicylate concentration was 35.9 μg/ml ± 4.9. The concentration was also relatively stable staying, on average, above 30 μg/ml throughout the studies, in none of the three studies were there any wild fluctuations.

Purged	Volunteer 4				Volunteer 5				Volunteer 6			
	Fibre 1		Fibre 2		Fibre 1		Fibre 2		Fibre 1		Fibre 2	
Time	MeS	S A	MeS	S A	MeS	S A	MeS	S A	MeS	S A	MeS	S A
0												
0.5	41.4	0.75	35.9	0.19	73	0	52.5	0.04	31.4	0.15		
1	30.1	0.72	19.3	0.77	54.7	0.08	36.2	0.37				
1.5	29.3	0.67	25.3	0.8	64.1	0.09	41.8	0.29				
2	27.7	0.68	21.1	0.79	58.8	0.19	48.5	0.15	35	0.25	29.6	0.25
2.5	14.8	0.54	22	0.69	54.2	0.11	40.5	0.12	25.8	0.2	39.3	0.25
3	24.6	0.56	22.3	0.66	57.5	0.1	43.7	0.15	27.2	0.17	38.3	0.14
3.5	22.8	0.53	22.5	0.55	61.2	0.18	44.4	0.23	28.4	0.21	25.6	0.12
4	0	0	19.3	0.93	61.3	0.1	34.5	0.21	32.4	0.18	36.6	0.15
4.5			18.7	0.67	28.1	0.06	46.8	0.45	31.6	0.16	31.5	0.28
5	0	0			65.6	0.05	71	0.03			35.7	0.18

Table 5.8: Summary of all methyl salicylate and salicylic acid concentrations from purged samples from delivery lines from final three volunteers. The values are concentration (μg/ml) in the dialysate for each line and at each collection point. The time is expressed in hours. Methyl salicylate was present only in the perfusate and not applied to the skin.

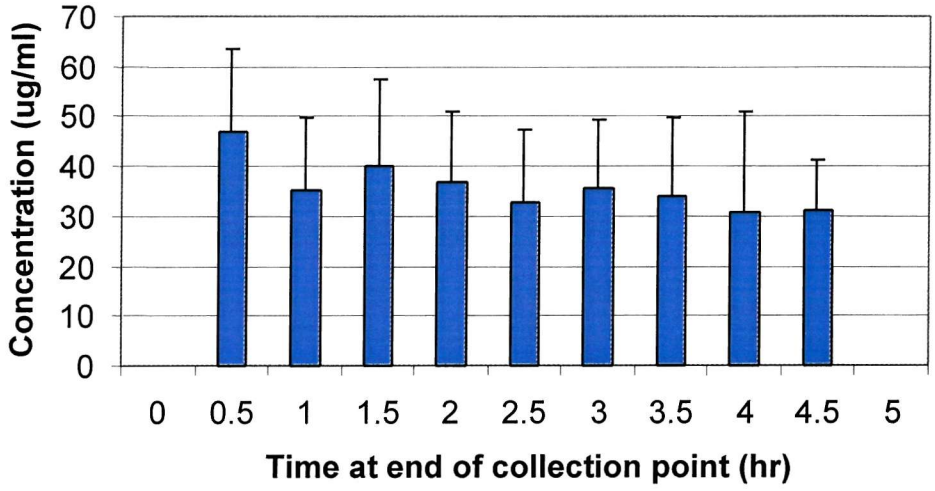

Figure 5.5: Average concentration of methyl salicylate (μg/ml) in purged samples for all collection points. Mean ± SD for 3 volunteers. Methyl salicylate was present only in the perfusate and not applied to the skin.

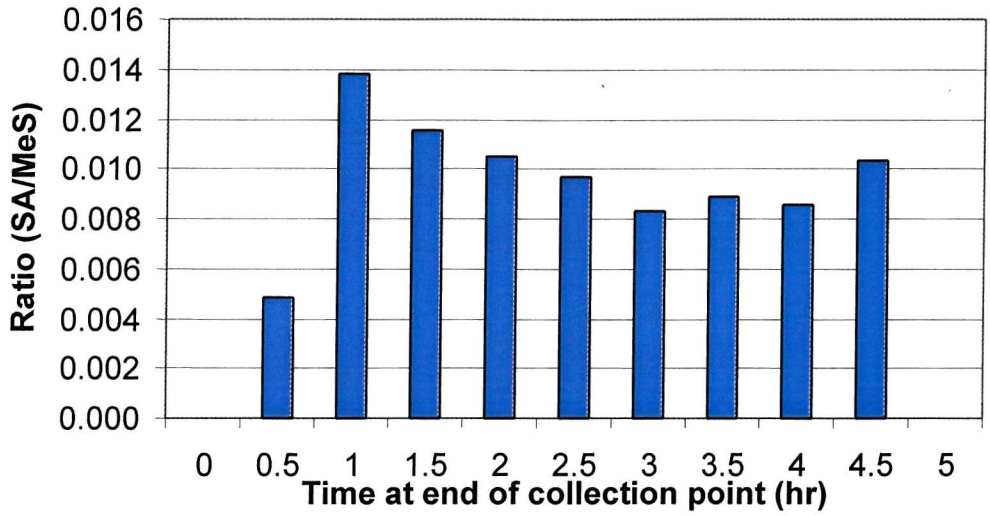

Figure 5.6: Graph showing the ratio of average salicylic acid to methyl salicylate concentration for all time points from purged samples. Methyl salicylate was present only in the perfusate and not applied to the skin. n = 3.

Figure 5.6 above shows the ratio of salicylic acid to methyl salicylate in the purged samples. The ratio did not appear to have any time dependence. The average ratio across all the collection points was 0.01 ± 0.002. Therefore the concentration of salicylic acid was on average only one percent of the methyl salicylate concentration in the samples. This was the same as the measured ratio for the perfusate before it had pumped through any tubing.

Table 5.9 and figure 5.7 below show the data from the perfused samples (0.4ml/hr) from the delivery lines. The average concentration of methyl salicylate over the whole study period was 20.6μg/ml ± 3.4 and for salicylic acid it was 1.7μg/ml ± 0.5. The ratio of salicylic acid to methyl salicylate concentration was on average 0.09 ± 0.04, see figure 5.8. This means that on average 9% of the total drug content in the samples is in the form of salicylic acid.

Supply	Volunteer 4				Volunteer 5				Volunteer 6			
	Fibre 1		Fibre 2		Fibre 1		Fibre 2		Fibre 1		Fibre 2	
Time	MeS	S A	MeS	S A	MeS	S A	MeS	S A	MeS	S A	MeS	S A
0												
0.5			17.4	1.07	44.1	1.58	49.3	3.29	20	1.33	10.4	0.67
1	18.8	0.8	11.9	1.24	35.4	1.85	42.8	2.24	12.9	0.95	13	0.76
1.5	22	0.8	18	1.38	36.1	1.78	10.2	1.91	13.5	1.08	13.9	0.84
2	15.4	1.14	14.9	1.39	5.93	1.11	17.7	3.07	18.8	1.17	29	0.97
2.5	20	0.61	18.8	0.83	33.7	0	31.6	3.21	15.3	1.51	7.31	1.11
3	18.6	1.28	15.2	1.46	34.5	2.29	22.4	2.69	15	1.87	23.1	1.3
3.5	16.8	1.09	15.2	1.46	27.1	2.63	27.2	2.86	7.77	1.23	24.2	1.45
4	15.4	1.27	11.6	1.6	24.2	2.51	10.7	8.9	17.1	1.67	23	1.41
4.5	14.5	1.2	9.1	1.59	28.9	2.9	22	4.02	15.2	1.77	25.7	1.68
5	16.5	0.71	14.7	0.8								

Table 5.9: Summary of all methyl salicylate and salicylic acid concentrations from supply samples from delivery lines from final three volunteers. The values are concentration (μg/ml) in the dialysate for each line and at each collection point. The time is expressed in hours. Methyl salicylate was present only in the perfusate and not applied to the skin.

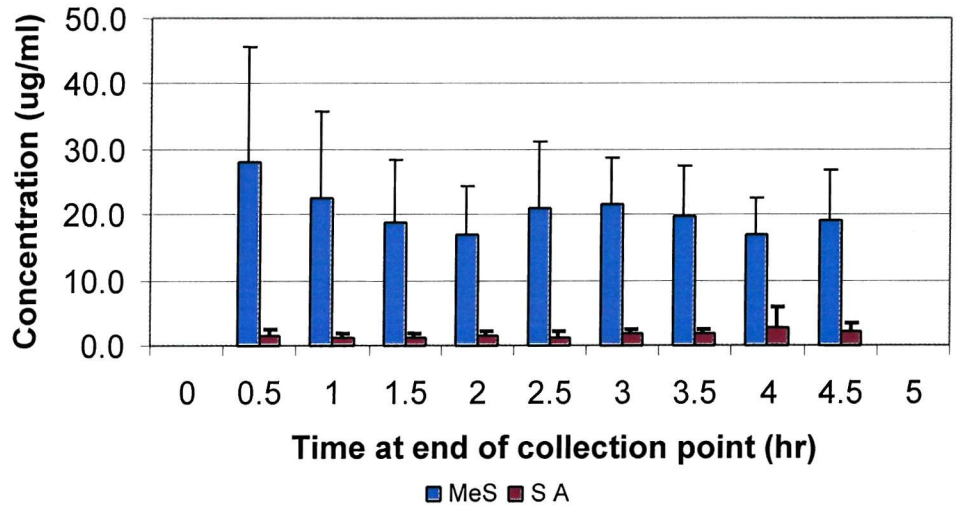

Figure 5.7: Average concentration of methyl salicylate and salicylic acid (μg/ml) in supply samples for all collection points. Methyl salicylate was present only in the perfusate and not applied to the skin. n = 3.

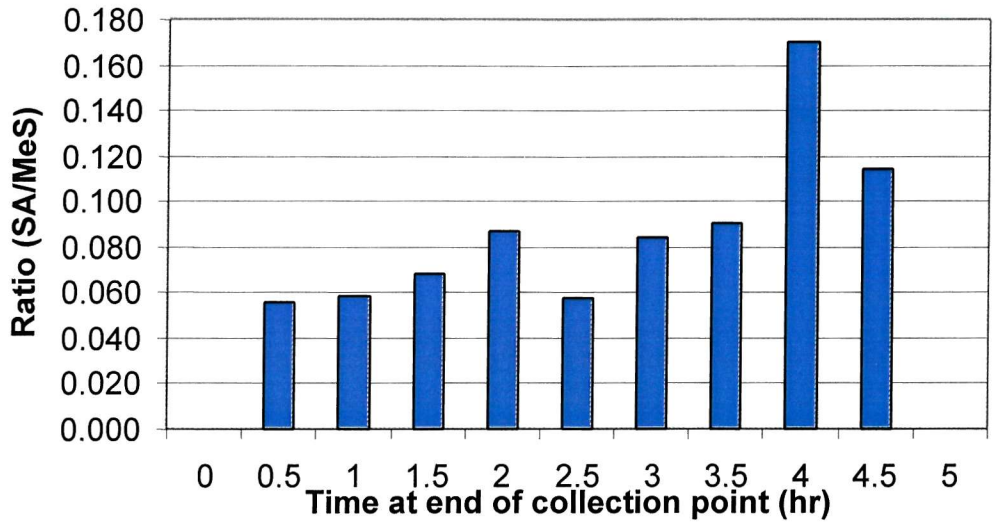

Figure 5.8: Graph showing the ratio of average salicylic acid to methyl salicylate concentration for all time points from supply samples. Methyl salicylate was present only in the perfusate and not applied to the skin.

Table 5.10 and figure 5.9 below show the data from the receptor lines for all volunteers and all collection points. The concentration of both methyl salicylate and salicylic acid appeared to be time dependent. Methyl salicylate concentration appeared to peak at about 1.5 hours and reached a plateau suggesting that a steady-state had been reached. In contrast salicylic acid concentration continually increased up to the final collection point.

Collection	Volunteer 4				Volunteer 5				Volunteer 6			
	Fibre 1		Fibre 2		Fibre 1		Fibre 2		Fibre 1		Fibre 2	
Time	MeS	S A	MeS	S A	MeS	S A	MeS	S A	MeS	S A	MeS	S A
0	0	0	0	0	0	0	0	0	0	0	0	0
0.5	0	0	0	0	0.11	0	0	0	0.11	0	0	0
1	0.08	0.02	0	0	0.33	0.04	0.15	0.05	0.17	0.03	0.07	0.06
1.5	0.08	0.03	0	0.06	0.48	0.13	0.27	0.05	0.17	0.07	0.13	0.07
2	0.06	0.09	0	0.09	0.19	0.3	0.28	0.2	0.09	0.09	0.21	0.1
2.5	0.12	0.11	0	0.05	0.3	0.33	0.14	0.2	0.16	0.12	0.12	0.16
3	0.1	0.15	0	0.15	0.29	0.36	0.35	0.31	0.14	0.18	0.19	0.16
3.5	0.11	0.19	0	0.14	0.36	0.43	0.37	0.39	0.08	0.21	0	0.08
4	0.29	0.21	0	0.23	0.26	0.42	0.13	0.33	0.21	0.26	0.31	0.27
4.5	0	0.18	0	0.24	0.34	0.43	0.33	0.36	0.12	0.22	0.25	0.35
5	0		0									

Table 5.10: Summary of all methyl salicylate and salicylic acid concentrations from samples from receptor lines from final three volunteers. The values are concentration (μg/ml) in the dialysate for each line and at each collection point. The time is expressed in hours. Methyl salicylate was neither present in the perfusate nor applied to the skin.

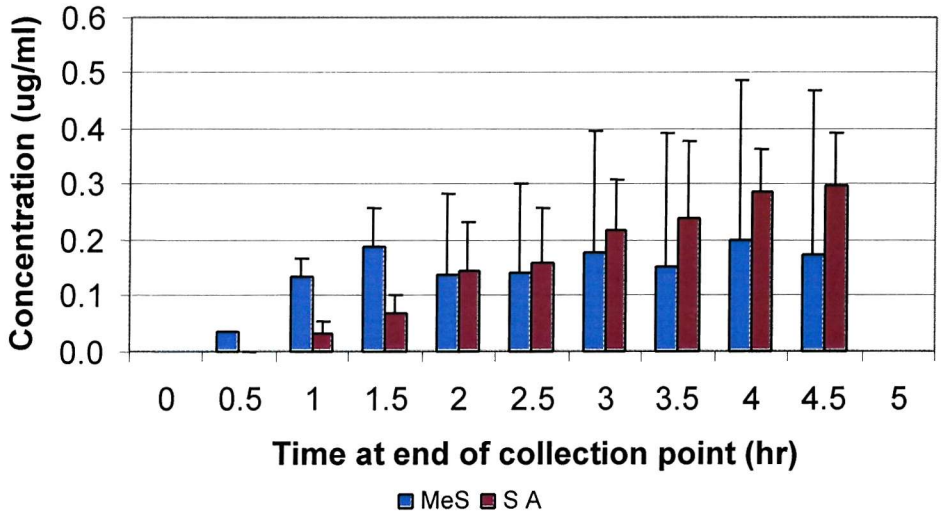

Figure 5.9: Average concentration of methyl salicylate and salicylic acid (μg/ml) in samples from receptor lines for all collection points. Methyl salicylate was neither present in the perfusate nor applied to the skin.

The ratio between the methyl salicylate and salicylic acid concentrations showed time dependence, the ratio increased throughout the study period, see figure 5.10.

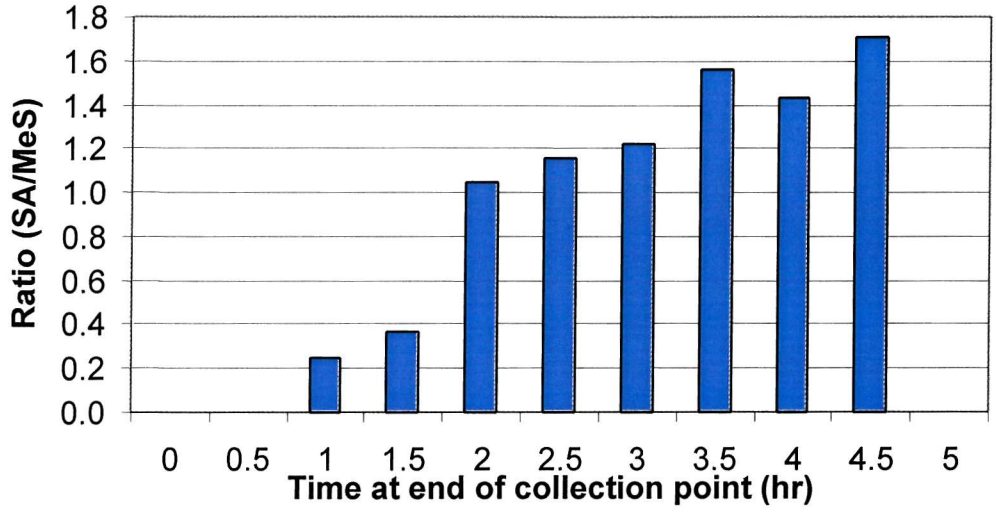

Figure 5.10: Graph showing the ratio of average salicylic acid to methyl salicylate concentration for all time points from receptor line samples. Methyl salicylate was neither present in the perfusate nor applied to the skin.

The average ratio was 0.97 ± 0.62 and the final collection point ratio was 1.71. The concentration of salicylic acid by the final collection point was almost double that of the methyl salicylate concentration.

Figures 5.11 and 5.12 below, compare the salicylic acid/methyl salicylate ratios of the purge, delivery and receptor line samples. The figures demonstrate that the ratio increases in the order of purge<delivery<receptor.

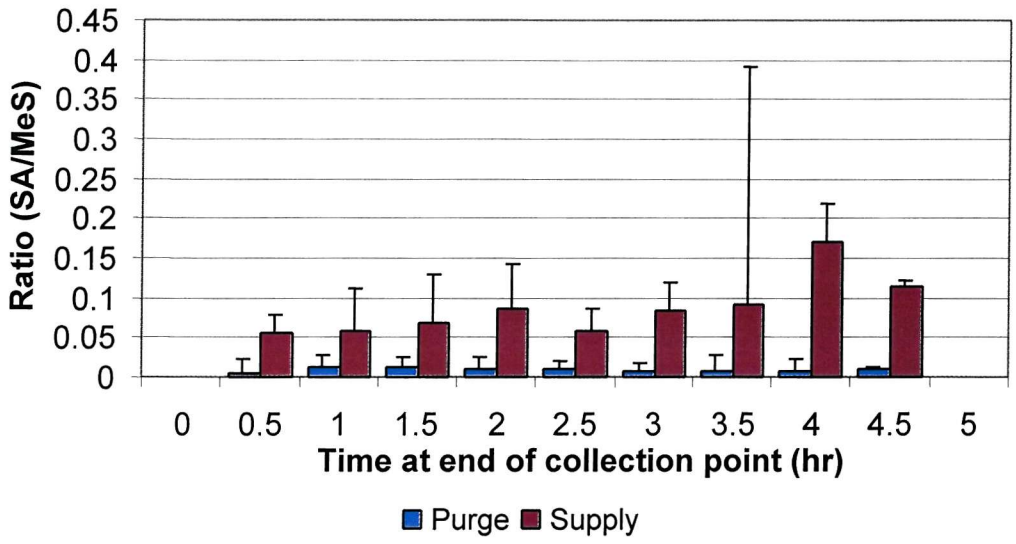

Figure 5.11: Comparison of average methyl salicylate/salicylic acid ratios from purged and supply samples.

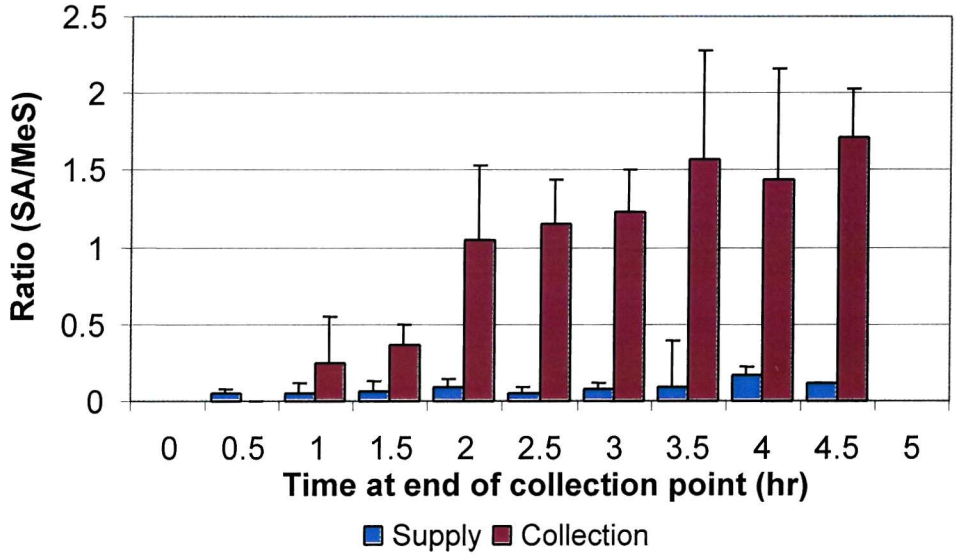

Figure 5.12: Comparison of average methyl salicylate/salicylic acid ratios from delivery and receptor line samples.

The average AUC ([μg/ml]. min) was calculated for the salicylic acid and methyl salicylate for the purge, delivery and receptor lines, see table 5.11.

Line	Purge		Delivery		Receptor	
Compound	MeS	SA	MeS	SA	MeS	SA
Volunteer 1	5721	133.5	4492.5	256.35	25.2	29.4
	6192	181.5	4183.5	372.6	0	28.8
Volunteer 2	16371	28.05	8097.9	499.5	79.8	73.2
	12732	60.75	7017	965.7	60.6	56.7
Volunteer 3	6354	39.6	4067.1	377.4	37.5	35.4
	6562.5	38.4	5088.3	305.7	38.4	37.5
Group Average	8989	80	5491	463	40	44
Group SD	4469	63	1675	260	28	18

Table 5.11: Table showing AUC values ([μg/ml].min) for all lines and all volunteers for both salicylic acid and methyl salicylate.

The change in AUC between the lines from purge to delivery and delivery to receptor was found to significant for both compounds ($p<0.05$) using a paired Student's t-test.

The ratio of the AUC's for salicylic acid and methyl salicylate were calculated and compared, see table 5.11.

Line	Purge	Delivery	Receptor
Volunteer 1	0.023	0.057	1.167
	0.029	0.089	NA
Volunteer 2	0.002	0.062	0.917
	0.005	0.138	0.936
Volunteer 3	0.006	0.093	0.944
	0.006	0.060	0.977
Group Average	0.012	0.083	0.988
Group SD	0.011	0.031	0.102

Table 5.11: Table of ratios of salicylic acid and methyl salicylate AUC's for all lines and all volunteers.

The ratios increase from purge to delivery to receptor and the increases are all significant ($p<0.01$) using a paired Students t-test. This indicates that relative to methylsalicylate, the salicylic acid concentration is increasing as it travels through the dermis.

5.6 Discussion

All studies carried out in this chapter had at least one control line that was used to measure the transdermal absorption of topically applied methyl salicylate. The protocol used was the same as for the noradrenaline-occluded lines in chapter 4. Both noradrenaline and occlusion were used to maximise the amount of methyl salicylate that penetrated to the dermis. The drug solution was also kept the same; a 50/50 water, propylene glycol mix that was saturated with methyl salicylate.

The absorption profile, figure 5.1, is almost identical to that shown in figure 4.5. The methyl salicylate concentration peaked within the first 1.5 hours and then begins reduce, which is probably explained by the formulation drying out. The salicylic acid concentration peaked later at approximately 3 hours where it reached a plateau region until the end of the study. The conclusions drawn in chapter 4 also apply to these data. Methyl salicylate is metabolised to salicylic acid as it passes through the skin and the delayed salicylic acid peak is representative of the time taken for methyl salicylate to be metabolised. The graph showing the ratio of salicylic acid to methyl salicylate concentration, figure 5.2, shows a steady increase over the time course of the study. This demonstrated that the salicylic acid concentration is constantly increasing relative to the methyl salicylate concentration. By the final collection point the concentration of salicylic acid is almost nine times greater than that of methyl salicylate. In the applied drug solution only 1% of the total drug content was in the form of salicylic acid.

The rest of the studies involving the double probes had major difficulties. The most important difficulty was delivering methyl salicylate directly to the dermis. The method used was to pump a solution containing methyl salicylate through a fibre inserted into the dermis. The methyl salicylate would diffuse into the dermis down a concentration gradient. A second fibre was placed adjacent and parallel to this delivery line and was perfused with a solution containing no methyl salicylate. Any methyl salicylate and salicylic acid present in the tissue would then diffuse into this second fibre and could be measured. A problem arose when it was discovered that methyl salicylate was strongly adsorbed on to the surface of the tubing used to connect the syringe to the

fibre. *In vitro* tests showed that under the conditions used *in vivo* less than 1% of the methyl salicylate in solution would reach the dialysis fibre. Two other sets of tubing were tested and a nylon tubing material was found to be better however the percentage of methyl salicylate reaching the fibre was still very low, approximately 2.5%.

To increase the amount reaching the tissue, the protocol was changed:

- A higher concentration of methyl salicylate was used
- The tubing length was reduced to 30 cm from 150 cm
- Prior to perfusing the fibre the tubing was purged in attempt to coat the inner surface
- The tubing was purged after every sample collection to ensure that fresh methyl salicylate solution was reaching the fibre.

This procedure did allow a much higher concentration of methyl salicylate to reach the fibre however it was still less than 20% of the starting solution and it introduced considerable variability. It is almost impossible to determine the exact concentration of methyl salicylate in the delivery fibre.
This is likely to be a problem for any highly lipophilic drug used in this way. For further studies to be undertaken a better solution needs to be identified this may include;

- Reducing tubing length to a minimum
- Identifying a better tubing material
- Coating the inside of the tubing to prevent adsorption, (a silicon based coating possibly)

The purge samples were analysed as they would give an indication of the methyl salicylate concentration reaching the fibre. The concentration measured would be higher than that reaching the fibres because the higher flow rate used in the purge would allow less time for the methyl salicylate to adsorb onto the surface of the tubing.
The average concentration of methyl salicylate was found to be 36μg/ml ± 5 only 18% of the original solution. The ratio of salicylic acid to methyl salicylate was found to be 0.01 i.e. 1% of the salicylate concentration was in the form of salicylic acid. As these samples had passed through the implanted fibre at a

very high flow rate (40ml/hr) this figure was used as a baseline to compare to the ratios from the other samples. The high flow rate would mean that the relative recovery or loss would be close to zero. The assumption was that if methyl salicylate were not metabolised within the dermis this ratio would remain low in the other two sets of samples.

The average recovery of methyl salicylate measured *in vitro* using a flow rate of 0.4ml/hr was approximately 12%. Therefore less than 12% of the methyl salicylate that reaches the delivery fibre enters the tissue space. The average sample concentration from the delivery line was 21μg/ml therefore the concentration lost to the tissue space was no more than 3μg/ml.
The concentration of both methyl salicylate and salicylic acid showed no time dependence and remained reasonably constant throughout the study period. The salicylic acid to methyl salicylate ratio was 0.09 ± 0.04, which was higher than the ratio in the purge samples. When the ratios of the AUC's of both samples were compared the difference was found to significant ($p<0.01$), see table 5.11. An increase in ratio was not expected in the delivery line samples. The average *in vitro* recovery of salicylic acid at 0.4ml/hr was approximately 36%, three times greater than methyl salicylate. This should mean that a greater proportion of the salicylic acid in the perfusate should enter the tissue space and the ratio in delivery samples should be lower than in the purged samples. The ratio increase would indicate that either salicylic acid is prevented from entering the tissue space or that salicylic acid is entering the delivery fibre from the tissue space. This would be caused by methyl salicylate being metabolised to salicylic acid in the area around the fibre. A build up of salicylic acid would reduce the concentration gradient across the fibre membrane causing less salicylic acid to move into the dermis. If the tissue concentration of salicylic acid became higher than the perfusate concentration then there would be a net movement of salicylic acid back into the fibre.

The receptor line samples showed a markedly different profile to either the purge samples or the delivery line samples. The methyl salicylate concentration initially increased and peaked at the 1.5 hour collection point, the concentration then remained approximately constant until the end of the

study period. The salicylic acid concentration steadily increased throughout the study period. The time taken for the methyl salicylate concentration to reach a plateau would be the time required for steady-state conditions to be reached between the two parallel fibres. However salicylic acid did not appear to reach steady-state within the time course of the study. This could mean that salicylic acid has a much lower diffusion constant than methyl salicylate or that the salicylic acid concentration was constantly increasing through methyl salicylate metabolism. The very high salicylic acid to methyl salicylate ratio tends to suggest the latter explanation. The ratio in the receptor lines also increased over the study period and by the final collection point it was 1.7 ± 0.3. Therefore the concentration of salicylic acid is almost double that of methyl salicylate, a large increase from the ratio in the delivery line samples of 0.09 ± 0.04. When the ratios of the AUC's of both samples were compared the difference was found to significant ($p<0.01$). This indicates that methyl salicylate has been extensively metabolised to salicylic acid as it diffused through the dermis.

The ratio of salicylic acid to methyl salicylate in the receptor line was then compared to the ratio calculated for the transdermal samples. The ratio for the 4.5hr collection point was 9.0 ± 5.8 and 1.71 ± 0.31 for the transdermal and receptor lines respectively. The average ratio of the transdermal and receptor lines were 5.2 ± 3.1 and 1.0 ± 0.6 respectively. When the ratios of both sets of samples were compared using a paired Students t-test the difference was found to significant ($p<0.01$). This suggests that the epidermis is more metabolically active in terms of methyl salicylate hydrolysis than the dermis.

5.7 Conclusion

The study indicates that the dermis is metabolically active towards methyl salicylate and this activity can be measured separately from both epidermal and hepatic metabolism. Comparison of the salicylic acid to methyl salicylate ratios from the transdermal lines and receptor lines suggests that the epidermis is more metabolically active towards methyl salicylate than the dermis.

For further studies to be carried out the procedure needs to be refined. The percentage of methyl salicylate adsorbing onto the tubing needs to be reduced and the concentration of methyl salicylate and salicylic acid that reaches the fibres needs to be known more accurately and to remain constant. This is also true of any other drug that is used with this method.

6 Results:Ketoprofen- protein binding study-*In vitro*

6.1 Introduction

The role of protein binding in transdermal drug absorption is poorly understood. In an attempt to investigate its affects a series of *in-vitro* and *in-vivo* experiments were carried out.

The drug chosen for these experiments was ketoprofen, a non-steroidal anti-inflammatory drug (NSAID), which is used both orally and topically for the relief of mild to moderate pain. It was chosen for several reasons. It is highly protein bound, most sources state that it is at least 95 % protein bound in plasma (Dollery, 1999), therefore variations caused by protein binding should be easier to detect than with methyl salicylate which is only 50% protein bound. Other reasons for its selection are that it has P_{ow} coefficient of 3, which several researchers suggest is near optimal for transdermal absorption (Singh & Roberts 1994) and there is a sensitive HPLC analysis method in the literature which allows detection to levels as low as 1ng/ml.

6.2 Methods

Initially *in vitro* experiments were run to investigate the relative recovery of ketoprofen under a variety of different parameters, flow rate, concentration, fibre type and the presence of protein. *In vitro* studies were carried out using both 2kDa and 3MDa probes, both probe types were constructed and sterilised as described in the materials and methods chapter.

The first *in vitro* studies used the 2kDa probes to discover the effect of varying the flow rate and drug concentration on the relative recovery. The next set of *in vitro* studies compared the relative recovery of the 2kDa and 3MDa fibres from drug solutions both with and without 2.25% human serum albumin (HSA). The relative recovery of HSA using 3MDa fibres was also measured.

6.3 Results: *2kDa* Fibres

The 2kDa *in vitro* experiments were all carried out following the procedure detailed in the materials and methods chapter, section 2.2.3.1.

6.3.1 *In vitro*: Flow Rate

The effect of varying the flow rate on the relative recovery of ketoprofen using the 2kDa fibres was studied. The experiments were carried out using the IVAC syringe pumps set at three different flow rates: 1, 2 and 4 ml/hr. A 5μg/ml ketoprofen bath solution was made in Ringer's solution. Perfusate samples were taken hourly and frozen at -20°c until analysis. Spaces in tables represent samples that were lost during sample preparation or analysis.

Time	a	b	c	d
0	0.0	0.0	0.0	0.0
1	28.7	25.4	22.7	18.3
2		27.4	22.0	23.4
3		24.0	33.3	17.6
4	25.0	20.8	24.3	18.4
Average	26.9	24.4	25.6	19.4
SD	2.6	2.8	5.3	2.7
	Total Av.	Total SD		
	23.7	4.3		

Table 6.1: Determination of percentage relative recovery (%) from a 5μg/ml bath solution of ketoprofen at a flow rate of 0.4 ml/hr, results from 4 separate fibres.

Time	Fibre 1	Fibre 2	Fibre 3	Fibre 4
0	0.0	0.0	0.0	0.0
1	27.8	5.7	25.3	32.3
2	31.1	37.9	27.7	29.0
3	37.9	29.3	32.9	44.6
4				
Average	32.3	24.3	28.6	35.3
SD	5.2	16.7	3.9	8.2
	Total Av.	Total SD		
	30.1	9.4		

Table 6.2: Determination of percentage relative recovery (%) from a 5μg/ml bath solution of ketoprofen at a flow rate of 0.2 ml/hr, results from 4 separate fibres.

Time	a	b	c	d
0	0.0	0.0	0.0	0.0
1	47.3	23.9	31.1	
2	48.5	37.2	45.4	43.4
3	45.0	48.0	35.9	36.1
4	39.8	28.7	35.2	24.5
Average	45.2	34.5	36.9	34.6
SD	3.9	10.6	6.0	9.5
	Total Av.	Total SD		
	37.8	8.3		

Table 6.3: Determination of percentage relative recovery (%) from a 5μg/ml bath solution of ketoprofen at a flow rate of 0.1 ml/hr, results from 4 separate fibres.

The average recovery for 0.4, 0.2 and 0.1 ml/hr was 23.7%, 30.1% and 37.8% respectively; see tables 6.1, 6.2 and 6.3 above. The average recoveries were found to be significantly different to each other ($p<0.05$) and show that recovery is inversely proportional to flow rate.

6.3.2 *In vitro*: Concentration

The effect of the concentration of ketoprofen on relative recovery was studied with three bath solutions: 1, 5 and 10 μg/ml. The experiments were carried out using the IVAC syringe pumps set at 0.2 ml/hr. The ketoprofen bath solution was made using Ringer's solution. Perfusate samples were taken hourly and frozen at -20°C until analysis. Spaces in tables represent samples that were lost during sample preparation or analysis.

Time	Fibre 1	Fibre 2	Fibre 3	Fibre 4
0	0.0	0.0	0.0	0.0
1	35.7	22.2	20.9	24.2
2	23.6	26.0	26.8	18.3
3	16.9	29.2	15.9	14.9
4	45.2	21.9	11.6	21.9
Average	30.3	24.8	18.8	19.8
SD	12.6	3.5	6.6	4.1
	Total Av.	Total SD		
	23.4	8.3		

Table 6.4 Determination of percentage relative recovery (%) from a 1μg/ml bath solution of ketoprofen at a flow rate of 0.2ml/hr, results from 4 separate fibres.

Time	Fibre 1	Fibre 2	Fibre 3	Fibre 4
0	0.0	0.0	0.0	0.0
1	27.8	5.7	25.3	32.3
2	31.1	37.9	27.7	29.0
3	37.9	29.3	32.9	44.6
4				
Average	32.3	24.3	28.6	35.3
SD	5.2	16.7	3.9	8.2
	Total Av.	Total SD		
	30.1	9.4		

Table 6.5: Determination of percentage relative recovery (%) from a 5μg/ml bath solution of ketoprofen at a flow rate of 0.2ml/hr, results from 4 separate fibres.

Time	Fibre 1	Fibre 2	Fibre 3	Fibre 4	Fibre 5
0	0.0	0.0	0.0	0.0	0.0
1	29.8	39.6	30.6	28.4	29.2
2	31.0	33.2	28.8	32.1	38.2
3	29.0	37.9	33.7	30.3	35.0
4	29.8	33.4	30.3	29.9	39.2
Average	29.9	36.0	30.9	30.1	35.4
SD	0.8	3.2	2.0	1.5	4.5
	Total Av.	Total SD			
	31.7	3.2			

Table 6.6: Determination of percentage relative recovery (%) from a 10μg/ml bath solution of ketoprofen at a flow rate of 0.2ml/hr, results from 5 separate fibres.

The relative recoveries from 1, 5 and 10μg/ml ketoprofen solutions were 23.4%, 30.1% and 31.7% respectively, see tables 6.4, 6.5 and 6.6 above. Only the results from the 1 and 10 μg/ml solutions were found to be significantly different ($p<0.05$).

6.4 Results: *2kDa* & 3MDa Fibres

In vitro experiments were run to investigate the difference in relative recovery of ketoprofen between the 3MDa and 2kDa fibres. The experiments were repeated in the presence of human serum albumin at physiological levels to determine the effect of protein binding on the relative recovery in both fibres. The procedures used are those detailed in the materials and methods chapter, sections 2.2.3.2 and 2.2.3.3. A peristaltic push/pull pump was used in all cases (for the reasons stated in the methods section) and set at a flow rate of 0.2ml/hr. The bath concentration of ketoprofen was 5μg/ml for all experiments and the protein binding experiments were run in the presence of 2.25% human albumin. The relative recovery of HSA was also determined using 3MDa fibres running at 0.2ml/hr in a solution containing 2.25 % HSA.

6.4.1 3MDa Fibre: With and without HSA

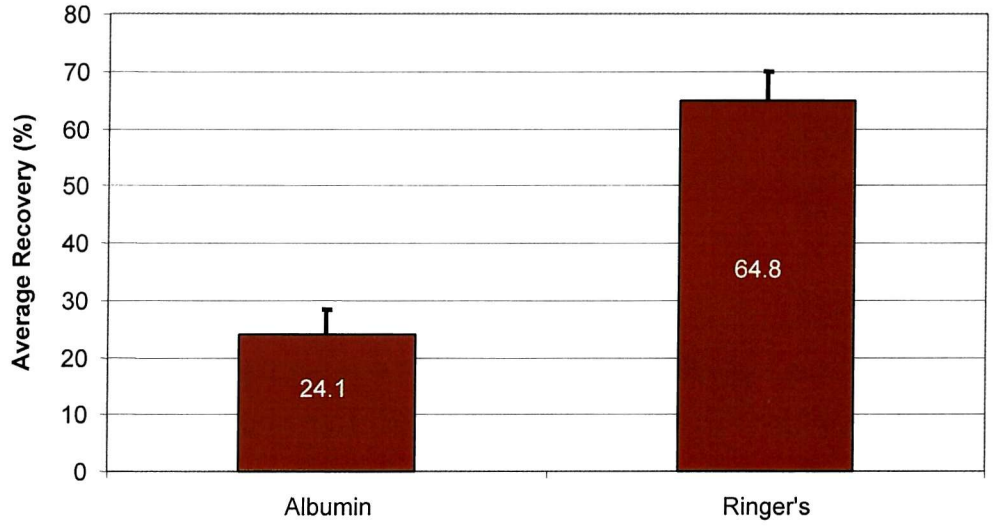

Figure 6.1: Comparison of percentage relative recovery (%) of ketoprofen from a 5ug/ml bath solution either with or without 2.25% HAS at a flow rate of 0.2ml/hr using 3MDa fibres. (Results are mean ± SD for 7 fibres)

The average relative recovery of a 3MDa fibre from a 5μg/ml solution of ketoprofen when using a flow rate of 0.2ml/hr was found to 64.8% ± 5.1, see figure 6.1 above. The recovery decreased to 24.1% ± 4.3 when 2.25% HSA

6.4 Results: *2kDa* & 3MDa Fibres

In vitro experiments were run to investigate the difference in relative recovery of ketoprofen between the 3MDa and 2kDa fibres. The experiments were repeated in the presence of human serum albumin at physiological levels to determine the effect of protein binding on the relative recovery in both fibres. The procedures used are those detailed in the materials and methods chapter, sections 2.2.3.2 and 2.2.3.3. A peristaltic push/pull pump was used in all cases (for the reasons stated in the methods section) and set at a flow rate of 0.2ml/hr. The bath concentration of ketoprofen was 5μg/ml for all experiments and the protein binding experiments were run in the presence of 2.25% human albumin. The relative recovery of HSA was also determined using 3MDa fibres running at 0.2ml/hr in a solution containing 2.25 % HSA.

6.4.1 3MDa Fibre: With and without HSA

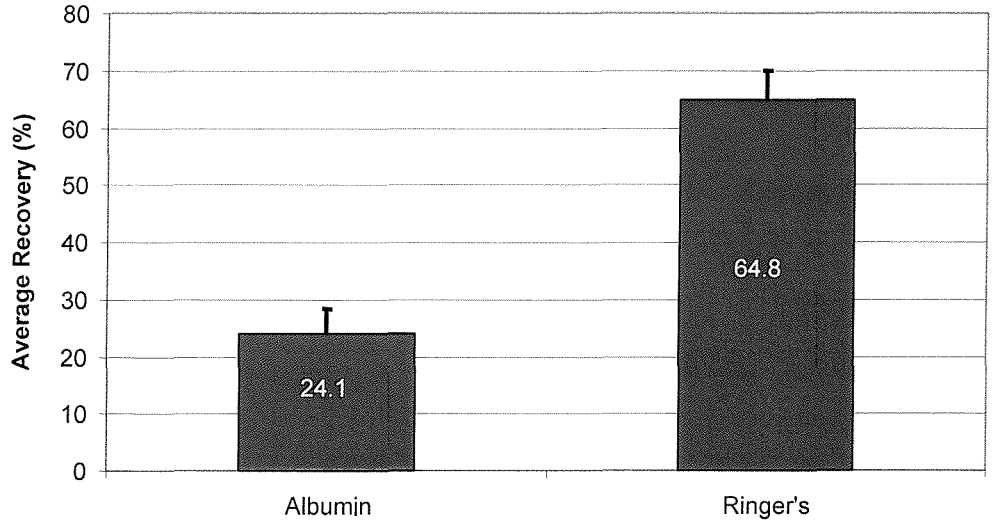

Figure 6.1: Comparison of percentage relative recovery (%) of ketoprofen from a 5ug/ml bath solution either with or without 2.25% HAS at a flow rate of 0.2ml/hr using 3MDa fibres. (Results are mean ± SD for 7 fibres)

The average relative recovery of a 3MDa fibre from a 5μg/ml solution of ketoprofen when using a flow rate of 0.2ml/hr was found to 64.8% ± 5.1, see figure 6.1 above. The recovery decreased to 24.1% ± 4.3 when 2.25% HSA

was included in the bath solution. The change in recovery (64% decrease) was statistically significant ($p<0.05$).

6.4.2 2kDa Fibre: With and without HSA

The average relative recovery of a 2kDa fibre from a 5μg/ml solution of ketoprofen when using a flow rate of 0.2ml/hr was 33.0% ± 10.5, see figure 6.2. The recovery decreased to 1.6% ± 1.1 when 2.25% HSA was included in the bath solution. The change in recovery was found to be significant ($p<0.05$).

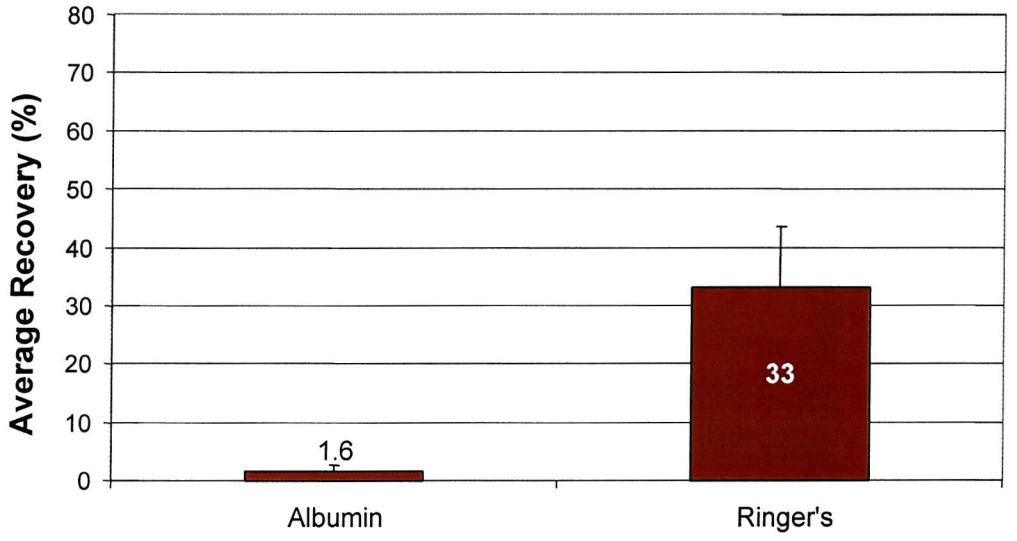

Figure 6.2: Comparison of percentage relative recovery (%) of ketoprofen from a 5ug/ml bath solution either with or without 2.25% HAS at a flow rate of 0.2ml/hr using 2kDa fibres. (Results are mean ± SD for 7 fibres)

6.4.3 3MDa Fibre: Relative recovery of HSA

The samples were analysed using a Coomassie® protein assay kit with the procedure detailed in the materials and methods section, 2.2.5.3. The average recovery was 21.2% ± 4.6

Time	Fibre 1	Fibre 2	Fibre 3	Fibre 4
0	0.71	0.90	1.31	0.79
1	16.42	22.16	21.57	15.51
2	16.09	27.48	19.91	18.58
3	16.29	28.62	20.13	17.09
4	13.18	46.31	19.84	19.28
Average	15.49	31.14	20.36	17.61
SD	1.55	10.50	0.81	1.68
All Fibre Average		21.15		
All Fibre SD		4.59		

Table 6.7: Relative recovery of HSA from a 2.25% solution using 3MDa fibres at a flow rate of 0.2ml/hr.

6.5 Discussion

The relative recoveries from the flow rate experiments indicate that recovery is inversely proportional to the flow rate. This mirrors the results from chapter 3 and from other researchers. As stated in chapter three the relationship is a result of the retention time of the dialysate within the fibre. As the flow rate increases the retention time within the fibre decreases allowing less time for the perfusate to reach equilibrium with the surrounding drug solution resulting in a lower relative recovery.
As for methyl salicylate, a flow rate of 0.4 ml/hr was initially chosen to be used in subsequent *in vivo* ketoprofen studies. Even though this flow rate does not give the highest relative recovery it has the advantage of giving a large enough sample to allow any analysis to be repeated, and also the potential to allow half hourly collections.

As stated previously, altering the drug concentration in the surrounding medium should not alter the relative recovery from a fibre, so long as all other parameters remain the same. However the results show that there was a significant reduction in the relative recovery between the lowest concentration and the highest. This, like the methyl salicylate studies, may be due to the loss of a constant amount of drug by adsorption onto the surface of the petri dish and/or the collecting Eppendorf. Bath samples were not routinely taken.

The relative recovery of the 2kDa fibre was significantly lower in the presence of HSA, decreasing from 33% down to 1.6%. The reduction indicates that ketoprofen is binding to the HSA resulting in a lower concentration of free drug. The 2kDa fibre has a maximum pore size of 2,000 daltons, albumin on average is 68,000 and so is unable to diffuse through the fibre material. Therefore the reduction is the result of dialysing from a greatly reduced concentration of free ketoprofen, but calculating the final recovery using the total drug concentration (both free and bound). The level of protein binding can be calculated assuming that the relative recovery of free drug remains independent of free drug concentration. The total drug concentration was 5μg/ml and the average recovery from the HSA containing solution was 1.6% therefore the average sample concentration was 1.6% of 5μg/ml which equals

0.08μg/ml. From the experiment without HSA we know that the average recovery of free drug is 33%. The total concentration of free drug in the HSA containing solution is therefore 0.24μg/ml. Therefore approximately 4.8% of a 5μg/ml solution of ketoprofen is unbound when it contains 2.25% HSA. This closely matches the literature derived values which mostly suggest that it is 95% protein bound.

The relative recovery of ketoprofen using the 3MDa fibres was also found to be lower in the presence of HSA although to a lesser extent than seen with the 2kDa fibres. Without protein the recovery was 64% almost twice the value seen with the 2kDa fibres. The increase can be ascribed to two differences between the fibres:

1. The 3MDa fibre has a larger external diameter than the 2kDa fibre(400μm compared to 200μm) therefore the surface area of the probe exposed to the drug solution is greater, this allows equilibrium to be approached faster.
2. The 3MDa fibre has a much higher density of pores that are large enough to allow the free diffusion of drug.

With the addition of protein the recovery was reduced to 24%. If the 3MDa fire were only capable of dialysing free drug the expected recovery would be approximately 3% (60% of 4.8 %). The high recovery shows that protein bound drug is dialysed with this fibre. If 3% of the recovery is the dialysis of free drug then 21% must be due to protein bound drug. The relative recovery for protein bound drug is equal to the relative recovery of HSA using the 3MDa fibres. This is to be expected, as albumin is a very large molecule in comparison to ketoprofen, (68,000 daltons compared to 254). The diffusional properties of a molecule of albumin will not be affected greatly by the binding of a ketoprofen molecule.

6.6 Conclusion

The combined 2kDa and 3MDa fibre studies showed that the relative recovery using 3MDa fibres was approximately double that of the 2kDa fibres, 64% compared to 33%. The use of HSA allowed the measurement of ketoprofen protein binding and showed it to agree with the published value of 95% bound. The studies also proved that the 3MDa fibres were capable of dialysing protein and protein bound drug and that the relative recovery of protein was equal to the relative recovery of protein bound drug when isolated from the contribution from the unbound drug.

Therefore using both 2kDa and 3MDa fibres in *in vivo* studies should allow the measurement of percentage protein binding and clarify the role of protein binding in transdermal absorption.

7 Results: Ketoprofen- protein binding study-*In vivo*

7.1 Introduction

As stated in the previous chapter ketoprofen was chosen as the model drug to look at protein binding in transdermal absorption for several reasons:

1. It is highly protein bound, most sources state that it is 95% bound to albumin after administration.
2. It is well tolerated topically and is available in several topical formulations to reduce localised pain, i.e. rheumatoid and muscle pain.
3. It has a P_{ow} value of 3, which several researchers suggest is near optimal for transdermal absorption.
4. There is a sensitive HPLC analysis method pre-existing in the literature, which allows detection to levels as low as 1ng/ml.

The *in vivo* studies were planned to be a simple comparison of recovery using two different fibre types; 2kDa fibres, which will only allow the measurement of free drug and 3MDa fibres which will allow the measurement of total drug concentration, i.e. both free and protein bound fractions. Comparisons would show how influence of protein binding on the relative recovery of ketoprofen.

7.2 Method

The 8 volunteers for the *in vivo* studies had an average age of 22.5 years, (max. age = 24, min. age = 21), two of the volunteers were men and the rest were women. All were students studying at Southampton University. Due to problems with the fibres remaining patent during the studies not all of the volunteers had the same number of functioning lines (Table 7.1).

Volunteer	Age	Sex	2kDa Fibres	3MDa Fibres
1	22	M	3	2
2	21	F	2	3
3	23	F	3	3
4	23	M	2	3
5	24	F	2	3
6	21	F	3	3
7	22	F	3	3
8	24	F	3	3

Table 7.1: Volunteer information: Age, sex and number of lines for each experimental condition.

All volunteers had six fibres inserted, three of which were 2kDa fibres identical to those used in the methyl salicylate studies and three were 3MDa fibres the construction of which is detailed in the methods chapter, section 2.2.2.2. The fibres were inserted as described in section 2.2.4.1 and 2.2.4.2 of the methods chapter; the only difference was that all fibres were inserted using a 21-gauge needle to reduce variation. The rest of the study was performed using the procedure detailed in the *in vivo* section of the methods chapter. The two sets of fibres were inserted alternately and the fibre type nearest the wrist being random. All fibres were perfused with Ringer's solution containing 5μg/ml noradrenaline at a flow rate of 0.4ml/hr. All drug wells were occluded after the ketoprofen formulation had been applied. All parameters apart from the fibre type remained constant.

Subject	Fibre Type	Pre dose			0-1 hours			1-2 hours			2-3 hours			3-4 hours			4-5 hours		
		Fibre 1	Fibre 2	Fibre 3	Fibre 1	Fibre 2	Fibre 3	Fibre 1	Fibre 2	Fibre 3	Fibre 1	Fibre 2	Fibre 3	Fibre 1	Fibre 2	Fibre 3	Fibre 1	Fibre 2	Fibre 3
1	2kDa Fibre	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.58	3.73
	3MDa Fibre	NA	0.00	0.00	NA	0.00	0.00	NA	0.00	0.00	NA	0.00	0.00	NA	0.33	0.11	NA	0.95	0.71
2	2kDa Fibre	NA	0.00	0.00	NA	0.00	0.00	NA	0.00	0.00	NA	0.00	0.00	NA	1.14	0.20	NA	6.82	0.84
	3MDa Fibre	0.00	0.00	0.00	0.00	3.38	0.00	0.00	0.55	0.00	0.44	0.05	0.16	3.56	1.20	1.82	11.03	4.32	4.08
3	2kDa Fibre	0.00	0.00	0.00	0.00	0.00	0.72	0.00	0.00	0.00	0.53	0.00	0.00	0.29	0.37	0.22	1.01	1.05	1.31
	3MDa Fibre	0.00	0.00	0.00	0.41	1.49	1.11	0.74	1.02	0.52	1.20	0.78	0.76	1.24	1.36	1.19	1.60	2.79	1.54
4	2kDa Fibre	NA	0.00	0.00	NA	0.00	0.00	NA	0.00	0.00	NA	0.00	0.00	NA	0.00	0.35	NA	0.27	0.29
	3MDa Fibre	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.44	0.17	0.00	0.29	0.65	0.00	0.00	1.47	0.74	0.84	3.08
5	2kDa Fibre	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.15	0.00	0.08	0.00	0.21	0.42	NA	NA	NA
	3MDa Fibre	NA	0.00	0.00	NA	0.00	0.00	NA	0.05	0.14	NA	0.11	0.30	NA	0.14	0.43	NA	NA	NA
6	2kDa Fibre	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	3MDa Fibre	0.00	0.00	0.00	0.60	0.00	2.31	0.00	0.00	0.00	0.44	0.00	0.00	NA	0.38	0.00	NA	0.00	0.00
7	2kDa Fibre	0.00	0.00	0.00	0.00	0.00	0.18	0.00	0.00	0.00	0.27	0.00	0.00	0.14	0.09	0.23	0.50	0.40	0.47
	3MDa Fibre	0.00	0.00	0.00	0.08	0.00	0.00	0.04	0.00	0.00	0.38	0.11	0.00	0.58	0.34	0.25	0.13	0.52	0.29
8	2kDa Fibre	0.00	0.00	0.00	0.00	0.00	0.36	0.00	0.00	0.00	0.27	0.00	0.00	0.14	0.18	0.11	0.50	0.53	0.65
	3MDa Fibre	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.08	0.14	0.28	0.25	0.57	0.55	0.84	1.16	0.80	1.14	1.52

Table 7.2: Summary of data from all volunteers and all lines. The values are concentration (μg/ml) of ketoprofen in the dialysate sample for each line and at each time point (NA= not available due to technical problems) following topical ketoprofen application.

7.3 Ketoprofen Absorption and Recovery

Table 7.2 above and table 7.3 and figure 7.1 below show the data from all the microdialysis probes from all the volunteers for the two different fibre types. The concentrations of ketoprofen in samples from both fibres types followed the same pattern. At collection time 0-1h there is a measurable concentration in both lines that were reduced by collection time 1-2.h The concentrations from both lines then increased until the end of the studies with each increase being larger than the previous increase. The average concentration at the final collection period was 1.76μg/ml for the 3MDa probes and 1.1μg/ml for the 2kDa probes.

Collection Time	3MDa Fibre		2kDa Fibre	
	Average	SD	Average	SD
Pre-dose	0.00	0.00	0.00	0.00
0-1	0.39	0.53	0.05	0.09
1-2	0.17	0.25	0.00	0.00
2-3	0.29	0.27	0.05	0.06
3-4	0.74	0.69	0.20	0.21
4-5	1.76	2.19	1.10	1.30

Table 7.3: Table comparing the average sample concentration from both the 3MDa and 2kDa fibres from all 8 volunteers following topical ketoprofen application. The values are expressed as μg/ml ± the standard deviation.

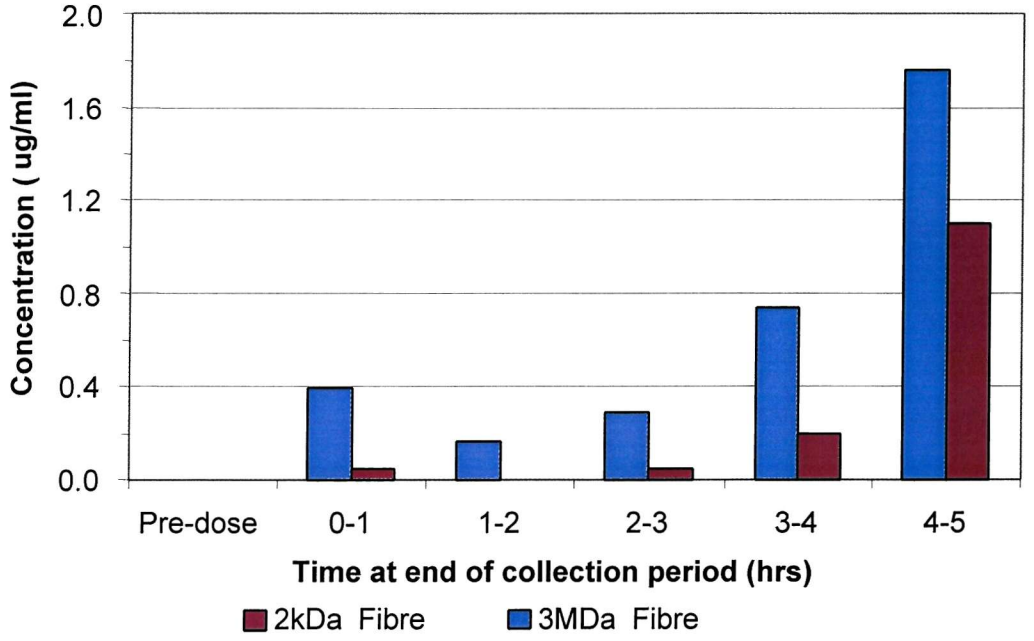

Figure 7.1: Comparison of average ketoprofen sample concentration from 3MDa and 2kDa fibres from 8 volunteers following topical ketoprofen application.

Figure 7.3 below compares the AUC values of both fibre types for each volunteer. Even though the inter-individual variation is very high (3MDa = 154μg/ml.min ± 134, 2kDa = 47μg/ml.min ± 49) a paired Student t-test showed that the 3MDa probes had a significantly higher AUC than the 2kDa probes ($p<0.05$). The ratio between the two AUC's was on average 3.2 ± 1.3 in the 8 volunteers.

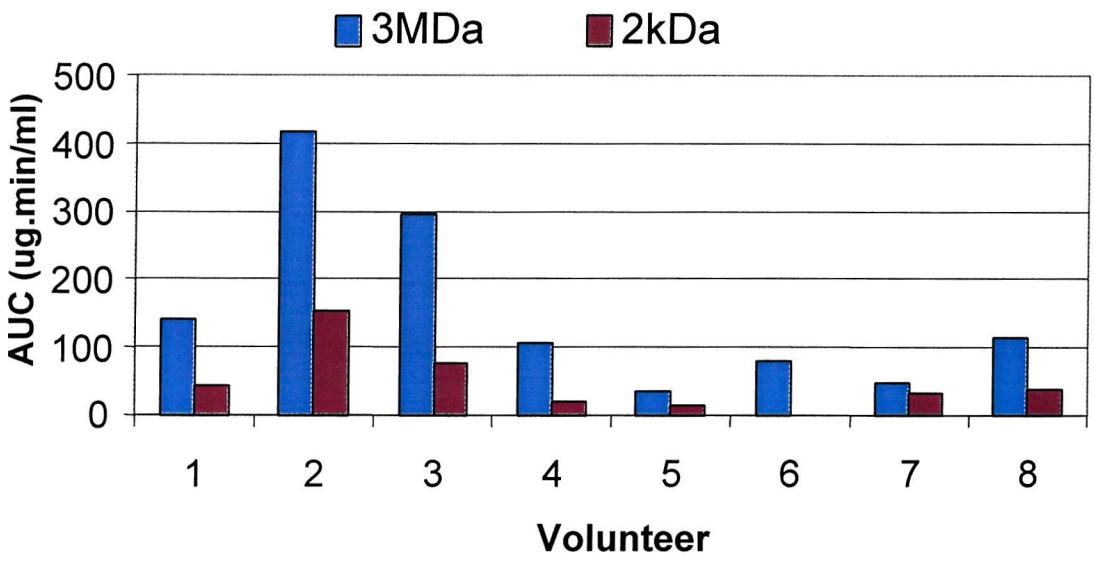

Figure 7.3: Comparison of AUC for ketoprofen (uncorrected for in vitro recovery) from both fibre types for each volunteer following topical ketoprofen application.

Table 7.4 below shows the C_{max} and the AUC for both fibre types. The C_{max} and AUC were found to be significantly larger from the 3MDa probes. The T_{max} for both fibres was not calculated because in each case the concentration continually increased throughout the course of the studies and a true maximum was not reached.

Parameters	3MDa Fibre		2kDa Fibre	
	Mean	St Dev	Mean	St Dev
C_{max} (ug/ml)	1.61[a]	2.07	0.98	1.25
AUC (ug.min/ml)	154[a]	133	47	49

Table 7.4: Table of pharmacokinetic values for topical ketoprofen absorption for both fibre types. Values with superscripts were significantly different ($p<0.05$-paired t-test). n = 8.

7.4 Probe Depth

At the end of each individual study the depth of the probes in the dermis was measured using an ultrasound scanner as described in the materials and methods section. Figures 7.4 and 7.5 show the relationship between the average measured probe depth and the AUC for each fibre type for each volunteer.

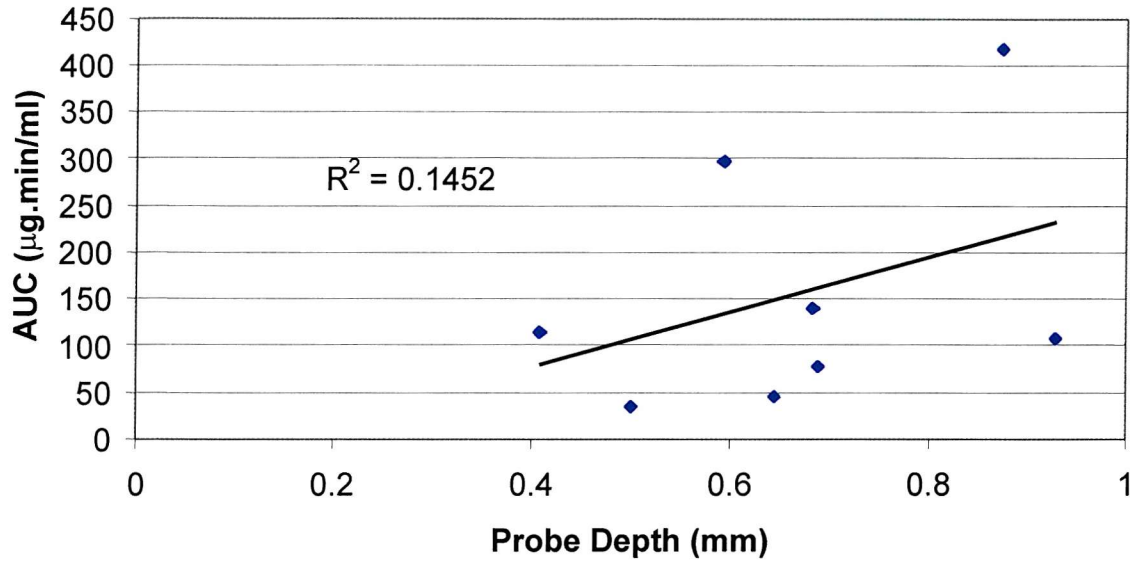

Figure 7.4: AUC (3MDa fibres) vs probe depth for 8 volunteers. R^2=0.15.

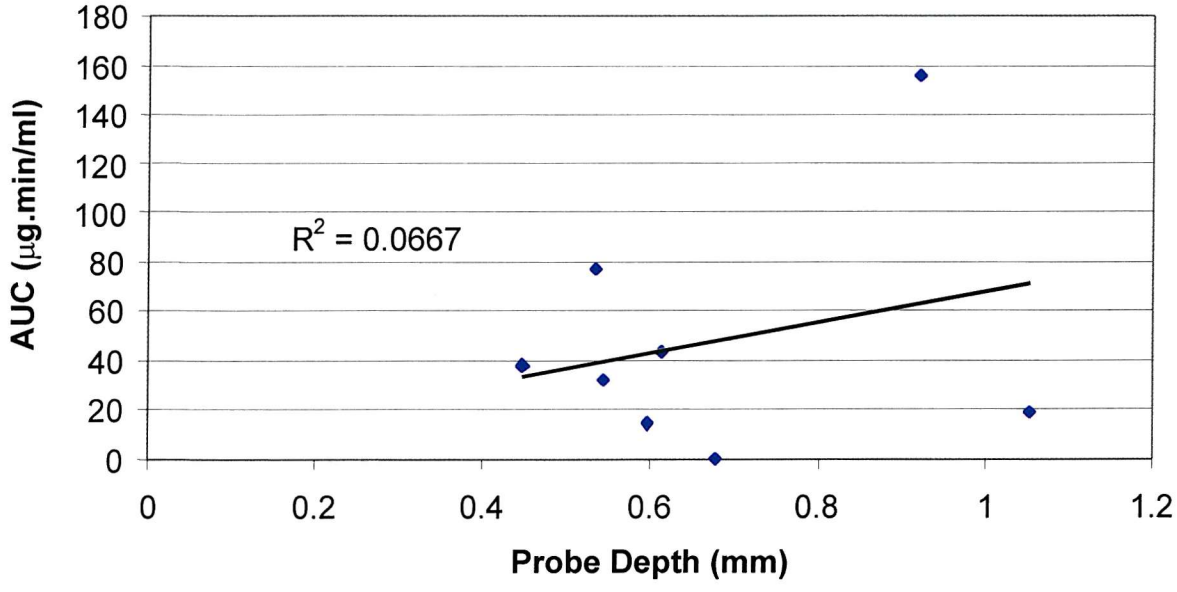

Figure 7.5: AUC (2kDa fibres) probe depth for 8 volunteers. R^2=0.07.

For both fibre types the linear regression trend line shows an increase in AUC as probe depth increases. However in both cases the R^2 value was so low that probe depth was not a significant variable. As for the methyl salicylate studies the majority of the fibres were implanted to a consistent range of approximately 0.4-0.7 mm, so that this conclusion only applies to this restricted experimental range of depth.

7.5 Discussion

The data from both the 3MDa and 2kDa fibres showed considerable inter-individual variation, in most cases the standard deviation exceeded the average concentration. This was expected as similar results were seen in previous chapters. This is probably due to differences in skin thickness and composition between volunteers.

The concentrations of ketoprofen removed from both the 3MDa and 2kDa fibres samples increased over time, and the increases between time points became larger as the studies progress. This would indicate that steady-state absorption had not been reached. At steady-state a plateau should be achieved where subsequent samples should have similar concentrations. These results differ from the methyl salicylate studies where steady-state appeared to be achieved within the time course of the study. As both methyl salicylate and ketoprofen have similar P_{ow} values and are of a similar size and lipophilicity it was expected that ketoprofen absorption would also reach steady-state within the study period. At the end of the ketoprofen studies when the drug wells were removed it was noticed that area of skin that had been exposed to the formulation was slightly raised which could be felt and also seen by the naked eye. When the fibres were scanned with ultrasound the epidermis directly above the fibres was found to be thicker and far less dense than the surrounding epidermis. Neither of these observations were seen in the methyl salicylate studies. The most likely explanation is that the ketoprofen commercial formulation has a much greater hydrating capacity than the water:propylene glycol mix that was used in the methyl salicylate studies. As the skin becomes hydrated the lipid bilayers in the stratum corneum would absorb water and swell, becoming less dense and more fluid. This allows compounds to diffuse faster through the stratum corneum and reduces the reservoir effect of the epidermis both of which allow a higher concentration to reach the dermis. The ketoprofen formulation also contains ethanol, which is known to disrupt the semi-crystalline structure of the lipid bilayer again increasing the speed of diffusion of absorbed compounds. The combination of developing skin hydration and ethanol absorption which would alter the nature of the stratum corneum barrier with time, would explain the

constant increase in concentration over time. If the study duration could have been extended the concentration would eventually have reached a plateau.

As expected the concentrations from the 3MDa lines were consistently higher than those from the 2kDa lines. This was confirmed by comparing the AUC's, which were significantly higher in the 3MDa lines in all volunteers. However the difference in concentration was much lower than expected based on the *in vitro* data. The *in vitro* studies in the previous chapter showed that ketoprofen is approximately 95% protein bound. The difference in relative recovery between the two fibre types from a ketoprofen solution containing a physiological concentration of HAS was approximately 15 fold. The difference between the AUC's of the two fibre types *in vivo* was only 3.5 fold. This difference is only slightly higher than the difference that would be expected if only free drug were present; the difference *in vitro* in the absence of albumin was 2 fold. A possible explanation is that the 3MDa fibre is dialysing protein faster than it can diffuse into the tissue space around the fibre. This would effectively strip the tissue space of mobile protein, reducing the extracellular protein concentration in the tissue to a fraction of normal levels. Once the protein levels had dropped the majority of the drug present around the fibre would be free drug, and the dialysate sample concentration would almost solely depend on the relative recovery of free drug. The difference in AUC was slightly higher than the difference dictated by the relative recovery of free drug. The protein levels in the tissue would take time to reduce and reach a lower steady-state and there would be a constant diffusion of protein from the surrounding tissue. This may explain the slightly elevated concentration of ketoprofen in the first collection point from the 3MDa fibres. At the start of the studies there is relatively high protein concentration around the fibres so the 3MDa fibres can dialyse both free and bound drug. However as protein levels decline only free drug is left around the fibres so that the sample concentration is reduced. No data for protein concentration was available for this study as the injection volume required for ketoprofen analysis left too small a sample for protein analysis. To remedy this for future studies an more sensitive analytical method for protein will be found.

The higher the protein concentration in the tissue space surrounding the fibres the nearer the 3MDa/2kDa ratio should be to the measured *in vitro* value of

15. As the protein levels drop so should the ratio as both fibres will be dialysing free drug. This was seen the ratio is high at the beginning, approximately 7.5 (although based on very inconsistent data) and as the study progresses the ratio gradually drops until by the final collection point it is only 1.6.

As for the methyl salicylate studies no correlation was shown between probe depth and AUC. Again the range of probe depths was very limited, 0.4-0.7mm, any difference in AUC caused by probe depth would small compared to inter-individual variations.

7.6 Conclusion

Ketoprofen penetrates the skin rapidly and was detected in some of the 3MDa fibre in the first collection point. However the 3MDa fibres appears to drain the tissue of mobile protein causing the concentration to drop by collection point two. The commercial formulation causes the skin to become hydrated and the stratum corneum to be disrupted, allowing an ever increasing amount of the drug to penetrate the stratum corneum and diffuse into the dermis. This caused the sample ketoprofen concentrations to rise increasingly from collection point two until the end of the study. The difference in AUC between the two fibres was much lower than suggested by *in vitro* experiments. This was probably due to low protein levels around the 3MDa fibres therefore both fibres are dialysing mostly free drug. The difference in concentration and AUC is therefore mainly dependent on the relative recovery of free drug by both fibre types. The decrease in the ratio of concentration between the two fibre types over time also suggests that the 3MDa fibre is draining the tissue space of mobile protein.

8 Results: Ketoprofen-stop-flow protein binding study- *In vivo*

8.1 Introduction

The studies from chapter 7 gave some unexpected results, because the drug concentrations detected using the 3Mda fibres and the 2kDa fibres were far closer than would have been predicted based on the *in vitro* data. The *in vitro* data proved that the 3Mda fibres were capable of dialysing protein and protein bound drug. The data also showed that *in vitro* the level of recovery of drug in the presence of protein was significantly higher for the 3Mda fibres (15 fold higher). Two possible reasons for the similar drug concentrations detected *in vivo* were postulated, either the concentration of diffusible protein within the skin was so low that protein bound drug did not form an appreciable component of the total drug concentration, or the large protein molecules have low diffusivity through the dermal tissue and that the microdialysis causes tissue depletion of the proteins in the volume around the fibres. Other researchers have shown that the extra-cellular fluid in skin tissue contains an appreciable concentration of albumin, approximately two thirds that of plasma and therefore any absorbed ketoprofen would be predicted to be significantly bound (Bart *et al*, 1986). Tissue depletion of protein is therefore the hypothesis that was tested in the following studies, which used a stop flow technique.

Tissue depletion would occur if the rate of removal of protein via diffusion into the microdialysis fibre was greater than the rate of diffusion of protein from the surrounding tissue space. If this hypothesis is correct and microdialysis was interrupted for a period within the study then the protein levels around the fibres should begin to return to normal tissue levels. If microdialysis was subsequently resumed the protein levels in the samples should be higher immediately after the interruption. There might also possibly be a rise in the concentration of drug recovered from the 3Mda fibres, as the level of protein bound drug should also increase.

8.2 Method

The 8 volunteers who took part in the *in vivo* studies had an average age of 27.4 years, (19-56 years), two of the volunteers were women and the rest were men. Six were students studying at Southampton University, one was a member of staff at the university and the oldest was retired. Due to problems with the fibres remaining patent during the studies not all of the volunteers had the same number of functioning lines (Table 8.1).

Volunteer	Age	Sex	2kDa Fibres	3MDa Fibres
1	19	F	3	3
2	22	M	3	3
3	22	M	3	3
4	31	F	3	3
5	56	M	2	3
6	22	M	2	3
7	21	M	3	3
8	26	M	3	3

Table 8.1: Volunteer information: Age, sex and number of lines for each experimental condition

All volunteers had six fibres inserted, three of which were 2kDa fibres identical to those used in the methyl salicylate studies and three were 3MDa fibres the construction of which is detailed in the methods chapter, section 2.2.2.2. The fibres were inserted as described in section 2.2.4.1 and 2.2.4.2 of the methods chapter, the only difference was that all fibres were inserted using a 21-gauge needle to reduce variation. The two sets of fibres were inserted alternately and the fibre type nearest the wrist being random. All fibres were perfused with Ringer's solution containing 5μg/ml noradrenaline at a flow rate of 0.4ml/hr. Ketoprofen gel (0.1ml) was applied to the skin within drug wells at the end of the first hour of microdialysis. All drug wells were occluded after the ketoprofen formulation had been applied. All parameters apart from the fibre type remained constant. The rest of the study was performed using the procedure detailed in the *in vivo* section of the methods chapter with the only exception being that perfusion of two of the 3MDa and 2kDa fibres was stopped between 2.0 and 2.5 hours after application of the gel. Microdialysis

was resumed after the half hour pause. Apart from the hour-long pre-dose collection, the collection periods were half an hour.
Where there were duplicate lines in the same subject, in this case the stop-flow lines, the values for the two lines were averaged prior to calculation of means and statistics between all 8 subjects.

The method of analysis for ketoprofen had been improved for this study; it was found that the wavelength (305nm) reported in by several papers for UV detection and used for chapter 7 was not the optimum for analysis of microdialysis samples. By changing the wavelength used to 260 nm a 10-fold increase in sensitivity was achieved, which meant that smaller collection periods could be employed allowing a greater temporal resolution whilst leaving enough volume of sample for protein analysis.

3MDa	Vol 1		Vol 2		Vol 3		Vol 4		Vol 5		Vol 6		Vol 7		Vol 8	
Time	Pause	Control	Pause	Control	Pause	Control	Pause	Control	Pause	Control	Pause	Control	Pause	Control	Pause	Control
0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0
0.5	0.06	0	0.09	0.07	0.21	0.07	0.52		0.00	0.32	0	0	0	0.04	0.04	0.04
1	0.11	0.05	0.11	0.07	0.44	0.08	1.58		0.00	0.62	0.01	0.02	0.03	0.16	0.20	0.23
1.5	0.27	0.05	0.14	0.09	0.82	0.16	1.67		0.11	0.69	0.01	0.09	0.10	0.38	0.45	0.47
2	0.43	0.15	0.16	0.30	1.26	0.35	1.79		0.25	1.03	0.04	0.16	0.27	0.50	0.51	0.36
2.5	Pause	0.26	Pause	0.34	Pause	0.86	Pause		Pause	1.48	Pause	0.25	Pause	0.74	Pause	0.55
3	1.22	0.49	0.39	0.35	4.40	1.28	3.96		1.54	2.73	0.09	0.33	0.64	1.09	1.11	0.73
3.5	0.85	0.90	0.21	0.43	4.16	2.65	1.96		1.63	2.61	0.06	0.47	0.52	1.64	0.86	0.91
4	1.18	1.07	0.35	0.49	5.09	2.89	2.76		2.25	2.93	0.16	0.48	0.80	1.99	1.17	0.92
4.5	1.45	1.21	0.44	0.51	5.73	3.35	3.11		2.70	3.43	0.23	0.62	1.13	3.22	1.18	0.96
5	2.17	1.17	0.47	0.63					3.05	3.17		0.69	1.64	5.66	1.77	1.06

Table 8.2: Summary of all data from all volunteers from all 3MDa fibres. The values are concentration (μg/ml) of ketoprofen in the dialysate sample for each line and each time point (spaces indicate that sample was lost) following topical ketoprofen application.

2kDa	Vol 1		Vol 2		Vol 3		Vol 4		Vol 5		Vol 6		Vol 7		Vol 8	
Time	Pause	Control	Pause	Control	Pause	Control	Pause	Control	Pause	Control	Pause	Control	Pause	Control	Pause	Control
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.5	0.04	0	0.02	0.00	0.00	0.00	0.00	0.00	0.04	0.03	0	0	0	0.03	0.05	0.00
1	0.10	0.12	0.01	0.10	0.02	0.00	0.12	0.25	0.09	0.24	0.00	0.00	0.02	0.12	0.06	0.00
1.5	0.10	0.00	0.04	0.07	0.19	0.00	0.18	0.27	0.17	0.04	0.00	0.00	0.03	0.22	0.04	0.00
2	0.07	0.00	0.12	0.12	0.24	0.08	0.30	0.41		0.37	0.00	0.00	0.02	0.18	0.04	0.00
2.5	Pause	0.04	Pause	0.32	Pause	0.17	Pause	0.47	Pause	2.33	Pause	0.00	Pause	0.26	Pause	0.00
3	0.20	0.07	0.12	0.27	0.93	0.42	0.42	0.55	0.88	2.53	0.00	0.00	0.11	0.26	0.07	0.03
3.5	0.27	0.13	0.16	0.24	1.54	0.49	0.47	0.58	1.41	2.84	0.00	0.00	0.25	0.32	0.19	0.10
4	0.44	0.20	0.23	0.31	0.65	0.65	0.52	0.63	2.40	3.74	0.00	0.00	0.33	0.36	0.21	0.19
4.5	0.51	0.38	0.54	0.36	1.35	0.99	0.53	0.68	2.26	5.00	0.01	0.01	0.55	0.51	0.34	0.46
5	0.84	0.38	0.53	1.07			0.52	0.72	2.24	5.00	0.02	0.02	0.85	0.62	0.53	0.47

Table 8.3: Summary of all data from all volunteers from all 2kDa fibres. The values are concentration (μg/ml) of ketoprofen in the dialysate sample for each line and each time point (spaces indicate that sample was lost) following topical ketoprofen application.

8.3 Ketoprofen Absorption and Recovery

Tables 8.2 and 8.3 above show all the data from all 8 volunteers and all fibre types. Figure 8.1 shows the averaged concentration and standard errors for the 8 volunteers.

The profiles of both control lines are very similar to the profiles seen in the previous chapter. Both show an increase throughout the course of the study period with no plateau region reached with either fibre type. The average ratio between the two control fibres (3MDa : 2kDa) for each time point was 2.16 ± 0.69.

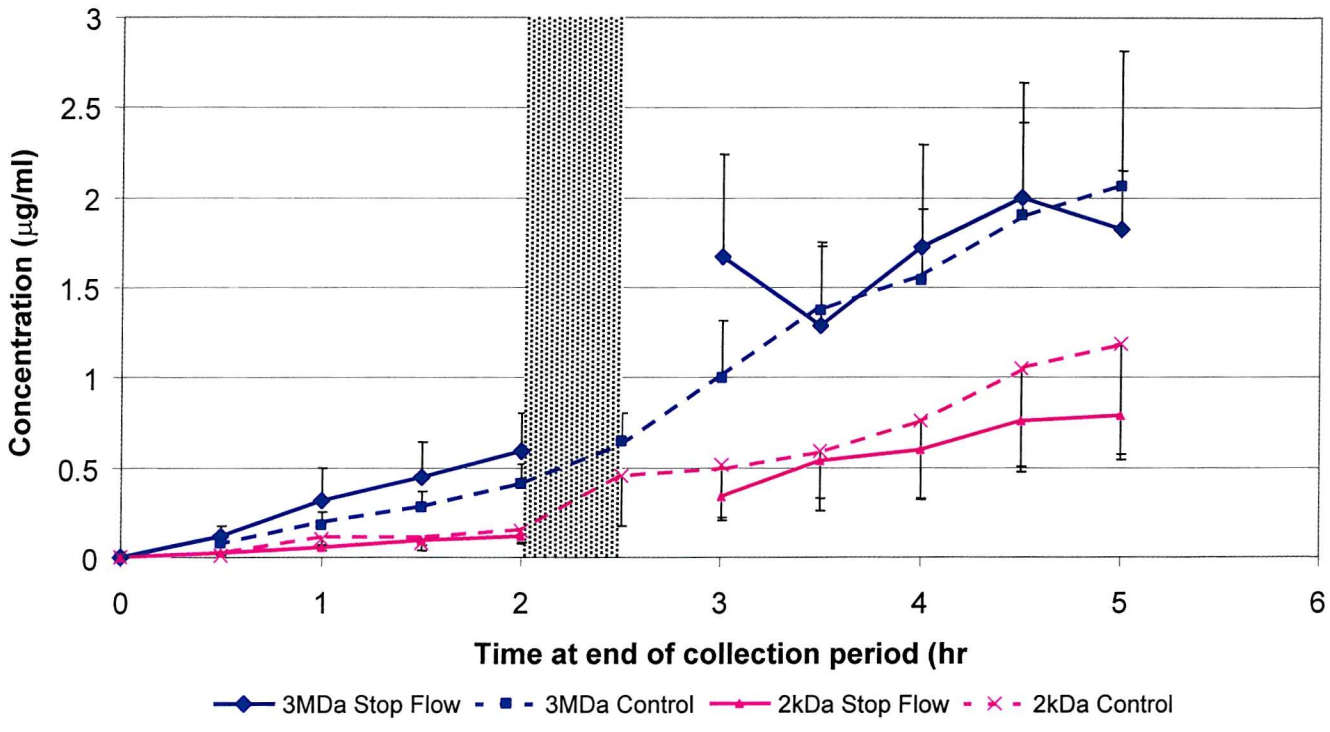

Figure 8.1: Comparison of sample concentration from 3MDa and 2kDa fibres from 8 volunteers following topical ketoprofen application. Shaded area denotes period of stop-flow.

The 2kDa fibre stop-flow lines follow a similar profile to the control lines i.e. a gradual increase over time with no steady-state reached by the end of the study period. The 3MDa stop-flow fibres initially show a similar profile to the control lines but the time point immediately following the stop-flow period showed a higher concentration (1.67 ± 1.62 µg/ml) than the control line (1.00

± 0.82 μg/ml), although this was not significantly different. The concentration decreased after this time point and the profile resembled the control line for the remainder of the study period. The average ratio between the two stop-flow fibres (3MDa : 2kDa) for each time point was 3.87 ± 1.45, there was found to no significant difference between the control line ratio and the stop-flow ratio.

Fibre Type	3MDa Fibre		2kDa Fibre	
Condition	Stop-Flow	Control	Stop-Flow	Control
AUC (μg.min/ml)	261 ± 225	248 ± 170	85 ± 79	126 ± 191

Table 8.4: AUC for each fibre type and both control and stop-flow. Both 3MDa fibres were significantly different to both 2kDa fibres (control and stop-flow), $p<0.05$. There was no significant difference between the control and stop flow line of same fibre type. n = 8.

Table 8.4 above, shows the AUC of each fibre type under control and stop-flow conditions plus or minus the standard deviation. There was found to be no significant differences between the stop-flow and control lines of the same fibre type using a paired t-test. Between fibre types however all values were significantly different, $p<0.05$.

Figure 8.2 below shows the average concentrations recovered for the 3MDa and 2kDa control fibres with the concentrations adjusted using the relative recovery for each fibre, found *in vitro* in the absence of protein. The average AUC's for the corrected data of the 3MDa and 2kDa line were 355 ± 277 μg.min/ml and 382 ± 572 μg.min/ml respectively. The results were not significantly different (paired T test, $p> 0.05$).

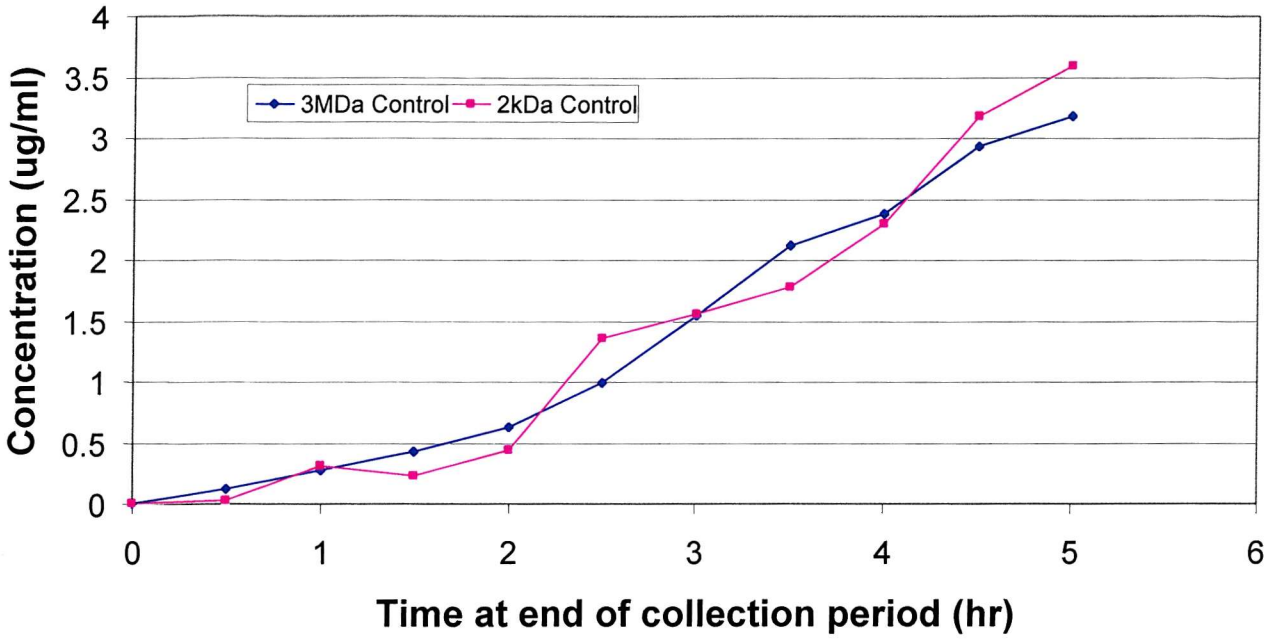

Fig 8.2: Comparison of corrected average ketoprofen concentrations for 3MDa and 2kDa control lines following topical ketoprofen application. n = 8.

Figure 8.3, below, shows the average concentration of both the 3MDa and 2kDa stop-flow lines corrected for *in vitro* recovery in the absence of protein. The average AUC's of the 3MDa and 2kDa line were respectively 403 ± 347 μg.min/ml and 259 ± 240 μg.min/ml. The results were not significantly different (paired T-test, $p>0.05$). The concentrations at individual time points were compared between the control and stop-flow lines and paired t-tests were used to identify any significant differences. None of the time points on either the 2kDa and 3MDa stop-flow lines were significantly different to their respective control line time points. This may have been because the higher level in the stop-flow fibres was compared with a rising level in the control fibre. In order to remove the effect of the constantly increasing concentration the t-test was repeated but this time the change in concentration between successive time points was compared. There were no significant differences between the 2kDa control and stop-flow lines. The same analysis applied to the 3MDa fibres showed a statistically significant difference immediately after the stop-flow period, with a significant decrease between time points 3 and 3.5 hours, and a subsequent increase between 3.5 and 4 hours ($p< 0.01$ and 0.01 respectively).

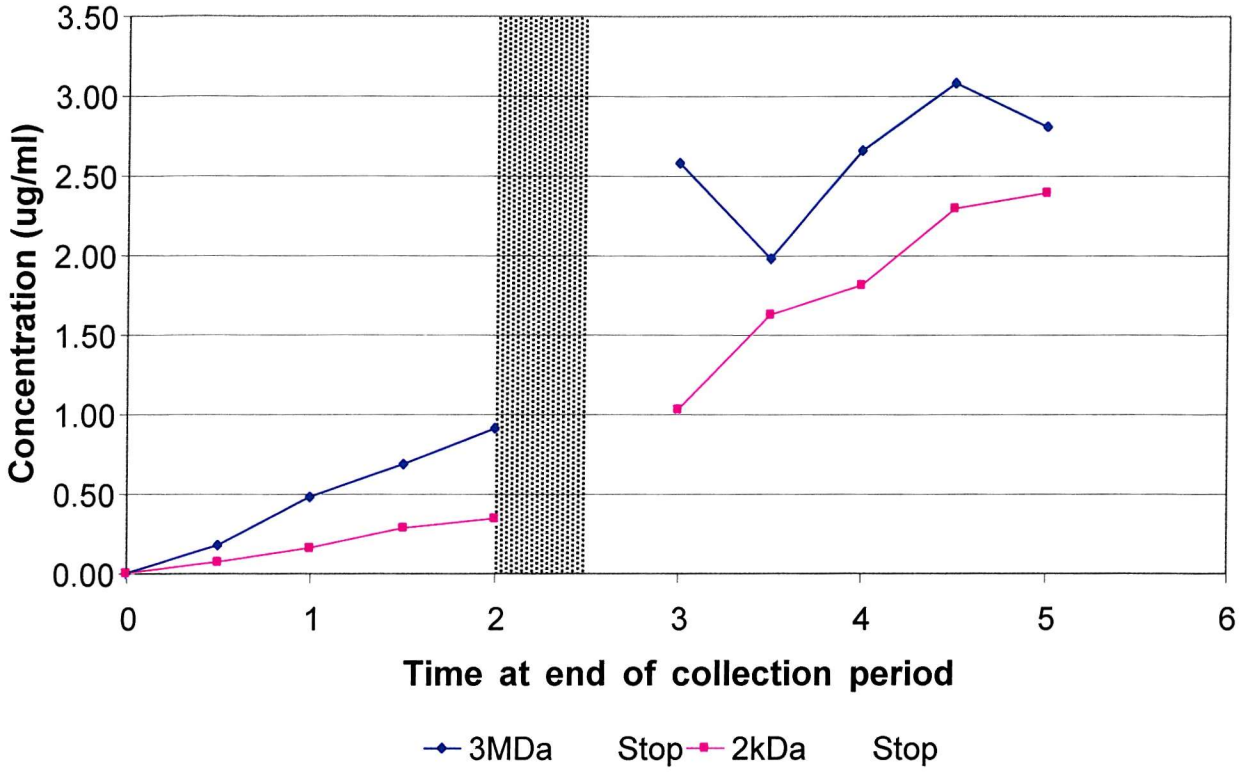

Fig. 8.3: Comparison of corrected average ketoprofen concentrations for 3MDa and 2kDa stop-flow lines following topical ketoprofen application. Shaded area denotes period of stop-flow. n = 8.

The data was also analysed using an assigned rank test. For each line in each volunteer (the average in the case of the stop-flow lines) the concentration at each time point was ranked with respect to the other time points for that line. The ranking for each time point for the stop-flow lines was then compared to the time point on the respective control line. There was no significant difference in the rankings between the stop-flow and control lines from the pre-dose sample until the stop-flow period. After the stop-flow period the first 3MDa stop-flow collection point had a significantly higher ranking than its respective control line samples ($p<0.01$). No difference in ranking was shown between any of the 2kDa lines.

8.4 Protein Recovery

Figure 8.4 below, shows the average concentration of protein recovered using 3MDa fibres at each collection point for all 8 volunteers. The profiles with and without stop-flow are similar, with initial concentrations of approximately 500μg/ml reduced over 3 hours and reaching a constant value after about 3.5 hours, at a concentration of approximately 200 μg/ml. The stop-flow line data showed a marked increase in the protein concentration after the stop-flow period, which returned to the steady-state concentration by 4 hours. The AUC's of the control and stop-flow lines were 78606 ± 23351 μg.min/ml and 94377 ± 25952 μg.min/ml respectively. A paired t test showed this to be not significant ($p>0.05$).

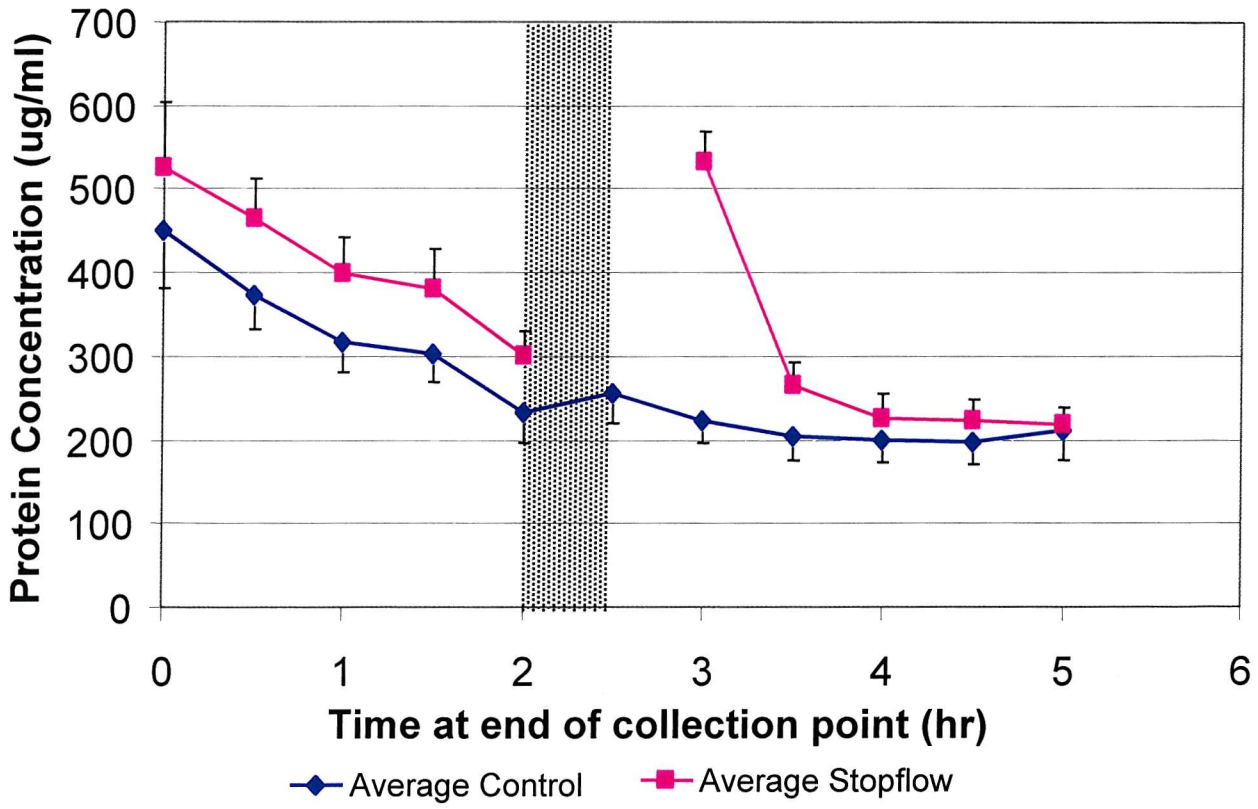

Fig. 8.4: Average protein concentrations from 3MDa control and stop-flow lines, from 8 volunteers following topical ketoprofen application. Shaded area denotes period of stop-flow.

Statistical analysis by paired t-test showed that the only significant difference was in the collection period immediately after the stop flow period ($p<0.001$). A similar difference was found when the changes in concentrations between successive collections were compared; the concentration after the stop-flow

period (3h *cf* 2h) was significantly higher in the stop-flow but not in the control lines (3h *cf* 2h) ($p<0.001$), no other collection points showed significance.

8.5 Probe Depth

Figures 8.5 and 8.6 (below) show the relationship between probe depth and calculated AUC for the 3MDa and 2kDa control lines. For both lines the R^2 value was below 0.2 indicating that for the range of probe depths measured there was no significant correlation with AUC.

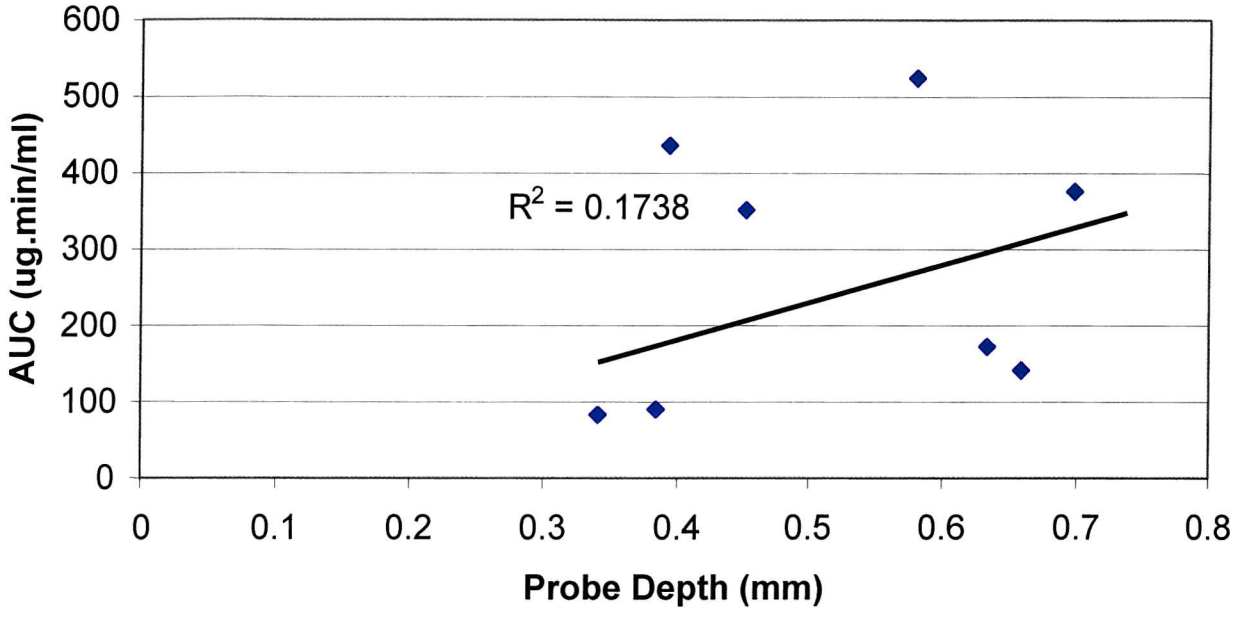

Figure 8.5: AUC (3MDa fibres) vs probe depth for 8 volunteers. R^2=0.17.

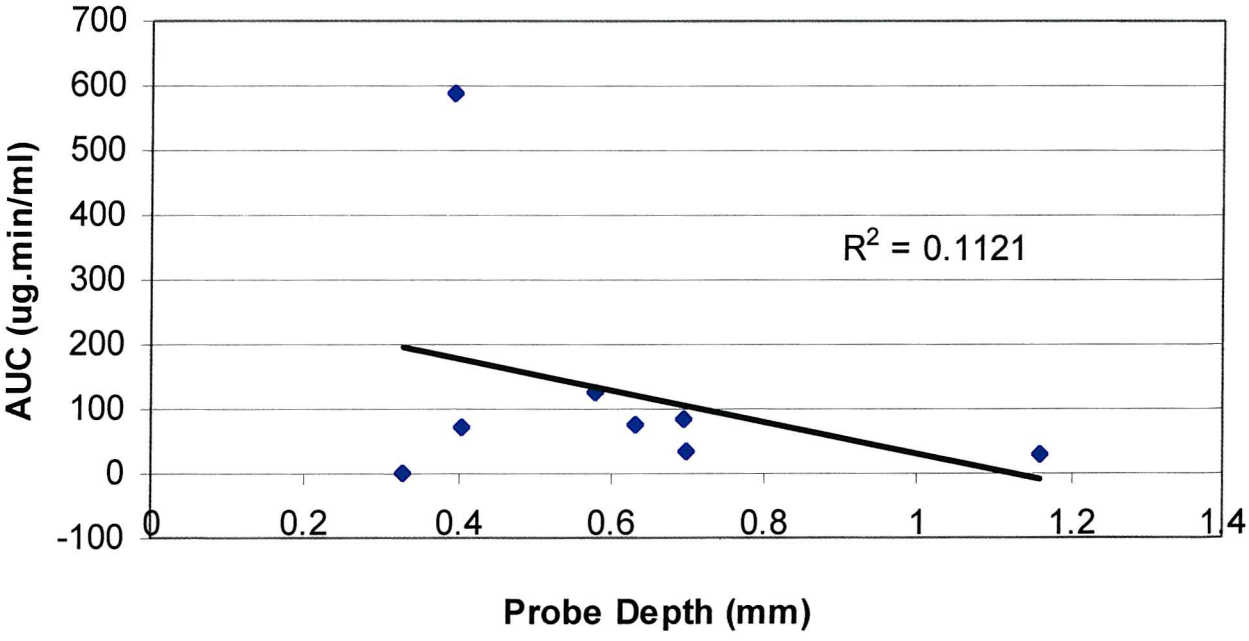

Figure 8.6: AUC (2kDa fibres) vs probe depth for 8 volunteers. R^2=0.11.

8.6 Discussion

As in the previous studies, the inter-individual variation was large, the average concentrations often had standard deviations that were greater than the actual values. However as each volunteer had both fibre types with both controls and stop-flow lines, the results could be analysed using paired t-tests, which minimises the impact of inter-individual variability.

The two control lines showed very similar profiles (fig 8.2), with a continuously increasing concentration with most increases appearing slightly greater than the last. The most obvious difference when compared to the results from the previous chapter was the initial high concentration found in the first post-dose sample (Chapter 7), which was not seen in this study (Chapter 8). This raises the possibility that the initial high concentration seen previously may have resulted from contamination while the formulation was being applied. However after this collection point the profiles followed similar trends with steadily larger increases in concentration over time and a steady-state not being reached within the time course of the study. At the end of the study, when the drug wells were removed, the area of skin where the formulation had been applied was visibly raised. Ultrasound scans (figs 8.7 and 8.8) showed that the epidermal tissue under the drug well was far less dense and was noticeably thicker than the surrounding epidermal tissue. This epidermal swelling was not found with the propylene glycol/water formulation used with the methyl salicylate studies, which suggests that it was caused either by the drug itself or by a combination of the ethanol content and occlusion (see figs 8.7 and 8.8). Ethanol is known to be a potent penetration enhancer (Guy *et al*, 1990), it disrupts the semi-crystalline structure of the lipid bilayer surrounding the keratinocytes in the stratum corneum allowing any applied drug or water to penetrate and diffuse through more easily. Water penetrating the lipid bilayer would further increase its fluidity and potentially increasing the rate of diffusion of compounds through to the dermis. It is possible that the continuously increasing concentrations and the fact that steady-state was not reached within the time course of the study are due to the barrier function of the epidermis being continuously reduced by the effects of ethanol penetration and hydration.

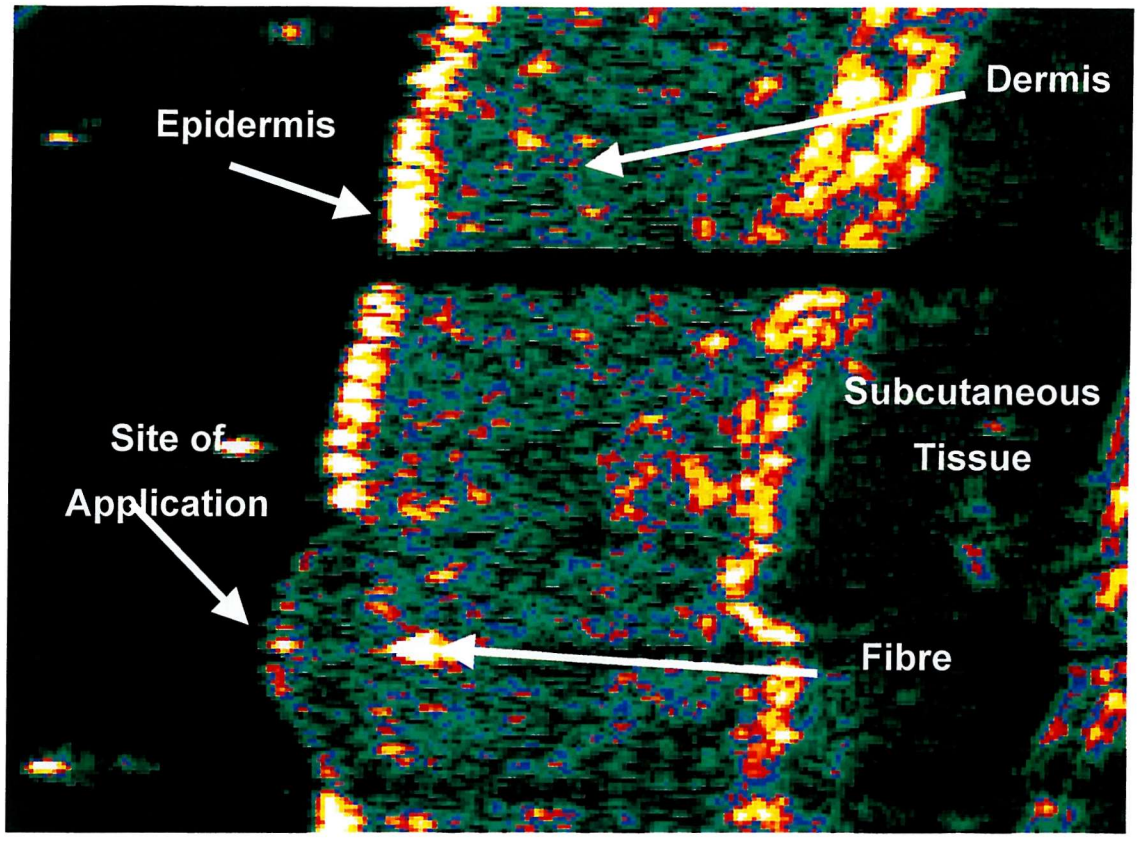

Fig 8.7: Ultrasound cross-section of skin on volunteers forearm showing implanted fibre and site of drug application. Drug applied was ketoprofen in ethanol/water gel.

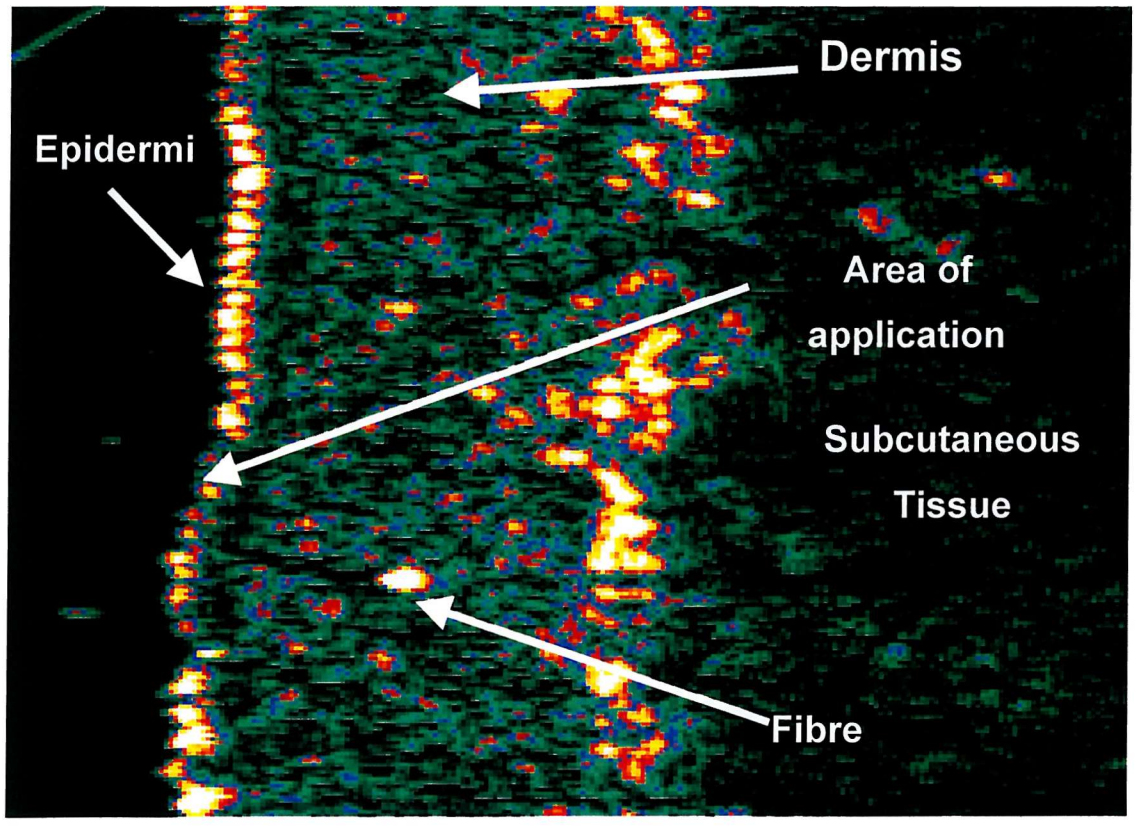

Fig 8.8: Ultrasound cross-section of skin on volunteers forearm showing implanted fibre and site of drug application. Drug applied was methyl salicylate in propylene glycol/water

The average ratio between 3MDa control lines and 2kDa control lines was found to be far smaller than the ratio seen *in vitro* between the two fibre types in the presence of protein. When the relative recovery was determined *in vitro* for both 3MDa and 2kDa fibres from a solution of ketoprofen containing a physiological solution of HSA the ratio between the sample concentrations was 15. However the ratio seen *in vitro* from solutions containing solely ketoprofen was 1.9. This is a clear indication that the 3MDa fibre types are dialysing mostly free drug *in vivo.* The control line data were recalculated using the relative recoveries established *in vitro* from solutions of ketoprofen only, this would give the minimum tissue concentrations assuming that only free drug was present (fig 8.2). The AUC's of the two lines were found to have no significant difference between them helping corroborate the hypothesis that mostly free drug is being dialysed.

The protein recovery data from the 3MDa lines (fig 8.4) showed similar profiles for both the stop-flow and control lines. The only exception was the collection point immediately after the stop-flow period, which appears to have far greater concentration of protein in the stop-flow line and was found to be significant. There are two important points of information to be noted; firstly from the start of the study period the tissue protein concentration reduces over time and reaches a steady state by just after half way through the study period and secondly when microdialysis is paused protein levels return to their initial level. Both facts taken together clearly indicate that microdialysis is directly causing tissue depletion of mobile protein and that only by pausing perfusion can tissue levels start to return to their normal resting concentrations. Similar results were seen in unpublished data from a co-researcher based at Southampton General Hospital (G.Clough, personal communication) the major difference in the study protocols was the collection period was only 5 minutes compared to 30 minutes in this study. The data showed much higher initial protein concentrations, typically 2-3000 μg/ml, however within half an hour the protein concentrations had reduced to a level approximately equal to that seen in the first collection period of this study. The concentrations from both studies then followed almost exactly the same profile; both had a statistically significant increase in concentration after the stop-flow period. This data shows that tissue depletion of protein is rapid and

that a concentration gradient around the fibre forms almost immediately. The relatively long collection periods used in this study have masked this, all concentrations are an average over the entire half hour collection period and therefore any rapid changes in concentration were not detectable.

The two stop-flow lines followed a similar profile to the control lines with one exception, the first sample collected after the stop-flow period from the 3MDa lines appeared to have a much higher concentration than its corresponding control line (fig. 8.1), and was found to be significant. As this was not seen in the 2kDa stop-flow line, the increase in concentration must be due to an increase in protein bound drug. Again the ratio between the two stop-flow lines was much lower than the *in vitro* ratio measured in the presence of protein, which like the control line data indicates that very little, if any, protein bound drug is being dialysed. The AUC's of the corrected stop-flow line data (fig. 8.3) were found to have no significant difference; this again adds weight to the proposal that the 3MDa fibres are dialysing mostly free drug.

T o summarise the findings so far from this chapter:

- Microdialysis using 3MDa fibres causes tissue depletion of dialysable protein in the tissue space around the inserted fibre.
- When microdialysis is paused for 0.5 hours the concentration of protein returns to its initial levels.
- The difference in ketoprofen concentration recovered from the 3MDa and 2kDa fibres is approximately equal to the difference in relative recovery of free drug from the two fibre types. This indicates that the 3MDa fibres are mostly dialysing free drug and very little protein bound drug.
- When microdialysis is paused for 0.5 hours the increase in ketoprofen concentration from the 3MDa fibres is significantly greater than the control line, but no increase is seen in the 2kDa fibres. Therefore there is not a measurable increase in free drug concentration after the pause in microdialysis but there is for the concentration of protein bound drug.

The findings of this study can be explained on the basis of the rates of diffusion of extracellular protein compared to free drug. Firstly the medium through which both have to move, the dermis, is a highly viscous environment

containing large structural and connective proteins such as collagen and elastin fibres. Compared to free drug, albumin proteins are large, polar molecules, the molecular weight of a typical albumin molecule is >30,000 whereas the molecular weight of ketoprofen is 254. The larger mass of the albumin means that the rate of diffusion in comparison with ketoprofen would be far lower. During cutaneous microdialysis both protein, protein bound drug and free drug are diffusing into the fibre and being constantly removed.

The fact that stop-flow did not affect the profile of the 2kDa fibres indicates that there was no significant depletion of free drug, see fig 8.9. This suggests that the concentration of free drug adjacent to the fibre and in tissue increasing distances away from the fibre are approximately equal during microdialysis i.e. the rate of flux or rate of diffusion is high enough to counter the rate of removal by microdialysis. Therefore when microdialysis is paused there is no measurable increase in the concentration of ketoprofen from the 2kDa fibres when perfusion is resumed, as there is no significant concentration gradient of free ketoprofen in the tissue around the fibre.

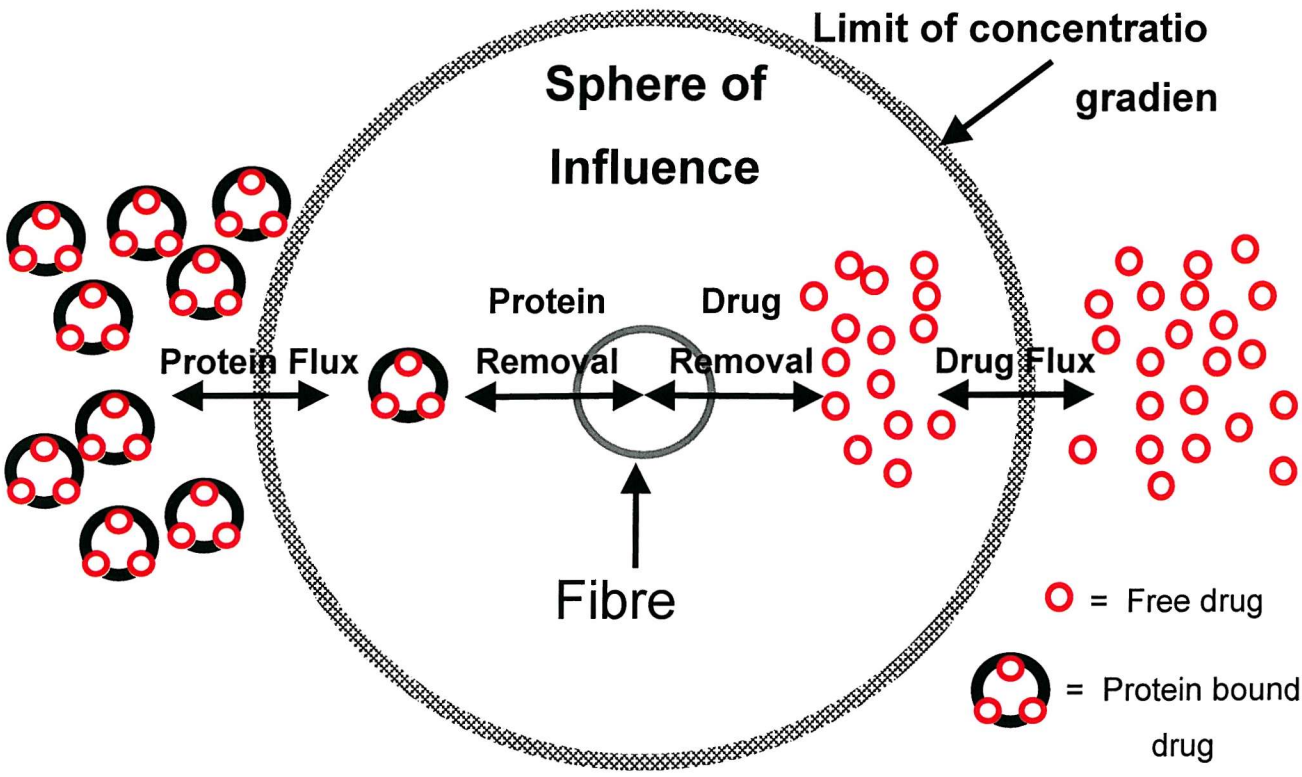

Fig 8.9: Comparison of free drug and protein/protein bound drug flux around inserted fibre. Drug Flux ≥ Drug Removal, sample concentration tissue concentration . Protein Flux < Protein Removal, leading to tissue depletion

The rate of diffusion of free drug is such that any decrease in free drug due to the increasing concentrations of protein is reversed by diffusion of free drug into the tissue adjacent to the fibre. With protein or drug bound to protein the rate of diffusion or flux from the adjacent does not appear to be high enough to counter the rate of removal via the fibre leading to a state of tissue depletion of protein around the fibre. When microdialysis in the 3MDa fibres is paused the concentration of protein around the fibre returns to its initial levels and when perfusion is resumed a higher concentration of protein and protein bound drug are measured. The practical offshoot of this is that using 3MDa fibres to study protein bound drugs routinely will require a great deal of care when designing a workable protocol. Flow rates will have to be kept low in order to maintain a higher relative concentration of protein within the tissue. The collection periods will need to be kept small e.g. 5 minutes or less with long pauses in microdialysis between them. The short collection periods will reduce the amount of tissue depletion and the regular pauses in microdialysis will mean that even with a low rate of diffusion an appreciable concentration gradient will not develop around the 3MDa fibres.

Alternatively the 3MDa could be used with continuous flow as a method of improving the detection of free drug, especially extremely lipophilic/hydrophilic and/or highly protein bound drugs as the larger surface area and higher density of dialyzable pores will mean that the relative recovery will always be higher than that from 2kDa fibres.

8.7 Conclusion

Microdialysis using 3MDa fibres leads to tissue depletion of protein whether bound to drug or not. However microdialysis using 2kDa fibres does not appear to lead to tissue depletion of free drug. The most likely explanation is due to the relative rates of diffusion of protein compared to free drug. Albumin is a large molecule with a high molecular mass, this results in a low rate of diffusion through the dermis, the much smaller, lighter ketoprofen has a far greater rate of diffusion. With respect to protein the rate of diffusion within the dermis appears to be less than rate of removal via the 3MDa fibre leading to tissue depletion and the formation of a concentration gradient around the fibre. When the perfusion was halted the rate of removal drops to zero but the rate of diffusion remains unchanged. Protein moves down the concentration gradient and the protein concentration around the fibre returns to pre-perfusion levels. When perfusion is resumed the concentration of protein or drug is higher than in the control line. In the case of the 2kDa fibres the rate of removal of free drug appears to be equal to or less than the rate of diffusion. Therefore the concentration around the fibre remains equal to the tissue concentration at progressively further distances away from the fibre. Therefore no concentration gradient is formed. When perfusion is halted the concentration of free drug around the fibre remains constant as there is no concentration gradient, when perfusion is resumed the concentration of the sample showed no significant change from the control sample.

To use 3MDa fibres to study highly protein bound drugs study protocols will have to designed to minimise tissue depletion of protein. This means incorporating low flow rates and short collection times with periods of zero flow in between every collection point. If 3MDa fibres are used with continuous flow they are effectively only measuring variations in free drug concentration albeit with a higher relative recovery than 2kDa fibres.

9 Results: fenbufen and biphenylacetic acid

9.1 *In vitro*

9.1.1 Introduction

A series of *in vitro* studies were performed to calculate the relative recovery of fenbufen and biphenylacetic acid at various flow rates and bath concentrations.

Several flow rates were tried to investigate the relationship between flow rate and the calculated relative recovery. As flow rate increases the residence time of the perfusate in the fibre is reduced which in turn would reduce the relative recovery. Theory suggests the relationship between flow rate and relative recovery should be linear, i.e. double flow rate and halve the relative recovery. According to Fick's law the relative recovery should be independent of bath concentration.

9.1.2 Methods

All studies were carried out using the protocol detailed in the materials and methods chapter. All lines used were made from the 2kDa fibres. The only modification to the method described was that the fibres used had a fine stainless steel wire inserted along their entire length.

9.1.3 Results

9.1.3.1 Concentration

Four concentrations of both fenbufen and biphenyl acetic acid were studied *in vitro*, 40, 20, 10 and 5μg/ml solutions. Six fibres were tested at each concentration and hourly collections were made for four hours.

With a bath concentration of 40μg/ml the average relative recoveries for fenbufen and biphenylacetic acid were 50% and 53% respectively, see tables 9.1 and 9.2. The two relative recoveries were not found to be significantly different using a t test ($p>0.05$).

Time	Line A	Line B	Line C	Line D	Line E	Line F
0	0.00	0.00	0.00	0.00	0.00	0.00
1	48.39	48.53	47.97	49.17	50.63	51.17
2	48.16	53.19	48.63	55.04	53.85	51.95
3	45.56	48.42	45.33	54.84	47.69	43.26
4	50.23	48.47	55.18	55.76	54.62	52.37
Average	48.09	49.65	49.28	53.70	51.70	49.69
SD	1.92	2.36	4.19	3.04	3.18	4.31
Group Average				50.35		
Group Standard Deviation				2.01		

Table 9.1: Percentage relative recoveries for fenbufen (%). Bath concentration = 40μg/ml Fenbufen. Flow rate = 0.2ml/hr.

Time	Line A	Line B	Line C	Line D	Line E	Line F
0	0.00	0.00	0.00	0.00	0.00	0.00
1	52.29	53.24	51.78	53.59	54.57	54.98
2	50.19	55.62	50.73	57.49	56.33	54.72
3	47.72	50.71	47.56	57.02	50.45	45.72
4	51.53	49.94	57.24	57.51	56.79	54.34
Average	50.43	52.38	51.83	56.40	54.54	52.44
SD	2.00	2.58	4.03	1.89	2.89	4.49
Group Average				53.00		
Group Standard Deviation				2.13		

Table 9.2: Percentage relative recoveries for biphenylacetic acid (%). Bath concentration = 40μg/ml biphenylacetic acid. Flow rate = 0.2ml/hr.

At a bath concentration of 20μg/ml the relative recoveries of fenbufen and biphenylacetic acid were 53% and 56% respectively, see tables 9.3 and 9.4. There was no significant difference between them (p>0.05).

Time	Line A	Line B	Line C	Line D	Line E	Line F
0	0.00	0.00	0.00	0.00	0.00	0.00
1	54.79	45.48	49.95	48.86	51.35	53.36
2	55.46	51.05	53.32	53.32	52.21	55.79
3	58.53	49.59	56.01	52.79	53.20	59.15
4	55.40	49.47	54.02	52.34	53.67	58.82
Average	56.04	48.90	53.33	51.83	52.61	56.78
SD	1.68	2.39	2.52	2.02	1.03	2.74
Group Average				53.25		
Group Standard Deviation				2.89		

Table 9.3: Percentage relative recoveries for fenbufen (%). Bath concentration = 20μg/ml Fenbufen. Flow rate = 0.2ml/hr..

Time	Line A	Line B	Line C	Line D	Line E	Line F
0	0.00	0.00	0.00	0.00	0.00	0.00
1	56.54	47.29	51.93	51.61	52.30	55.09
2	57.01	52.51	56.24	73.95	54.41	57.64
3	60.51	51.11	57.81	55.04	54.09	60.79
4	57.28	50.82	56.41	54.72	55.04	60.94
Average	57.83	50.43	55.60	58.83	53.96	58.62
SD	1.81	2.22	2.54	10.20	1.18	2.80
Group Average				55.88		
Group Standard Deviation				3.27		

Table 9.4: Percentage relative recoveries for biphenylacetic acid (%). Bath concentration = 20μg/ml biphenylacetic acid. Flow rate = 0.2ml/hr.

At a bath concentration of 10μg/ml the relative recoveries of fenbufen and biphenylacetic acid were 50% and 50% respectively, see tables 9.5 and 9.6. There was no significant difference between them (p>0.05).

Time	Line A	Line B	Line C	Line D	Line E	Line F
0	0.00	0.00	0.00	0.00	0.00	0.00
1	47.13	51.11	51.03	49.69	43.39	44.08
2	48.20	52.65	54.19	52.50	47.89	45.66
3	45.37	53.22	53.97	51.31	49.98	50.89
4	50.41	52.29	52.95	50.63	47.07	47.70
Average	47.78	52.32	53.03	51.03	47.08	47.08
SD	2.11	0.89	1.44	1.18	2.75	2.94
Group Average				49.72		
Group Standard Deviation				2.73		

Table 9.5: Percentage relative recoveries for fenbufen (%). Bath concentration = 10μg/ml Fenbufen. Flow rate = 0.2ml/hr.

Time	Line A	Line B	Line C	Line D	Line E	Line F
0	0.00	0.00	0.00	0.00	0.00	0.00
1	47.39	51.51	50.05	49.14	43.18	44.38
2	48.18	53.05	52.80	52.02	47.65	45.68
3	45.17	53.08	52.73	50.81	49.98	51.30
4	50.67	52.75	51.72	50.08	46.91	47.99
Average	47.85	52.60	51.82	50.51	46.93	47.34
SD	2.27	0.74	1.28	1.21	2.82	3.03
Group Average				49.51		
Group Standard Deviation				2.45		

Table 9.6: Percentage relative recoveries for biphenylacetic acid (%). Bath concentration = 10μg/ml biphenylacetic acid. Flow rate = 0.2ml/hr.

At a bath concentration of 5μg/ml the relative recoveries of fenbufen and biphenylacetic acid were 46% and 48% respectively, see tables 9.7 and 9.8. There was no significant difference between them ($p>0.05$).

Time	Line A	Line B	Line C	Line D	Line E	Line F
0	0.00	0.00	0.00	0.00	0.00	0.00
1	47.61	45.51	46.52	46.72	40.28	47.23
2	50.20	38.78	54.49	48.63	36.18	36.84
3	51.42	43.15	47.43	45.01	48.52	46.87
4	47.25	43.21	47.14	49.27	47.57	47.56
Average	49.12	42.66	48.90	47.41	43.14	44.62
SD	2.02	2.81	3.75	1.93	5.92	5.20
Group Average				45.97		
Group Standard Deviation				2.87		

Table 9.7: Percentage relative recoveries for fenbufen (%). Bath concentration = 5μg/ml Fenbufen. Flow rate = 0.2ml/hr.

Time	Line A	Line B	Line C	Line D	Line E	Line F
0	0.00	0.00	0.00	0.00	0.00	0.00
1	46.97	45.06	48.71	49.70	43.68	46.88
2	50.40	37.99	60.15	52.35	38.90	39.08
3	52.14	42.90	52.00	48.21	52.67	47.36
4	46.82	42.70	51.07	49.50	51.61	47.60
Average	49.08	42.16	52.98	49.94	46.71	45.23
SD	2.63	2.98	4.98	1.73	6.58	4.11
Group Average				47.68		
Group Standard Deviation				3.81		

Table 9.8: Percentage relative recoveries for biphenylacetic acid (%). Bath concentration = 5μg/ml biphenylacetic acid. Flow rate = 0.2ml/hr.

The relative recoveries of both fenbufen and biphenylacetic acid were significantly lower at a bath concentration of 5μg/ml compared to the higher concentrations (t-test, $p<0.05$), see figure 9.1. Biphenylacetic acid relative recovery at a bath concentration of 10μg/ml was also found to be significantly lower than the two higher concentrations (t-test, $p<0.05$).

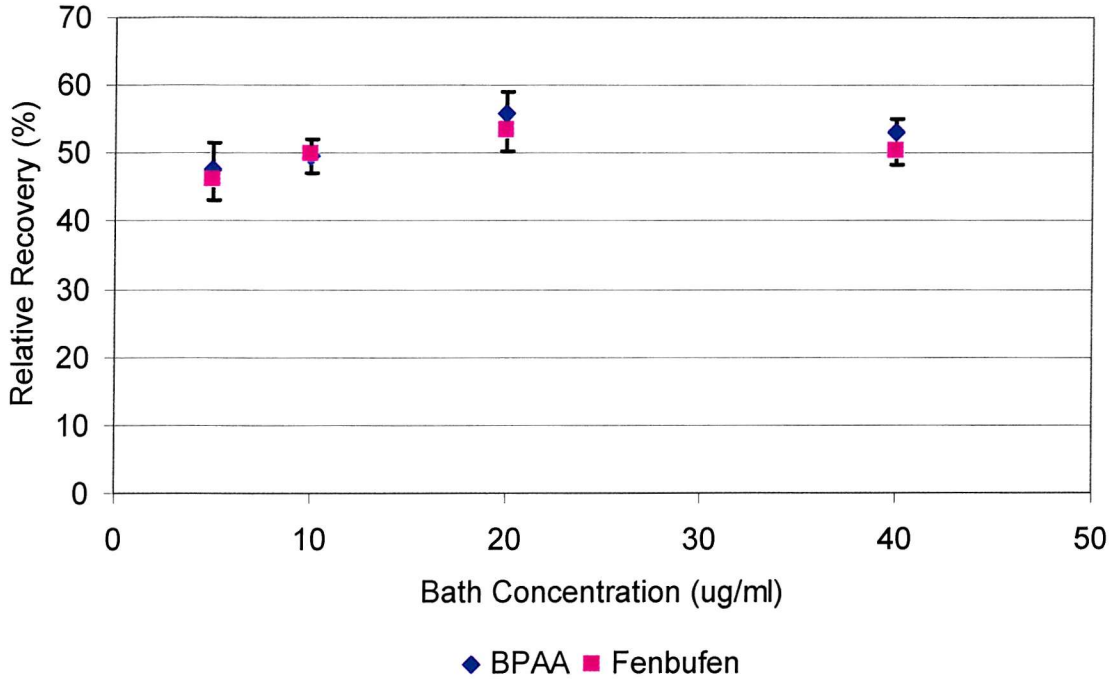

Figure 9.1: Relationship of relative recovery to bath concentration.

9.1.3.2 Flow Rate

Four dialysate flow rates were tested *in vitro*, 0.4, 0.3, 0.2 and 0.1 ml/hr for both fenbufen and biphenylacetic acid at a bath concentration 5μg/ml. Six fibres were tested at each flow rate and hourly collections were made for four hours.

At a flow rate of 0.4ml/hr the relative recoveries of fenbufen and biphenylacetic acid were 24% and 25% respectively, see tables 9.9 and 9.10. There was no significant difference between them ($p>0.05$).

Time	Line A	Line B	Line C	Line D	Line E	Line F
0	0.00	0.00	0.00	0.00	0.00	0.00
1	21.44	26.59	25.14	25.25	21.55	26.53
2	21.81	25.99	31.94	24.27	21.64	24.89
3	20.12	27.37	23.49	22.77	23.03	25.47
4	20.51	27.16	24.55	23.74	24.17	24.97
Average	20.97	26.78	26.28	24.01	22.60	25.47
SD	0.79	0.62	3.84	1.03	1.25	0.75
Group Average				24.35		
Group Standard Deviation				2.26		

Table 9.9: Percentage relative recoveries for fenbufen acid(%). Bath concentration = 5μg/ml fenbufen. Flow rate = 0.4ml/hr.

Time	Line A	Line B	Line C	Line D	Line E	Line F
0	0.00	0.00	0.00	0.00	0.00	0.00
1	21.81	26.87	25.40	25.56	21.77	27.06
2	22.31	26.41	31.58	24.66	21.90	25.24
3	20.58	27.59	23.63	23.01	23.49	25.98
4	20.92	27.59	24.72	24.20	24.51	25.55
Average	21.40	27.12	26.33	24.36	22.92	25.96
SD	0.80	0.58	3.57	1.06	1.32	0.80
Group Average				24.68		
Group Standard Deviation				2.20		

Table 9.10: Percentage relative recoveries for biphenylacetic acid acid(%). Bath concentration = 5μg/ml biphenylacetic acid. Flow rate = 0.4ml/hr.

At a flow rate of 0.3 ml/hr the relative recoveries of fenbufen and biphenylacetic acid were 37% and 41% respectively, see tables 9.11 and 9.12. There was found to be a significant difference between the relative recoveries of the two compounds (t-test, $p<0.05$).

Time	Line A	Line B	Line C	Line D	Line E	Line F
0	0.00	0.00	0.00	0.00	0.00	0.00
1	33.89	41.27	37.34	37.43	33.95	36.20
2	32.45	38.87	39.39	36.56	33.38	37.90
3	39.70	37.82	39.33	44.23	37.61	31.26
4	38.84	40.43	41.15	37.95	34.06	34.34
Average	36.22	39.60	39.30	39.04	34.75	34.92
SD	3.59	1.55	1.56	3.51	1.93	2.84
Group Average				37.31		
Group Standard Deviation				2.26		

Table 9.11: Table 9.9: Percentage relative recoveries for fenbufen acid(%). Bath concentration = 5μg/ml fenbufen. Flow rate = 0.3ml/hr.

Time	Line A	Line B	Line C	Line D	Line E	Line F
0	0.00	0.00	0.00	0.00	0.00	0.00
1	36.82	45.69	41.37	41.02	37.37	39.22
2	35.01	42.56	42.96	39.84	36.54	42.11
3	42.84	40.02	41.70	47.81	40.64	33.30
4	42.59	44.66	45.17	41.05	36.86	37.05
Average	39.31	43.23	42.80	42.43	37.85	37.92
SD	4.00	2.51	1.72	3.63	1.89	3.71
Group Average				40.59		
Group Standard Deviation				2.51		

Table 9.12: Percentage relative recoveries for biphenylacetic acid acid(%). Bath concentration = 5μg/ml biphenylacetic acid. Flow rate = 0.3ml/hr.

At a flow rate of 0.2 ml/hr the relative recoveries of fenbufen and biphenylacetic acid were 46% and 48% respectively, see tables 9.7 and 9.8. There was found to be no significant difference between the relative recoveries of the two compounds ($p>0.05$).

When the flow rate was reduced to 0.1 ml/hr the relative recoveries of fenbufen and biphenylacetic acid were 72%, see tables 9.13 and 9.14. There was no significant difference between the two values ($p<0.05$).

Time	Line A	Line B	Line C	Line D	Line E	Line F
0	0.00	0.00	0.00	0.00	0.00	0.00
1	76.06	67.62	52.01	68.48	70.07	64.07
2	81.23	74.58	75.27	75.97	78.45	67.70
3	84.27	72.12	68.21	72.65	76.30	64.62
4	78.51	71.89	70.77	69.75	74.01	66.99
Average	80.02	71.55	66.57	71.71	74.71	65.85
SD	3.53	2.89	10.13	3.33	3.58	1.77
Group Average				71.73		
Group Standard Deviation				5.27		

Table 9.13: Table 9.9: Percentage relative recoveries for fenbufen acid(%). Bath concentration = 5μg/ml fenbufen. Flow rate = 0.1 ml/hr

Time	Line A	Line B	Line C	Line D	Line E	Line F
0	0.00	0.00	0.00	0.00	0.00	0.00
1	77.42	68.70	51.80	69.16	70.25	65.15
2	82.32	75.25	75.79	76.32	79.73	68.56
3	84.33	72.37	68.52	73.15	76.64	65.13
4	78.66	71.99	70.96	69.77	74.30	67.42
Average	80.69	72.08	66.76	72.10	75.23	66.57
SD	3.20	2.68	10.43	3.32	4.00	1.71
Group Average				72.24		
Group Standard Deviation				5.34		

Table 9.14: Percentage relative recoveries for biphenylacetic acid acid(%). Bath concentration = 5μg/ml biphenylacetic acid. Flow rate = 0.1ml/hr.

The relationship between flow rate and relative recovery for both fenbufen and biphenylacetic acid was found to be approximately linear. The R^2 value for both compounds was approximately 0.915.

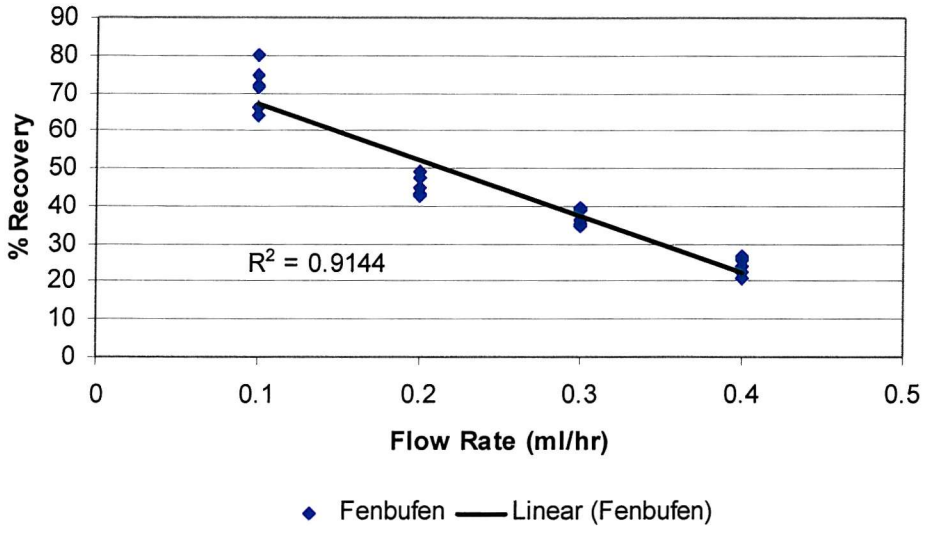

Figure 9.2: Relationship of flow rate to relative recovery for fenbufen.

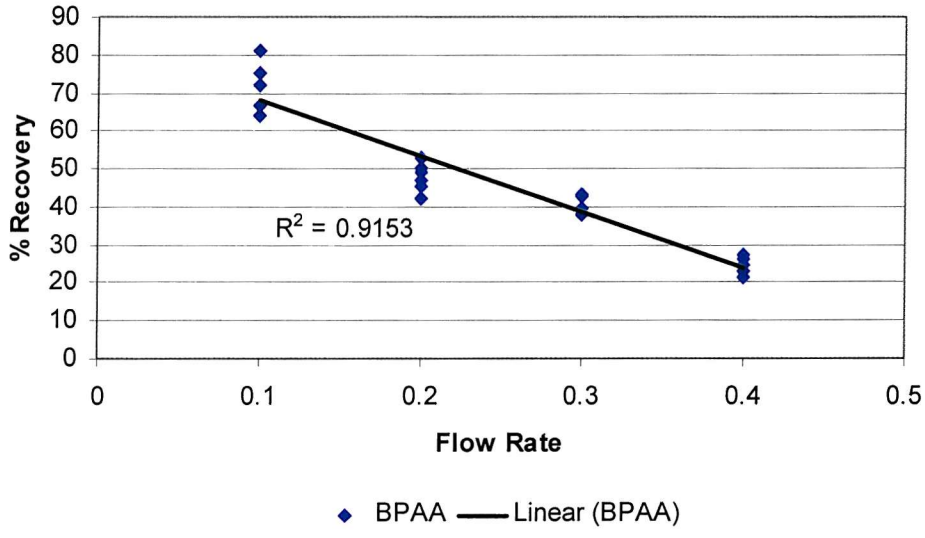

Figure 9.3: Relationship of flow rate to relative recovery for biphenylacetic acid.

9.1.4 Discussion

At the three highest concentrations tested the relative recovery of both fenbufen and biphenylacetic acid was largely independent of the concentration. However the relative recovery at the lowest concentration tested (5μg/ml) was significantly lower for both compounds. A similar result had also been seen with the 3 compounds tested *in vitro* in the previous studies. This reduction in relative recovery had previously been attributed to the compounds becoming adsorbed onto the surface of the petri dishes, used *in vitro*, due to the lipophilic nature of the drugs. During the earlier *in vitro* studies bath samples had not been taken at every time point and the bath concentration was assumed to be the concentration of the solution originally prepared. Any reduction in bath concentration caused by adsorption of the compound would result in the calculation of a reduced relative recovery. At the lowest bath concentration it was assumed that a greater proportion of the drug in solution would be become attached to the plastic of the petri dish resulting in a larger percentage decrease in bath concentration. The resulting perfusate concentration would be also be lower, and therefore when the perfusate concentration was divided by the assumed bath concentration the resulting relative recovery would be correspondingly lower. However for the current set of *in vitro* studies bath samples were taken at every time point so that an accurate relative recovery was calculated. The reason for the reduced relative recovery at low concentrations therefore cannot be solely due to adsorption onto the petri dish surface. All samples were collected and stored in plastic eppendorf vials prior to analysis. It is possible that the compounds were adsorbed onto the surface of the vials before they were transferred to the glass HPLC vials. If a higher proportion of the perfusate samples were sticking to the plastic vials compared to the bath samples then the calculated relative recovery would be lower than the actual relative recovery. This effect would be most noticeable at low concentrations whereas at higher concentrations the proportion of drug lost would be too small to detect undetectable.

The calculated relative recovery was found to have an inversely proportional relationship to the flow rate and that for the concentration used the relationship was linear.

9.2 *In vivo* Introduction

Fenbufen is a commonly prescribed NSAID used to treat pain and inflammation in rheumatic disease and other musculoskeletal disorders (Chiccarelli *et al*, 1980). Fenbufen itself is inactive and requires metabolism to form the active metabolite, felbinac (biphenylacetic acid), which is a potent inhibitor of prostaglandin synthesis. Fenbufen is used orally rather than felbinac because it has a lower ulcerogenic potential and undergoes rapid hepatic metabolism via β-oxidation to biphenylacetic acid (Chiccarelli *et al*, 1980; Hosie & Bird, 1994). Topically, however felbinac is used because there is no risk of ulcer formation and the metabolic activity of skin compared to liver is very low and significant metabolism of fenbufen might not occur.

Fenbufen and biphenylacetic acid were chosen for study primarily due to their closely related structures, both are biphenyl structures but fenbufen has a 4 carbon chain attached whereas biphenylacetic acid has only 2, see figure 9.4. The difference in structure results in fenbufen having a lower solubility in water than biphenylacetic acid i.e. it is more lipophillic and as such might cross the skin more readily. By applying both drugs on each volunteer it is possible to compare the effect of the structural differences on the percutaneous absorption. Secondly fenbufen is a pro-drug and by monitoring for both compounds it should be possible to detect the presence of any biphenylacetic acid within the upper layers of the skin, which would indicate that metabolism had taken place locally. Literature searches show that there are no reports that this metabolic pathway exists in the dermal or epidermal tissue to any significant extent, however β-oxidation is a common pathway for fatty acid metabolism. Therefore it is not possible to predict whether any metabolism of fenbufen to biphenylacetic acid would take place and whether it would be detectable.

9.3 *In vivo*: Method

The 8 volunteers who took part in the *in vivo* studies had an average age of 22 years, (19-30 years), two of the volunteers were men and the remainder were women. All eight were students studying at Southampton University. Due to problems with the fibres remaining patent during the studies not all of the volunteers had the same number of functioning lines (Table 9.15).

Volunteer	Age	Sex	Fenbufen Fibres	Biphenylacetic acid Fibres
1	19	M	3	3
2	20	M	2	1
3	24	F	3	3
4	20	F	2	3
5	30	F	3	3
6	19	F	3	2
7	20	F	2	2
8	24	M	3	3

Table 9.15: Volunteer information: Age, sex and number of lines for each compound

All volunteers had six 2kDa fibres inserted, which were identical to those used in the methyl salicylate studies except that they all had steel wire inserts throughout their length. The fibres were inserted as described in section 2.2.4.1. All fibres were perfused with Ringer's solution containing 5μg/ml noradrenaline at a flow rate of 0.4ml/hr. Either fenbufen gel (1%) or biphenylacetic acid gel (1%) was applied to the skin within drug wells at the end of the first hour of microdialysis. The production of the gel is detailed in the methods section, it contained a 50/50 water ethanol mixture into which was dissolved 1% of the drug and 3% carboxy-methylcellulose. All drug wells were occluded after the formulations had been applied. All parameters apart from the drug applied remained constant. The rest of the study was performed using the procedure detailed in the *in vivo* section of the methods chapter. After the one hour pre-dose collection, the collection periods were half an hour.

Where there were duplicate or triplicate lines in the same subject the values for the lines were averaged prior to calculation of means and statistics between all 8 subjects.

The method of analysis for both fenbufen and biphenylacetic acid is that used for ketoprofen, the internal standard used was ketoprofen.

9.4 *In vivo*: Results-fenbufen application.

Fenbufen Application						
Volunteer 1	BPAA			Fenbufen		
Time	Line 1	Line 2	Line 3	Line 1	Line 2	Line 3
0	0.000	0.000		0.000	0.000	
0.5	0.000	0.016		0.212	0.164	
1	0.024	0.130		0.039	0.054	
1.5	0.009	0.026		0.035	0.015	
2	0.003	0.002		0.006	0.004	
2.5	0.005	0.006		0.009	0.020	
3	0.000	0.029		0.030	0.000	
3.5	0.000	0.009		0.010	0.026	
4	0.008	0.012		0.016	0.034	
4.5	0.023	0.011		0.015	0.034	
5	0.015	0.009		0.026	0.019	
Volunteer 2	BPAA			Fenbufen		
Time	Line 1	Line 2	Line 3	Line 1	Line 2	Line 3
0	0.000	0.000	0.000	0.000	0.000	0.000
0.5	0.005	0.009	0.020	0.173	0.047	0.131
1	0.044	0.011	0.019	0.160	0.097	0.083
1.5	0.017	0.011	0.087	0.392	0.063	0.073
2	0.028	0.016	0.009	0.259	0.330	0.079
2.5	0.012	0.007	0.006	0.130	0.071	0.093
3	0.013	0.010	0.006	0.093	0.070	0.106
3.5	0.019	0.005	0.010	0.127	0.079	0.083
4	0.007	0.014	0.007	0.086	0.096	0.173
4.5	0.009	0.006	0.008	0.125	0.120	0.096
5	0.008	0.010	0.013	0.220	0.153	0.155
Volunteer 3	BPAA			Fenbufen		
Time	Line 1	Line 2	Line 3	Line 1	Line 2	Line 3
0	0.000	0.000		0.194	0.000	
0.5	0.000	0.000		0.192	0.193	
1	0.000	0.000		0.191	0.193	
1.5	0.000	0.000		0.382	0.000	
2	0.000	0.000		0.186	0.199	
2.5	0.000	0.000		0.195	0.216	
3	0.000	0.000		0.205	0.335	
3.5	0.000	0.000		0.280	0.593	
4	0.000	0.000			0.706	
4.5	0.000	0.000		0.182	0.466	
5	0.000	0.000			0.796	
Volunteer 4	BPAA			Fenbufen		
Time	Line 1	Line 2	Line 3	Line 1	Line 2	Line 3
0	0.000	0.000	0.000	0.000	0.000	0.000
0.5	0.013	0.017	0.014	0.013	0.000	0.000
1	0.011	0.011	0.008	0.005	0.000	0.000
1.5	0.004	0.011	0.000	0.000	0.000	0.000
2	0.000	0.000	0.000	0.000	0.000	0.108
2.5	0.000	0.000	0.000	0.016	0.092	0.328
3	0.000	0.000	0.000	0.079	0.255	0.811
3.5	0.000	0.000	0.000	0.250	0.563	1.146
4	0.000	0.000	0.000	0.475	0.887	1.276
4.5	0.000	0.000	0.000	0.686	1.195	1.772
5	0.000	0.000	0.000	0.995	1.452	2.468
Volunteer 5	BPAA			Fenbufen		
Time	Line 1	Line 2	Line 3	Line 1	Line 2	Line 3
0	0.000	0.000		0.000	0.000	
0.5	0.000	0.000		0.000	0.000	
1	0.000	0.000		0.000	0.000	
1.5	0.000	0.000		0.000	0.000	
2	0.000	0.000		0.010	0.009	
2.5	0.000	0.005		0.022	0.026	
3	0.000	0.006		0.073	0.102	
3.5	0.000	0.006		0.166	0.266	
4	0.005	0.005		0.454	0.495	
4.5	0.005	0.007		0.489	0.625	
5	0.005	0.006		0.608	0.520	
Volunteer 6	BPAA			Fenbufen		
Time	Line 1	Line 2	Line 3	Line 1	Line 2	Line 3
0	0.000	0.000	0.000	0.000	0.000	0.000
0.5	0.000	0.000	0.000	0.000	0.000	0.000
1	0.000	0.000	0.000	0.000	0.000	0.000
1.5	0.000	0.000	0.000	0.000	0.000	0.000
2	0.000	0.000	0.000	0.000	0.000	0.000
2.5	0.000	0.000	0.000	0.000	0.000	0.009
3	0.000	0.000	0.000	0.010	0.023	0.029
3.5	0.000	0.000	0.000	0.038	0.102	0.076
4	0.000	0.000	0.000	0.155	0.323	0.212
4.5	0.000	0.000	0.004	0.351	0.635	0.482
5	0.000	0.000	0.000	0.439	0.787	0.517
Volunteer 7	BPAA			Fenbufen		
Time	Line 1	Line 2	Line 3	Line 1	Line 2	Line 3
0	0.000	0.000	0.000	0.000	0.000	0.000
0.5	0.000	0.000	0.000	0.000	0.000	0.000
1	0.000	0.000	0.000	0.000	0.000	0.000
1.5	0.000	0.000	0.000	0.000	0.000	0.000
2	0.000	0.000	0.005	0.000	0.000	0.008
2.5	0.000	0.000	0.000	0.000	0.000	0.013
3	0.000	0.000	0.000	0.000	0.051	0.001
3.5	0.000	0.000	0.000	0.019	0.037	0.008
4	0.000	0.000	0.000	0.026	0.043	0.012
4.5	0.000	0.000	0.000	0.068	0.065	0.022
5	0.000	0.000	0.000	0.080	0.072	0.057
Volunteer 8	BPAA			Fenbufen		
Time	Line 1	Line 2	Line 3	Line 1	Line 2	Line 3
0	0.000	0.000	0.000	0.000	0.000	0.000
0.5	0.037	0.007	0.010	0.228	0.229	0.233
1	0.000	0.014	0.004	0.226	0.199	0.222
1.5	0.000	0.008	0.005	0.294	0.383	0.226
2	0.000	0.007	0.008	0.799	1.021	0.270
2.5	0.000	0.007	0.007	1.498	1.389	0.965
3	0.000	0.013	0.005	1.859	2.272	1.551
3.5	0.000	0.008	0.007	2.100	1.306	2.017
4	0.050	0.006	0.005	1.970	1.204	2.097
4.5	0.000	0.005	0.006	1.644	1.146	1.701
5	0.000	0.008	0.004	1.294	0.939	1.506

Biphenylacetic Acid Application						
	BPAA			Fenbufen		
Time	Line 1	Line 2	Line 3	Line 1	Line 2	Line 3
0	0.000			0.000		
0.5	3.179			0.000		
1	2.624			0.000		
1.5	0.480			0.000		
2	0.654			0.000		
2.5	1.816			0.000		
3	1.890			0.000		
3.5	0.371			0.000		
4	0.494			0.000		
4.5	0.554			0.000		
5	0.575			0.000		
	BPAA			Fenbufen		
Time	Line 1	Line 2	Line 3	Line 1	Line 2	Line 3
0	0.000	0.000	0.049	0.000	0.000	0.000
0.5	0.007	0.017	0.006	0.000	0.000	0.000
1	0.027	0.026	0.031	0.000	0.000	0.000
1.5	0.030	0.042	0.048	0.000	0.000	0.000
2	0.052	0.063	0.137	0.000	0.000	0.000
2.5	0.096	0.127	0.351	0.000	0.000	0.000
3	0.175		0.834	0.000	0.000	0.000
3.5	0.251	0.707	0.967	0.000	0.000	0.000
4	0.434	1.312	1.480	0.000	0.000	0.000
4.5	0.597	1.862	1.737	0.000	0.000	0.000
5	0.785	2.470	2.103	0.000	0.000	0.000
	BPAA			Fenbufen		
Time	Line 1	Line 2	Line 3	Line 1	Line 2	Line 3
0	0.000	0.000	0.000	0.000	0.000	0.000
0.5	0.000	0.000	0.000	0.000	0.000	0.000
1	0.000	0.004	0.016	0.000	0.000	0.000
1.5	0.000	0.014	0.000	0.000	0.000	0.000
2	0.020	0.084	0.269	0.000	0.000	0.000
2.5	0.102	0.299	0.712	0.000	0.000	0.000
3	0.281	0.857	1.283	0.000	0.000	0.000
3.5	0.729	1.635	1.824	0.000	0.000	0.000
4	1.157	2.198	2.088	0.000	0.000	0.000
4.5	1.342	2.512	2.137	0.000	0.000	0.000
5	1.328	2.249	1.751	0.000	0.000	0.000
	BPAA			Fenbufen		
Time	Line 1	Line 2	Line 3	Line 1	Line 2	Line 3
0	0.000	0.000		0.000	0.000	
0.5	0.004	0.010		0.000	0.000	
1	0.013	0.015		0.000	0.000	
1.5	0.103	0.104		0.000	0.000	
2	0.788	0.613		0.000	0.000	
2.5	1.802	1.796		0.000	0.000	
3	3.377	3.063		0.000	0.000	
3.5	5.019	4.014		0.000	0.000	
4	6.222	5.001		0.000	0.000	
4.5	7.326	6.010		0.000	0.000	
5	7.508	7.061		0.000	0.000	
	BPAA			Fenbufen		
Time	Line 1	Line 2	Line 3	Line 1	Line 2	Line 3
0	0.000	0.007		0.000	0.000	
0.5	0.000	0.016		0.000	0.040	
1	0.005	0.014		0.000	0.000	
1.5	0.030	0.059		0.000	0.000	
2	0.085	0.185		0.000	0.000	
2.5	0.253	0.484		0.000	0.000	
3	0.690	1.208		0.000	0.000	
3.5	1.426	2.344		0.000	0.000	
4	2.092	3.751		0.000	0.000	
4.5	2.955	5.072		0.000	0.000	
5	3.499	4.685		0.000	0.000	
	BPAA			Fenbufen		
Time	Line 1	Line 2	Line 3	Line 1	Line 2	Line 3
0	0.000	0.000	0.000	0.000	0.000	0.000
0.5	0.000	0.000	0.000	0.000	0.000	0.000
1	0.000	0.005	0.008	0.000	0.000	0.000
1.5	0.009	0.023	0.042	0.000	0.000	0.000
2	0.031	0.079	0.107	0.000	0.000	0.000
2.5	0.068	0.244	0.248	0.000	0.000	0.000
3	0.132	0.828	0.578	0.000	0.000	0.000
3.5	0.347	2.122	1.330	0.000	0.000	0.000
4	0.861	3.859	2.741	0.000	0.000	0.000
4.5	1.668	4.850	4.049	0.000	0.000	0.000
5	2.295	5.272	4.566	0.000	0.000	0.000
	BPAA			Fenbufen		
Time	Line 1	Line 2	Line 3	Line 1	Line 2	Line 3
0	0.000	0.000	0.000	0.000	0.000	0.000
0.5	0.000	0.040	0.000	0.000	0.000	0.000
1	0.000	0.143	0.000	0.000	0.000	0.000
1.5	0.010	0.207	0.007	0.000	0.000	0.000
2	0.042	0.287	0.033	0.000	0.000	0.000
2.5	0.115	0.348	0.071	0.000	0.000	0.000
3	0.195	0.423	0.128	0.000	0.000	0.000
3.5	0.350	0.515	0.244	0.000	0.000	0.000
4	0.612	0.768	0.430	0.000	0.000	0.000
4.5	1.045	0.997	0.824	0.000	0.000	0.000
5	1.979	1.221	1.489	0.000	0.000	0.000
	BPAA			Fenbufen		
Time	Line 1	Line 2	Line 3	Line 1	Line 2	Line 3
0	0.000	0.000	0.000	0.000	0.000	0.000
0.5	0.009	0.000	0.000	0.000	0.000	0.000
1	0.054	3.313	0.030	0.000	0.000	0.000
1.5	0.211	3.349	0.260	0.000	0.000	0.000
2	1.319	7.218	1.900	0.000	0.000	0.000
2.5	4.132	8.193	4.754	0.000	0.000	0.000
3	7.006	6.877	6.042	0.000	0.000	0.000
3.5	9.212	7.290	6.741	0.000	0.000	0.000
4	10.044	5.901	6.698	0.000	0.000	0.000
4.5	7.966	4.499	5.874	0.000	0.000	0.000
5	6.615	3.820	4.819	0.000	0.000	0.000

Table 9.16: Summary of all data from all volunteers from all fibres. The values are concentration (μg/ml) of fenbufen and biphenylacetic acid (BPAA) in the dialysate samples for each line and each time point (spaces indicate that sample was lost).

Table 9.16 above details all the concentrations measured from all volunteers at all time points and with either fenbufen or biphenylacetic acid applied to the skin.

Table 9.17 below shows the averaged data from all volunteers for sites where fenbufen was applied, both fenbufen and biphenylacetic acid concentrations were measured in the perfusate samples. As with other studies the data shows very large inter-individual variations as shown by the large standard deviations. The profile for fenbufen absorption showed a steady increase in concentration throughout the time course of the study indicating that steady state had not been reached. Biphenylacetic acid was present at all post dose times in some of the samples however compared to fenbufen the concentrations were very small, the concentration appeared to reach a peak at around one hour even though the concentration of fenbufen continues to rise throughout the study. When the applied gel was tested there was no detectable concentration of biphenylacetic acid present.

Time at end of collection period (hr)	Fenbufen		BPAA	
	Mean	SD	Mean	SD
0	0.012	0.034	0.000	0.000
0.5	0.091	0.101	0.007	0.007
1	0.071	0.091	0.015	0.027
1.5	0.087	0.119	0.008	0.014
2	0.146	0.240	0.003	0.006
2.5	0.221	0.436	0.003	0.003
3	0.347	0.639	0.004	0.006
3.5	0.415	0.606	0.003	0.004
4	0.527	0.589	0.006	0.007
4.5	0.534	0.549	0.004	0.006
5	0.637	0.576	0.004	0.005

Table 9.17: Comparison of fenbufen and biphenylacetic acid concentrations (ug/ml) following topical fenbufen gel application. n = 8.

Figure 9.4 below shows the concentrations of fenbufen and biphenylacetic acid from the fenbufen application sites corrected using the *in vitro* relative recovery. The relative recovery determined for fenbufen and biphenylacetic acid at a flow rate of 0.4ml/hr was used to convert the sample concentrations into minimum tissue concentrations. The applied concentration of fenbufen was 10mg/ml, the highest mean tissue concentration found was 2.5μg/ml,

which represents a 4000 fold decrease in concentration as it crosses the skin and enters the dermal tissue space.

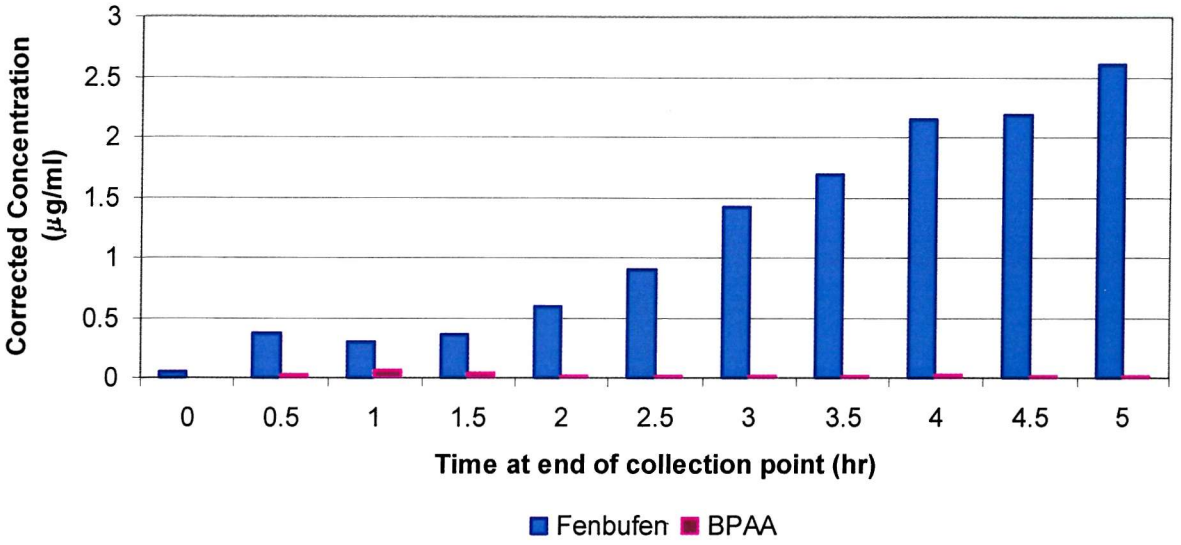

Figure 9.4: Relative recovery corrected concentrations of fenbufen and biphenylacetic acid following topical fenbufen gel application. n = 8.

Table 9.18 below shows the averaged data from all volunteers for sites where biphenylacetic acid was applied, both fenbufen and biphenylacetic acid concentrations were measured in the perfusate samples. As with other substrates the data showed very large inter-individual variations as indicated by the large standard deviations. The profile for biphenylacetic acid absorption showed an a slowly increasing sample concentration throughout the time course of the study with steady state not being reached. Essentially fenbufen was not seen in the samples at concentrations above the limit of detection, one sample from one volunteer showed a very small concentration of fenbufen at the half hour collection point, however the baseline for that sample was relatively noisy and the peak probably represented as an analytical artefact.

Time at end of collection period (hr)	Fenbufen		BPAA	
	Mean	SD	Mean	SD
0	0.000	0.000	0.003	0.006
0.5	0.003	0.000	0.403	1.122
1	0.000	0.000	0.483	0.949
1.5	0.000	0.000	0.256	0.439
2	0.000	0.000	0.671	1.164
2.5	0.000	0.000	1.325	1.900
3	0.000	0.000	1.847	2.168
3.5	0.000	0.000	2.274	2.587
4	0.000	0.000	2.819	2.525
4.5	0.000	0.000	3.153	2.330
5	0.000	0.000	3.276	2.245

Table 9.18: Comparison of fenbufen and biphenylacetic acid concentrations (ug/ml) following topical biphenylacetic acid gel application. n = 8.

Figure 9.5 below shows the corrected concentrations of fenbufen and biphenylacetic acid from the biphenylacetic acid application sites. The relative recovery determined for fenbufen and biphenylacetic acid at a flow rate of 0.4ml/hr was was used to convert the sample concentrations into minimum tissue concentrations. The corrected concentrations for biphenylacetic acid showed that it achieved a far higher maximum tissue concentration than fenbufen. At the final collection point the minimum tissue concentration for fenbufen (figure 9.4) was 2.6μg/ml and 13.4μg/ml for biphenylacetic acid (figure 9.5).

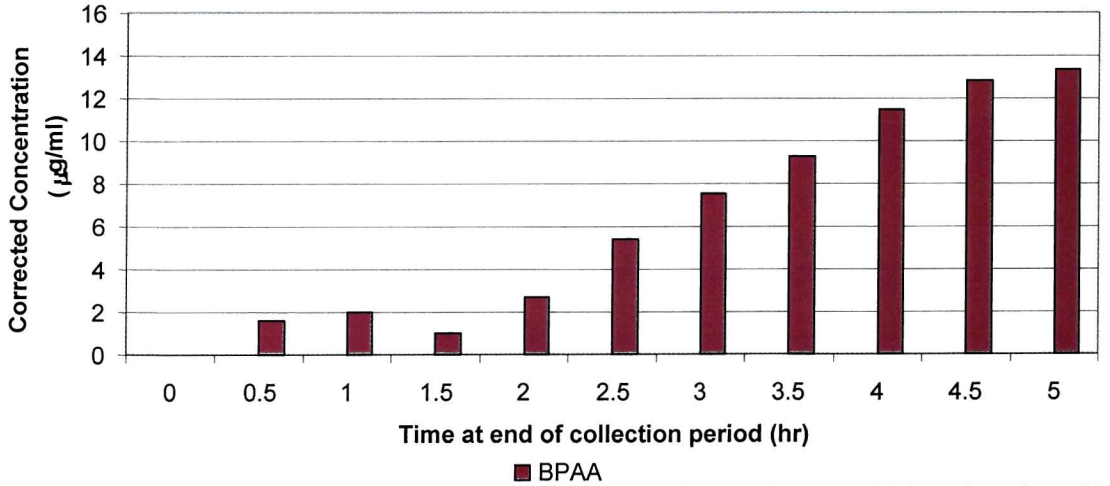

Figure 9.5: Relative recovery corrected concentrations of fenbufen and biphenylacetic acid following topical biphenylacetic acid gel application. n = 8.

Table 9.19 below shows the C_{max} and AUC for both compounds from both application sites. In the fenbufen application sites the AUC and C_{max} for fenbufen was significantly higher than for biphenylacetic acid ($p<0.05$). When the fenbufen concentrations from the fenbufen application sites were compared to the biphenylacetic acid concentrations from the biphenylacetic acid applications sites it was found that the Cmax and AUC for biphenylacetic acid was significantly higher ($p<0.05$), indicating greater absorption of this substrate. The greater absorption and low extent of metabolism of fenbufen to biphenylacetic acid meant that the concentration of biphenylacetic acid after application of biphenylacetic acid was significantly greater ($p<0.05$) than after application of fenbufen.

	Fenbufen Application				Biphenylacetic acid Application			
	Fenbufen		Biphenylacetic Acid		Fenbufen		Biphenylacetic Acid	
Parameters	Mean	St Dev	Mean	St Dev	Mean	St Dev	Mean	St Dev
Cmax (ug/ml)	0.74	0.68	0.02	0.02	0.002	0.007	3.96	2.4
AUC (ug/ml.min)	82.92	99.98	1.64	1.98	0.08	0.21	446.11	392.51

Table 9.19: Pharmacokinetic parameters for fenbufen and biphenylacetic acid absorption following either topical biphenylacetic acid or fenbufen gel application. n = 8.

The HPLC traces from the analysis of the fenbufen application site samples showed an unidentified peak that increased in size during the course of the study period. It was found in every volunteer but only in the fenbufen site application samples. The retention time for the compound was shorter than that for fenbufen indicating it was more polar, and in fact the retention time was even shorter than that for biphenylacetic acid, possibly the result of some oxidation reaction in the skin. In an attempt to identify the compound samples containing the largest concentrations of the compound were pooled and re-injected using the same HPLC procedure. The eluted mobile phase was collected when the UV detector peaked at the retention time for the unknown compound. However when the fractions were run on a LC-MS system using the appropriate conditions no peak with approximately the correct mass could be detected. The samples were also rerun on the original HPLC system and no peak was seen at the retention time of the unidentified metabolite from the original analysis. Two possible conclusions could be drawn either the compound degraded or the mobile phase fraction collected did not contain the unidentified compound. Unfortunately the initial analysis and the subsequent

fraction collection used all the samples which contained the compound leaving none for a repeat fraction collection.

9.5 *In vivo*- Discussion

As per the other results chapters the inter-individual variation was considerable with the standard deviation in most cases exceeding the mean. Again this is most likely due to differences in skin thickness and composition between volunteers.

For both fenbufen and BPAA the measured concentrations increased over time without reaching a plateau indicating that steady state had not been reached within the time course of the study. At the end of the studies when the drug wells were removed the areas of skin where the drug formulation had been applied were slightly raised relative the surrounding skin, this could be both felt and seen with the naked eye. When the fibres were scanned with ultrasound the epidermis directly above the fibres was found to be thicker and far less dense than the surrounding epidermis. The possible reasons for this and the effect on the penetration have been discussed fully in the discussion sections of the previous two chapters.

The penetration of fenbufen into the dermis was found to less than for biphenylacetic acid, both the C_{max} and the AUC were significantly larger for biphenylacetic acid (see table 9.19). This was an expected result based on their P_{ow} values, fenbufen has a value $\approx$3.5 whereas biphenylacetic acid is $\approx$3. Even though fenbufen has the highest lipophilicity and therefore will probably have a greater flux into the stratum corneum, biphenylacetic acid will be more able to partition into the viable epidermis and dermis due to its greater hydrophilicity.

Very low levels of biphenylacetic acid were detected after the application of fenbufen. However the concentration reached a peak after on hour and then remained steady at very low levels for the duration of the study period. This would indicate that there maybe some minor β-oxidation activity within the

upper layers of the skin. The level of activity however is so low that it would not generate therapeutic concentrations of biphenylacetic acid.

More interestingly an unidentified metabolite was detected in the samples from the fenbufen application sites from every volunteer. The unknown compound was not present in the pre-application samples and undetectable in the 2-3 samples post application and the peak area increased throughout the time course of the study indicating that fenbufen was being metabolised. Unfortunately the compound could not be identified using LC-MS as was hoped, however the compound was not seen after biphenylacetic acid application indicating that the compound is probably derived from the pre β-oxidation structure. In a study conducted by Chiccarelli *et al* (1980) the metabolites of fenbufen after oral dosing were identified and are shown below in fig 9.6.

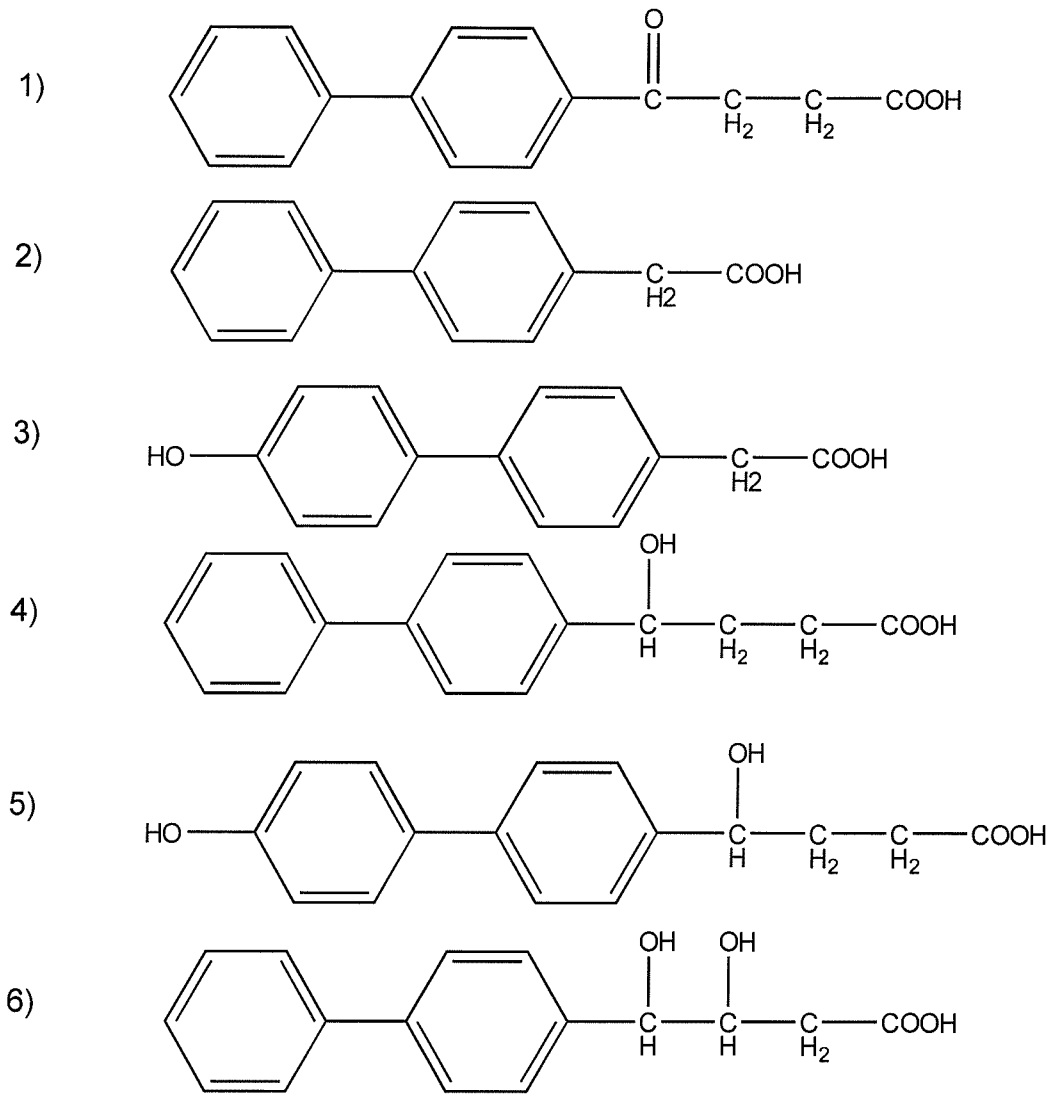

Figure 9.6: Fenbufen and its identified metabolites in man. 1) Fenbufen, 2) biphenylacetic acid , 3) 4-hydroxy-1,1-biphenyl-4-acetic acid, 4) γ-hydroxy-1,1-biphenyl-4-butanoic acid, 5) γ,4-dihydroxy-1,1-biphenyl-4-butanoic acid, 6) erythro, erythro,β,γ-dihydroxy-1,1-biphenyl-4-butanoic acid

The most likely candidates for the unknown compound are structures 4-6 as they have not undergone β-oxidation and they are more polar than the parent compound (fenbufen). However for an unequivocal identification more studies would need to be run and the correct fraction containing the unknown collected and analysed using mass spectrometry.

9.6 *In vivo*- Conclusion

In conclusion microdialysis has shown that despite being structurally very similar biphenylacetic acid penetrates the skin to the depth of the implanted fibre in far greater amounts than fenbufen. This increase in penetration is most likely due to biphenylacetic acid being more hydrophilic relative to fenbufen. This should allow biphenylacetic acid to partition to a greater extent into the viable epidermis and dermis than fenbufen. Both compounds are lipophilic so will be able to penetrate the stratum corneum. However it is likely that fenbufen, as it is more lipophilic, is forming a reservoir in the stratum corneum, which is preventing it from reaching the implanted fibre.

There appears to some very low level β-oxidation activity in the skin towards fenbufen. Very low concentrations of biphenylacetic acid were detected in the post-application samples from the fibres where fenbufen had been topically applied. However it is very unlikely that the metabolic action of the skin could generate therapeutic concentrations of biphenylacetic acid.

The upper layers of the skin did generate an unexpected metabolite which unfortunately could not be identified in this series of studies. However the evidence suggests it has the pre β-oxidation structure and is more hydrophilic than the parent compound suggesting some oxidation reaction.

10 Final Discussion

At the present time transdermal drug absorption is being recognised as a viable alternative to other routes of drug delivery (e.g. oral and intravenous) for particular drugs. These include:

- Drugs that are potent but have a narrow therapeutic window – transdermal application results in steady state absorption avoiding the peaks and troughs seen with oral and intravenous dosing.
- Drugs that are subject to a high rate of first pass metabolism – topically absorbed drugs do not pass through the liver prior to reaching the general circulation.
- Drugs that are poorly absorbed from the gastro-intestinal tract or cause gastro-intestinal problems when taken orally (such as aspirin and many other NSAID compounds). Transdermal drug absorption avoids the gastro-intestinal tract completely.

However there are problems associated with transdermal drug absorption, the flux across the skin is generally low therefore any applied drug either has to act locally or must have a high potency for systemic effects. Topical drug absorption is associated with a high degree of variability not only between individuals (see results from earlier chapters) but also between different sites of the body on the same individual (Roberts *et al.*, 1982; Feldman & Maibach 1967; Rougier *et al.*, 1987). Maximal drug absorbance is a fine balance between a molecule's lipo- and hydro-philicity and is also affected by the molecule's overall size (Idson, 1983; Pugh *et al*, 1996; Roberts *et al*, 1996; Roberts *et al*, 1998; Goosen C *et al*, 1998). Penetration is also influenced by the vehicle in which the compound is applied and whether penetration enhancers are co-applied (Guy *et al*, 1990; Tanojo *et al*, 1998; Barry, 1987; Taguchi *et al*, 1999). Therefore the pharmaceutical industry is constantly attempting to modify either the active compound or the vehicle in order to achieve a level of absorption required to treat the condition, whether it be local or distant to the application site. In order for companies to measure the effect of any modifications or alterations made to a drug formulation, a suitable sampling technique is required which must show:

- High accuracy
- High temporal resolution, the ability to take samples every few minutes rather than every few hours.
- Site specificity, sampling from the dermis or subcutaneous tissue.
- Practicability and minimally invasive.

Sampling methods employed previously have all had problems, either they were remote from the site of application (i.e. blood, urine, saliva or faeces) or they did not give an absorption profile with any temporal resolution (i.e. skin stripping, essentially only gives one time point) or are extremely invasive such as skin biopsies .

Cutaneous microdialysis at present is the only sampling technique that meets all the criteria mentioned above (Schnetz & Fartasch, 2000). Microdialysis was assessed in this thesis as a possible replacement/addition to the presently used sampling techniques for transdermally applied drugs.

10.1 Microdialysis to assess the dermal concentrations of various topically applied compounds

Through the course of this research microdialysis has been used to assess the transdermal absorption of 5 different compounds: methyl salicylate, salicylic acid, ketoprofen, fenbufen and biphenylacetic acid.

Methyl salicylate and salicylic acid were assessed in the same series of studies. Salicylic acid is the immediate product of methyl salicylate metabolism and is also an active compound used topically in its own right. The two compounds were used to assess several different parameters.

- The ability of microdialysis to measure cutaneous metabolism of topically applied compounds.
- The effect of occlusion on speed and duration of penetration.
- The effect of perfusing the fibres with low concentrations of noradrenaline in order to reduce dermal blood flow.

- The effect of changing the application vehicle from a propylene glycol based formulation to an ethanol based formulation.
- The difference in absorption of two structurally similar compounds with different lipophilicities.

Cutaneous microdialysis was shown to allow the detection of methyl salicylate metabolism via the build up of salicylic acid concentrations over time. The ratio of salicylic acid to methyl salicylate increased over the time course of the study, and the concentration of salicylic acid measured was far higher than could be explained by the small fraction of salicylic acid present in the applied formulation of methyl salicylate (esterase activity in the dermis is discussed below).

Occluding the application site was found to aid the duration of penetration of both methyl salicylate and salicylic acid (increased AUC) though it had little effect on the maximum concentration measured and the time at which it was achieved. The prolongation of absorption was thought to be a result of preventing the formulation from drying out rather than skin hydration, which is often stated to be reason for penetration enhancement in topical studies (Behl *et al*, 1980; Bucks *et al*, 1988; Ryatt *et al*, 1988). As the formulation dried out the viscosity would increase, thereby reducing diffusion through the applied formulation and reducing penetration. It also would increase the proportion of propylene glycol in the applied formulation, increasing the solubility of the compounds in the formulation, and thereby reduce the partition of the compounds into the stratum corneum.

Reducing the dermal blood flow by introducing noradrenaline to the perfusate resulted in increased concentrations of both compounds throughout the course of the studies giving an increased AUC and C_{max}. Reducing the blood flow reduced the removal of absorbed compounds by the dermal vasculature allowing higher tissue concentrations to be maintained (Siddiqui *et al*, 1989; Singh & Roberts, 1993; Singh & Roberts, 1994).

The vehicle used made a large difference to the measured tissue concentration, the ethanol based formulation resulted in tissue concentrations of methyl salicylate approximately 10 times greater than those seen with the propylene glycol based formulation. Ethanol is a proven penetration enhancer (Guy *et al*, 1990) that disrupts the packing of the lipid bilayer in the stratum

corneum. The disruption causes the bilayer to become more fluid and less crystalline which increases rates of diffusion and therefore drug flux.
When the absorption of methyl salicylate and salicylic acid were compared directly salicylic acid was found to give far higher tissue concentrations (approximately four times higher) even though structurally they are very similar. At first sight the result seems counter-intuitive as the compound with the higher lipophilicity (methyl salicylate) has the lower tissue concentrations. However maximal absorption is a result of a balance between lipo- and hydrophilicity. Compounds must have lipophilic properties to penetrate the stratum corneum but must have enough hydrophilic properties to diffuse into the dermis. Although methyl salicylate should be better at penetrating the stratum corneum it probably forms a reservoir there: salicylic acid on the other hand has a better balance, a far greater proportion of the absorbed compound is capable of diffusing into the more aqueous dermal tissue where it can be measured.

Ketoprofen was used to assess the ability of microdialysis to detect and measure highly protein-bound lipophilic drugs. Two fibre types were used in these studies, 2kDa and 3MDa fibres, the 2kDa fibres were only capable of dialysing free drug whereas the larger pored 3MDa fibres dialysed both protein bound and free drug. The initial *in vitro* studies confirmed that the 2kDa fibres didn't dialyse protein and that the 3MDa fibres could, they also showed that ketoprofen was approximately 95 % protein bound in the presence of human plasma albumin. The initial *in vivo* studies surprisingly indicated that there was almost no protein bound drug present in the skin even though it had been shown that albumin levels in the dermis were comparable to those in plasma (Bert *et al*, 1986). A second set of studies which employed a period of zero flow half way through the study indicated that microdialysis using 3MDa fibres depleted the dermis of mobile protein to such an extent that essentially only free drug could be measured. This highlighted the limited usefulness of microdialysis to investigate the effect of protein-binding on dermal drug concentration. However there remains the possibility of adding human albumin to the perfusate to prevent tissue depletion of albumin (Carneheim & Stahle, 1991). Another alternative is to employ short collection periods separated by periods of zero flow to minimise

the depletion and to allow tissue protein concentrations to return to basal levels.

Fenbufen is the pro-drug form of biphenylacetic acid with only biphenylacetic acid having any activity. To form biphenylacetic acid, fenbufen must undergo β-oxidation, this occurs readily in the liver but there have been no reports of activity in the skin. Both compounds were applied to volunteers in the same set of studies, firstly to investigate whether skin tissue has any β-oxidation activity and secondly to see the effect of a minor structural difference which results in biphenylacetic acid being more hydrophilic than fenbufen. The results indicated that there was practically no intrinsic β-oxidation activity in skin tissue and that biphenylacetic acid was far better at penetrating to the dermal tissue than fenbufen (approximately 5 fold). This again illustrates the balance required between a compound's lipophilic and hydrophilic nature; biphenylacetic acid is the more hydrophilic compound so it will be less capable of penetrating the stratum corneum, however its hydrophilic nature allows it diffuse into the dermis at a greater rate than fenbufen.

All the studies have demonstrated that cutaneous microdialysis is an excellent technique for comparing the relative tissue concentrations between two or more sets of experimental conditions. Areas where microdialysis has been shown to be effective include:

- Testing whether skin tissue has metabolic activity towards the applied compounds.
- The effect of changing the vehicle formulation, including the addition of penetration enhancers.
- The effect of altering the application procedure, i.e. occlusion and rubbing.
- The effect of altering the physiological condition of the skin on drug uptake, i.e. increasing or decreasing dermal blood flow with the use of glycerol tri-nitrate or noradrenaline (Barkve *et al*, 1986), studying disease states.
- The effect of altering the structure of the applied compound.

Gaining absolute tissue concentrations is more difficult than measuring change in concentration. The method used in these studies used a relative recovery gained from *in vitro* experiments to convert measured concentration in dialysate into estimated concentration in skin. Correcting the sample concentrations using the *in vitro* relative recoveries gives a minimum tissue concentration. The major area of difficulty for cutaneous microdialysis was the study of the effect of protein binding on tissue concentration. The studies have shown it to be extremely difficult to measure any protein bound drug in dermal tissue due to the depletion effect caused by dialysis. It may be possible to study protein binding in the dermis by adding human albumin to the perfusate to combat the depletion or by using periods of zero flow followed by short collection periods to allow protein levels to remain at normal levels.

10.2 Factors affecting transdermal absorption and dermal drug concentrations

10.2.1 Cutaneous metabolism

There are very few reported studies where cutaneous microdialysis has been used to study metabolism either cutaneous or systemic. There are several reasons for this, firstly as long as there is a ready supply of either fresh or frozen human skin, a series of *in vitro* experiments can be carried out far quicker than a set of *in vivo* microdialysis studies, historically *in vitro* experiments required less ethical approval (though this is no longer the case) and didn't require as much effort to obtain volunteers. Secondly the concentrations that could be used with *in vitro* systems were far higher, allowing for easier analysis, plus the total concentration was measured and not merely a percentage of the non-protein bound fraction, as is the case with most microdialysis studies.

The concentration of recovered metabolites of topically applied drugs using cutaneous microdialysis is always going to be relatively low for several reasons. The concentration applied to the skin cannot ethically be higher than

that medically approved and the concentration that is measured in the dermis can be 1000 fold or more lower than that applied, as shown in all the *in vivo* results in this thesis. This is compounded by the fact that the relative recovery of the drug is likely to be less 50% and is often lower than 20% and only the non-protein bound fraction is measured. Although skin is a metabolically active organ the rates are relatively low when compared to liver, kidney and lung.

A recent study used cutaneous microdialysis to measure the levels of penciclovir an anti-viral drug after oral dosing of famciclovir in adult human volunteers (Borg *et al*, 1999). Famciclovir is the pro-drug form of penciclovir and is converted by de-acetylation. The study showed that penciclovir reached the skin in concentrations high enough to inhibit herpes virus replication. Although the study had clearly indicated that metabolism had taken place, the vast majority if not all would have taken place at the intestinal wall and liver (Crumpacker, 1996 and Clarke *et al* 1995) and not within the skin.

Microdialysis has been successfully used in other tissues to study metabolic activity. For example lactate and glycerol turnover was measured in human adipose tissue (Jansson *et al*, 1995) and skeletal muscle insulin and glucose levels have been monitored following glucosamine infusion (Holmang *et al*, 1999). Nitric oxide production has been measured in rat hippocampus (Togashi *et al*, 1998) and noradrenaline and dopamine production has been measured in the prefrontal cortex of rats (Kawahara *et al*, 2001). However these and most other microdialysis studies were all looking at endogenous compounds not xenobiotic metabolism.

In vitro many cutaneous metabolism studies have investigated a wide range of enzyme systems and substrates. The studies used either fresh or frozen excised skin with a diffusion chamber, cell culture or skin homogenates. The problem with all *in vitro* systems is the relevance to the *in vivo* model. This is more the case with cell culture, skin homogenates and cultured keratinocytes.

One recent *in vitro* study (Hewitt *et al*, 2000) was a risk assessment of a commonly used herbicide fluroxypyrmethylheptyl ester (FPMH) and two related compounds fluroxypyrmethyl ester (FPM) and fluroxypyr (FP). The three compounds were applied to fresh excised human and rat skin mounted on diffusion cells. Both FPMH and FPM were completely metabolised to the hydrolysis product FP with no parent compound detected at all and there was no detectable metabolism of FP. Similar results were also found with frozen skin indicating a robust enzyme system. Overall the study showed that the esterase activity of human skin was high with respect to FPMH and FPM. It agrees with the initial study of this thesis that also showed that human skin had considerable esterase activity with respect to methyl salicylate metabolising it to salicylic acid. It is highly likely that cutaneous microdialysis will prove to be useful for studying the fate of topically applied compounds that are metabolised by esterases. The general esterase activity in skin appears to be high therefore there is more chance of generating a detectable level of metabolites.

Another study looking at esterase activity examined the penetration and metabolism of salicylic acid derivatives across hairless rat skin (Higo *et al*, 1995). The study showed that all the derivatives underwent significant hydrolysis to salicylic acid however the greatest level of activity was towards n-butyl salicylate, but this was the least permeable and most lipophilic. The authors reasoned that this was could be due to n-butyl salicylate having a greater reservoir effect in the stratum corneum, therefore the flux into the viable epidermis would be reduced and the resident esterases less likely to become saturated. Alternatively, it may simply be attributable to enzyme specificity.

In general microdialysis is potentially a useful technique for following *in vivo* cutaneous metabolism as it allows the measurement of metabolites created solely within the upper layers of the skin. However the relatively low recoveries of parent compounds and metabolites means that unless the enzyme system under investigation is particularly active and the applied dose relatively high, extremely sensitive methods of analysis would have to be used.

10.2.2 Drug structure and characteristics

Assuming topically applied compounds are relatively small (i.e. less than 500 daltons) their most important physico-chemical property in terms of absorption is their degree of lipophilicity (Potts & Guy, 1992; Goosen *et al*, 1998; Roberts & Walters 1998). There is a large body of evidence which suggests that good topical absorption is a balance between lipophilic and hydrophilic characteristics. An applied chemical must be able to penetrate the stratum corneum which, as it is a lipid rich environment, requires it to be lipophilic. However to be able to partition out of the stratum corneum and into the viable epidermis and dermis which is far less lipid rich requires it to possess a degree of hydrophilicity. The standard measure of lipophilicity of a compound is its P_{OW} value which is a ratio of the compounds solubility in octanol and water (Idson, 1983). The higher the value the greater its solubility in octanol and therefore the greater its lipophilicity. Several studies have shown that the P_{OW} value and the ability of a compound to penetrate the skin appears to have a parabolic relationship (Goosen *et al*, 1998; Roberts & Walters 1998; Hadgraft, 1999). The ideal P_{OW} value appears to be ≤ 2. Compounds with values greater than 2 tend to form reservoirs within the stratum corneum (Hewitt *et al*, 1993) as they are less able to partition into the viable epidermis (a more aqueous environment). Compounds with values less than two will poorly partition into the stratum corneum and remain on the surface of the skin. This effect was seen with both the methyl salicylate/salicylic acid study and the fenbufen/biphenylacetic acid study. In both cases the most lipophilic compound had the least cutaneous absorption despite being applied in the same vehicle and at the same concentration. Salicylic acid has a P_{OW} of approximately 2.5 whereas methyl salicylate is over 3 therefore even though methyl salicylate will partition more efficiently into the stratum corneum it is less able to partition into the viable epidermis. The same is true of the fenbufen/biphenylacetic acid study, fenbufen has a value of approximately 3.5 whereas biphenylacetic acid is nearer 3.

10.2.3 Vehicle

The selection of vehicle used to apply the compound of interest is very important, and can have a large influence on the flux through the upper layers of the skin. These effects are caused by several different factors, the partition coefficient of the compound between the vehicle and the stratum corneum, the level of saturation of the compound in the vehicle, the presence of any absorption enhancers in the vehicle and the ability of the vehicle to occlude the application site (Roberts *et al*, 1999),. The majority of recent research is directed at the effect of including absorption enhancers in the vehicle on the permeation of the test compound.

In a recent human *in vivo* study by Alberti *et al* (2001) the use of ethanol as a component of the vehicle used to apply terbinafine topically was investigated. The permeation of terbinafine into the stratum corneum was found to be greatest when the vehicle contained ethanol compared to control vehicles. The assumption was that ethanol helped disrupt the close packing of the stratum corneum lipids allowing a greater flux of terbinafine. A different study (Oh,J *et al*, 2001) compared vehicles containing a buffer mixed with either ethanol, propylene glycol (PG) or polyethylene glycol (PEG). Compared to buffer alone only ethanol and PG increased the permeation of the test compound (melatonin) through excised hairless mouse skin, the effect of ethanol was greater than that of propylene glycol.

Both of these studies agree with the findings of the methyl salicylate study from chapter 4 which showed a significant increase in the dermal concentration of methyl salicylate when applied in an ethanol based vehicle relative to the propylene glycol based vehicle.

The effect of disrupting the semi-crystalline state of the stratum corneum lipids was demonstrated in a study by Smith & Irwin (2000). The penetration of salicylic acid through excised human skin was studied at a range of vehicle pH values and with and without various penetration enhancers. The highest penetration was at pH values where the predominant species was unionised i.e. the species that had the greatest partitioning coefficient towards the stratum corneum. When penetration enhancers, including azone and oleic acid were used at these pH levels increased penetration occurred. As the

effect could not be attributable to ion-pairing the effect was most likely caused by the enhancer disrupting the lipids of the stratum corneum.

Many compounds have been studied as penetration enhancers (Sinha *et al*, 2000). These can be either part of the solvent system of the vehicle such as ethanol and propylene glycol (Alberti *et al*, 2001; Oh *et al*, 2001; Larrucea *et al*, 2001), or added to the vehicle along with the active ingredient at various concentrations; oleic acid, azone, phloretin, lauric acid, linoleic acid, linolenic acid and fatty acids of varying chain lengths (Valenta *et al*, 2001; Oh *et al*, 2001; Larrucea *et al*, 2001; Fujii *et al*, 2000; Santoyo *et al*, 2000). All are thought to act by increasing the liquidity of the stratum corneum lipids allowing greater drug flux through the upper layers of the skin.
Oleic acid is the compound that has probably been used the most to enhance penetration (Cooper *et al*, 1985; Loftsson *et al*, 1989; Golden *et al*, 1987; Francoeur *et al*, 1990; Mak *et al*, 1990). It is essentially a mono-unsaturated fatty acid which has a kinked structure caused by the single double bond. As oleic acid is not a straight chain fatty acid it disrupts the close packing of the straight chain lipids of the stratum corneum reducing its crystalline nature.

10.2.4 Physiological properties of the skin

The dermal vasculature is the predominant system for clearing the dermis and epidermis of topically absorbed compounds. Once compounds have reached the upper part of the dermis they encounter the upper papillary plexus, a dense region of small blood vessels. At this point a significant portion will enter the systemic circulation and be unavailable for further diffusion through the dermis (Hadgraft, 1999). Altering the level of blood flow in the cutaneous vasculature will have a major effect on the clearance of applied compounds. Decreasing the blood flow with the use of a vasoconstrictive agent, such as noradrenaline, will reduce clearance resulting in increased dermal concentrations (Singh & Roberts, 1994 and Roberts & Walters, 1998). This was clearly seen in the early *in vivo* chapters of this thesis the use of nor-adrenaline in the perfusate consistently resulted in significantly higher AUC values when compared to controls. Conversely the opposite is also true and

an increase in the cutaneous blood flow will lower dermal concentrations but systemic concentrations will be higher (Karatassas *et al*, 1993).

10.2.5 Protein binding

Drug-protein binding is a major determinant of drug action; the effective drug concentration is that of the unbound portion, as bound drug cannot interact with its specific target (Grandison *et al*, 2000). The extent of protein binding is dependant on the drug and protein concentrations, the affinity constant for the drug-protein interaction and the number of protein binding sites. The degree of plasma-protein binding has been studied for a large number of drugs, this is because the systemic concentration of free drug is the active portion of the dose. The level of protein binding may also affect the drug's pharmacokinetics (Wright *et al*, 1996). More specifically the bound form of the drug may be partially protected from the metabolic fate of the unbound drug, which may lead to a longer half-life (Talbert *et al*, 2002).

Almost all the reported studies looking at drug protein binding have looked at plasma protein binding of orally given drugs not topically applied drugs or protein binding in the ECF of skin tissue.

However studies have been completed that show that plasma-protein binding can be altered by various methods. A recent study looked at the effect of exercise on drug protein binding (Tesseromatis *et al*, 2001). Exercise increased the circulating levels of free fatty acids in laboratory rats, which are thought to interfere with drug protein binding. When ampicillin was administered the levels of free drug were found to be higher in the exercised groups with respect to the non-exercised control group. However the study gave no indication whether protein binding would be reduced within the ECF of skin tissue.

Another method of altering the degree of protein binding is to produce active structural analogues of the compound of interest. These can then be screened to test for the level of protein binding. This was done in a recent study, which examined the protein binding of analogues to the anti-retroviral

drug AZT (Quevedo *et al* 2001). The compounds were incubated with human serum albumin (HSA) at varying concentrations, and the level of protein binding measured. The point was to identify a derivative that had a higher affinity for HSA than AZT as this could improve upon its pharmacokinetic properties, the authors felt that increasing the level of protein binding would result in a longer half life, all the derivatives tested had greater affinities for HSA. This may have implications for topically applied drugs. By increasing the level of protein binding of an applied drug or an analogue it may be possible to form a reservoir of the bound form in the upper layers of the skin which may lengthen the duration of action from a single applied dose.

The above studies highlight the potential importance of measuring the level of protein binding in a tissue after dosing. It has implications for the metabolism, clearance, therapeutic concentration and disposition of an applied dose whether it be oral, intravenous or topical. It is somewhat surprising that there are no reported studies that have attempted to use microdialysis to study cutaneous drug-protein interactions. It has been shown that there is relatively high concentration of HSA in skin compared to plasma levels (Bart *et al*, 1986). Protein binding will have important implications on the sink conditions in the upper layers of the skin, the potential for metabolism and diffusional characteristics of the applied compound. In fact it is the diffusional characteristics of protein that resulted in difficulties in measuring protein binding *in vivo*. Until the protein depletion problems can be addressed cutaneous microdialysis may have a limited role in measuring cutaneous drug-protein binding. However using 2kDa fibres allows the direct measurement of free drug and this may be a real advantage as this is the active fraction of the total tissue concentration.

10.3 Further studies for the assessment of cutaneous microdialysis

Leading on from the work in this thesis there are 4 areas where work is warranted to assess further cutaneous microdialysis and to use it productively.

1) Esterase activity in the upper layers of human skin is relatively high both according to the results in this thesis and several reported studies (Hewitt *et al*, 2000; Cross *et al*, 1998). However the studies have all used excised human skin. If a series of microdialysis studies were conducted using different ester based compounds, especially if a homologous series of esters were used it may be possible to describe the specificity of the substrates of the ester activity in the skin. This would potentially allow a structure-activity relationship to be created that would both describe the ability of given ester to penetrate the upper layers of the skin and also predict the degree of metabolism it would undergo.
2) The parabolic relationship of P_{OW} to cutaneous absorption has been described in several papers however it has been based on *in vitro* data and mathematical models. If a homologous series of compounds could be identified that had an increasing (or decreasing) lipophilicity then a series of microdialysis studies could be run to validate the relationship *in vivo*.
3) Penetration enhancers have been studied extensively either *in vitro* or with *in vivo* systems that do not allow either a high temporal resolution or measurement at the site of application. It would be interesting to test the inclusion of various penetration enhancers (such as oleic acid, linoleic acid, lauric acid and fatty acids of varying chain lengths) on the absorption of previously studied compounds.
4) The chapters looking at the protein binding of ketoprofen highlighted the difficulty of dialysing protein even when 3MDa fibres are used. It may still prove to be possible to study protein binding within the skin if the right sampling regime could be established. The combination of small sampling times at low flow rates followed by long periods of zero flow may allow accurate measurement of both protein and protein bound drug. Alternatively using perfusate that contained varying concentrations of HSA could be used in a zero flux method to calculate the true resting concentration of both protein and protein-bound drug.

10.4 Final Conclusion

In summary cutaneous microdialysis is a very useful technique that is reproducible, simple, minimally invasive and allows comparisons between relative tissue concentrations resulting from different experimental conditions. Most importantly because multiple lines can be used in each volunteer it allows all experimental conditions to be paired within the same subject at the same time allowing a much stronger statistical analysis of the results. Therefore relatively fewer studies need to be carried out despite the large inter-individual variation encountered. Another advantage is that by using small pored fibres it is possible to directly measure the concentration of unbound drug present in the dermal tissue.

A major drawback is that it can be difficult to calculate absolute tissue concentrations. Also, due to low relative recovery, sample concentrations can be very low especially when sampling highly protein bound drugs or metabolites from enzyme systems of low activity. If the rate of diffusion of the target compound/protein is lower than the rate of dialysis tissue depletion of relevant species can occur.

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