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Evaluation of Microdialysis as a Tool for Studying Percutaneous Drug Absorption

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Abstract

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This thesis investigates the technique of cutaneous microdialysis as a means of exploring the factors that affect the absorption of topically applied drugs. Following a description of the factors affecting percutaneous absorption and methods used for studying percutaneous absorption the technique of microdialysis is described. The main advantage of cutaneous microdialysis is that it continuously samples from the dermal extracellular space *in vivo* and so enables a detailed pharmacokinetic profile to be built up of the drug under investigation under both normal and abnormal conditions. Using microdialysis to measure the dermal concentrations of the antiherpes drugs penciclovir (PCV) and aciclovir (ACV) following topical application, it was found that in their commercial preparations they are very poorly absorbed through normal skin. Therefore they represent suitable probes for investigating the different aspects of percutaneous absorption both in terms of the 'penetrant' and the barrier it has to cross. By adding compounds such as dimethyl sulphoxide and sodium lauryl sulphate to PCV in an aqua base gel the action of penetration enhancers on hydrophilic drug absorption was examined.

The role of the stratum corneum barrier was investigated using tape stripping with transepidermal water loss measurement used as a measure of barrier impairment. PCV and ACV absorption were measured through different degrees of barrier impairment. Cutaneous blood flow was manipulated using noradrenaline as a vasoconstrictor and glyceryl trinitrate as a vasodilator to assess the effect of cutaneous blood flow on washing away percutaneously absorbed drug. Dermal levels of systemically absorbed PCV were compared with topically applied PCV. Finally microdialysis fibres have been uniquely used to continuously deliver muscarinic agonists to sweat glands to both clarify the physiology and pharmacology of eccrine sweat glands, and to investigate the absorption of PCV via skin appendages.

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Chapter 1

Introduction

General introduction

The absorption of drugs through the skin is becoming increasingly important to the pharmaceutical, cosmetic and chemical industries. The transdermal route has become well established for the administration of substances whose site of action may be within the skin or systemic. Equally, as the skin is the barrier protecting us from our environment it is essential to understand and be able to measure how well chemicals present in our environment can be absorbed through the skin. This thesis investigates the factors surrounding percutaneous absorption. The role and effectiveness of the stratum corneum as the main barrier to percutaneous absorption is examined and the possibility of transappendageal absorption explored. Having established the mechanisms of absorption, the factors affecting established drug levels in the dermis are examined.

The following introduction describes the structure of the skin and possible pathways of percutaneous absorption. Particular emphasis is given to those factors known to affect percutaneous absorption. The techniques for investigating percutaneous absorption are outlined - including cutaneous microdialysis and the reasons for its use in the present study. Transepidermal water loss as a measurement of barrier function is defined and the use of laser Doppler perfusion imaging and high frequency dermal ultrasound explained. Finally the rationale for using the antiherpes drug penciclovir, as a probe to examine the factors surrounding percutaneous absorption is defined.

The structure and function of the skin

The skin forms the interface between the organism and the external environment. In man it provides protection against the inward and outward passage of water and electrolytes, the entry of toxic substances and microorganisms, ultraviolet radiation, mechanical forces, extreme temperatures and low voltage electric current. When intact, the barrier function of the skin is extremely effective. However, when damaged or defective, there is a risk of infection, entry of environmental substances and loss of body water.

In man, the skin is the most extensive organ of the body covering an area of 18,000cm^2 in an average sized adult (Katz *et al*, 1971). It varies in thickness according to anatomical site – from 1000μm on the palms and soles, to 16μm on the eyelids (Katz *et al*, 1971). It consists of two layers; a stratified, avascular squamous cellular epidermis and an underlying dermis of connective tissue. Both layers contribute to the protective function of the skin:

The epidermis provides the main barrier to the inward and outward movement of substances. It consists of a basal layer of mitotically active keratinocytes which migrate upwards undergoing keratinization and cornification to produce the stratum corneum. Keratinocytes make up 95% of the total cell numbers of the epidermis. There are three other cell types present in the epidermis; melanocytes which protect against UV radiation; antigen presenting Langerhans' cells which constitute an important part of the skin immune system and Merkel cells which play a role in cutaneous sensation.

The dermis provides tensile strength to the skin. It consists of fibres of collagen and elastin embedded within an extracellular ground substance made of polysaccharides and proteins. The dermis acts as a support system providing the epidermis with nutrients and oxygen, and removing waste products. It contains blood vessels, lymphatic vessels,

pilosebaceous units, sensory nerves and sweat glands all contained within the fibroelastic connective tissue (Uitto *et al*, 1986). The dermal component of the skin plays a major role in thermoregulation of the human body: Via a series of arteriovenous shunts, blood flow to the skin can be controlled thus regulating heat loss through the skin surface. The stimulation of eccrine sweat glands in high environmental temperatures promotes the evaporation of sweat from the skin surface which also lowers surface temperature (Archer 1998).

Below the dermis lies a layer of lipid (subcutaneous fat). This acts as an insulator, shock absorber and provides a reserve depot of calories (Figure 1.1).

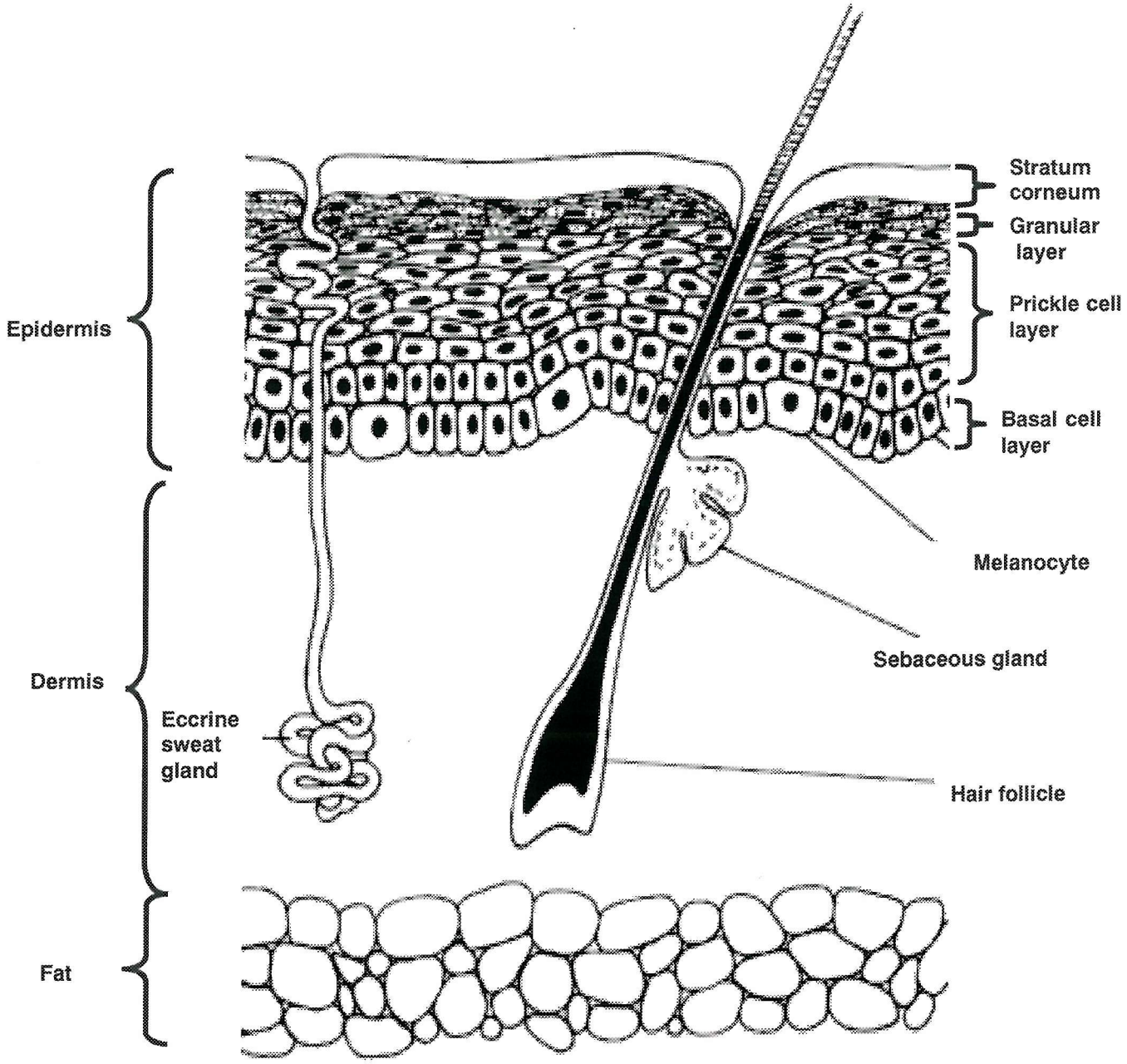


Figure 1.1 Simplified diagram of the skin

(*Reprinted with permission (Ashton R et al, 1992)*)

The stratum corneum provides the main barrier to permeability (Blank 1965). Monash and Blank showed that skin from which stratum corneum had been removed was highly permeable, whilst the removed stratum corneum was nearly as impermeable as the entire skin. The stratum corneum is approximately 10μm thick and consists of 8 to 16 layers of flattened, stratified and fully keratinized dead cells (corneocytes) embedded within a continuous heterogenous lipid matrix – this is the 'two compartment system' or 'bricks and mortar' model of the stratum corneum (Chapman *et al*, 1991; Elias *et al*, 1981). The keratinocytes are formed and continuously replaced by the basal layer or *stratum germinativum*. As keratinocytes differentiate and migrate outwards within the epidermis they synthesize the fibrous protein keratin and filaggrin (keratohyaline), a histidine rich protein (Friedmann 1986). On reaching the epidermis they are called corneocytes on account of possessing a cornified envelope that is resistant to proteolytic enzymes, organic solvents, acid and alkali solutions. This cornified envelope forms a rigid exoskeleton and is the site of insertion of intracellular keratin filaments. The keratins account for up to 80% of the dry mass of a corneocyte and contribute to the stratum corneum barrier against water transport, although a greater role is played by the intercellular lipid matrix (Jackson *et al*, 1995). In addition to the keratin filaments, corneocytes contain several low molecular weight polar compounds such as amino acids and urocanic acid, which help to maintain the hydration and elastic properties of the stratum corneum (Redelmeier *et al*, 1999). It takes 12 to 14 days for a keratinocyte to become fully differentiated and migrate from the basal epidermis to the stratum corneum. The lifespan for such a cell on the surface is 2 to 3 weeks (Katz *et al*, 1971). Keratinocyte differentiation speeds up following damage to the stratum corneum. This is of vital importance in maintenance of the stratum corneum barrier.

The lipid matrix of the stratum corneum originates from the secreted contents of lamellar bodies (membrane coating granules, Odland bodies, keratinosomes) (Jackson *et al*, 1995). These are first identified in keratinocytes of the spinous layer where they migrate to the cell periphery and, by exocytosis, discharge their contents, a mixture of lipids and hydrolytic enzymes, into the extracellular space. The lipids undergo structural and chemical transformations resulting in a planar lipid bilayer (Swartzendruber *et al*, 1989). In addition to the extracellular lipid bilayer, lipid is present bound to the envelope surrounding each corneocyte. The primary function of the lipid in the stratum corneum is protection against excessive water loss. Absence or alteration of the lipid results is pathological skin conditions that are associated with increased water loss (Feingold *et al*, 1991). Damage to the stratum corneum causes an increase in synthesis of sphingolipid, ceramide cholesterol and fatty acid within the skin to repair the damaged barrier (Elias *et al*, 1988). Recent work has suggested that both the corneocyte envelope-associated lipid as well the extracellular lipid is required for permeability barrier homeostasis (Behne *et al*, 2000).

Histologically the stratum corneum can be divided into two layers; the outer stratum disjunctum and the inner stratum compactum. It is thought that corneosomes (which are differentiated desmosomes unique to corneocytes) are mainly responsible for intercorneocyte cohesion (Behne *et al*, 2000; Chapman *et al*, 1991). There is a decrease in the number of corneosomes across the stratum corneum from compactum to disjunctum (Chapman *et al*, 1990). Hence, cell cohesion is higher in the lower levels of the stratum corneum. This is mirrored in the stratum corneum barrier, and it is well known that the lower layers of the stratum corneum provide the greatest contribution to the barrier (Blank 1965). The little barrier function provided by the stratum disjunctum was demonstrated over 50 years ago by the application of dyes to the

outer surface of the stratum corneum – there was colouration of the stratum disjunctum but not of the stratum compactum (Mackee *et al*, 1945).

Routes of Percutaneous Absorption

1. Absorption through the stratum corneum

There are three potential pathways across the stratum corneum (Scheuplein 1965):

a. Intercellular

b. Transcellular

c. Appendageal (via eccrine sweat glands or the pilosebaceous apparatus)

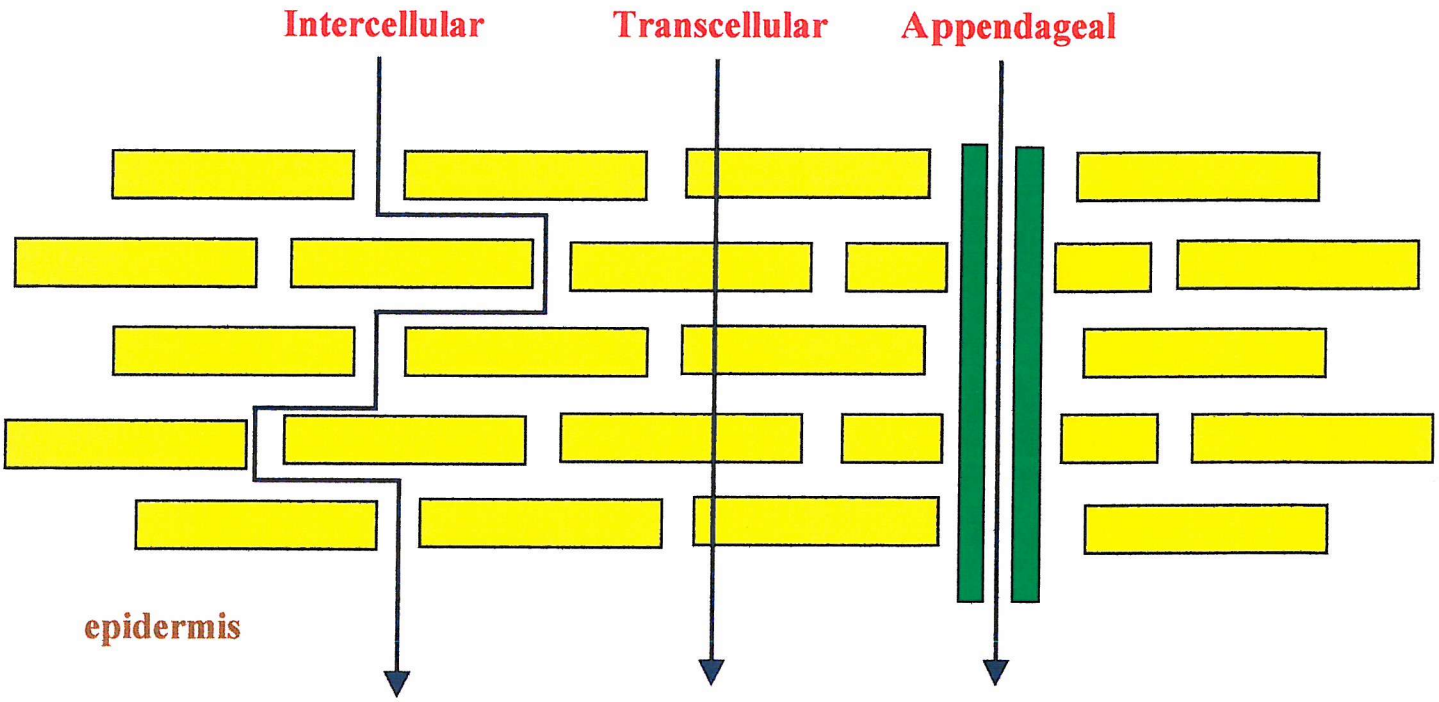


Figure 1.2 The three proposed pathways for movement of molecules across the stratum corneum. The yellow blocks represent corneocytes embedded in a lipid matrix.

Specialized transport systems have not been identified for cells of the stratum corneum and it is not known whether percutaneous absorption is influenced by specialized transport mechanisms present in the epidermis (Malkinson 1964). It has long been assumed that substances cross human skin by passive diffusion.

a) It is thought that for many compounds the tortuous intercellular route is predominant. This is essentially a non-polar lipid channel which favours the passage of hydrophobic molecules. It has been demonstrated that hydration of the stratum corneum increases the penetration of polar molecules more than non polar ones, and this is thought to be due to hydration of the lipid channels enhancing absorption of polar molecules rather than hydration of keratinized cells (Behl *et al*, 1980).

b) The transcellular route offers the greatest surface area to absorption, however, it involves multiple partitioning steps between the hydrophilic densely packed corneocytes and the intercellular lipid. Evidence regarding the importance of this route is conflicting; it has been proposed that for polar drugs the transcellular route is the main pathway of percutaneous absorption (Barry 1987). The observation that certain very polar molecules such as ethanol and methanol, that are unable to partition through a lipid medium, have comparable rates of passage through intact skin *in vitro* suggests the existence of aqueous pores within the extracellular lipid (Flynn 1989). The existence of such pores would allow penetration of water soluble molecules without multiple partitioning steps. Conversely, *in vivo* studies by Albery and Hadgraft have deemed the transcellular pathway physicochemically implausible (Albery *et al*, 1979).

c) Transappendageal absorption describes movement of molecule through the eccrine sweat glands or hair follicles:

Eccrine sweat glands are simple tubular glands that possess a coiled section located in the lower dermis. There are up to 440 sweat glands per cm^2, although there is great anatomical variation (Kuno 1934). Their average diameter at the skin surface is 70μm and in total they make up less than 1/10,000 of the skin surface area. Drugs applied to the skin reach the orifices of the sweat ducts and hair follicles directly, and therefore there is the potential for direct shunting of molecules through the skin. Sweat is 99% water and it would be expected that hydrophilic molecules may be preferentially absorbed by this route, however as with the transcellular route of percutaneous absorption, evidence for absorption through eccrine sweat glands is conflicting. Abramson in 1940 demonstrated that a 1% solution of methylene blue was absorbed into the eccrine duct when applied by electophoresis and was visible for several weeks, however he did not perform histological studies to look for evidence of penetration into the dermis (Abramson *et al*, 1940). Scheuplein considered on theoretical grounds that sweat ducts (and hair follicles) provided shunt pathways for slowly penetrating polar steroids and other molecules with low diffusion constants both in the early phase of topical drug application and at steady state (Scheuplein *et al*, 1969). *In vivo* work examining the absorption of topically applied lead found that sweat glands and hair follicles were responsible for rapid uptake of lead with much slower absorption via the transepidermal route (Stauber *et al*, 1994). This mechanism is echoed by some workers who consider that transappendageal absorption is only important in the early phase of drug absorption and that drugs that penetrate rapidly by this route reach steady state by slower diffusion through the epidermis (Katz *et al*, 1971). Other researchers conclude that the very low surface area of sweat glands precludes their importance in percutaneous absorption.

More work has been done to elucidate the role of hair follicles as a potential route of percutaneous absorption. Studies on rat skin (with and without intact follicles and sebaceous glands) examined the absorption of a range of substances; hydrocortisone, caffeine, niflumic acid and p-aminobenzoic acid. It was found that there was significant appendageal diffusion despite the relatively small surface area of the appendages and that the shunt pathway was important for both poorly absorbed polar compounds and also for caffeine which is usually well absorbed (Illel *et al*, 1991). Other researchers using animal models have confirmed the importance of the transfollicular route in passive drug absorption (Hueber *et al*, 1994a; Kao *et al*, 1988). Some work has been done using human skin *in vitro* to examine transappendageal routes (Barry, 1991). Hueber *et al* found the absorption of the steroids progesterone, oestradiol, testosterone and hydrocortisone was significantly higher through normal skin than appendage free scar skin (Hueber *et al*, 1994b). There is no other work documented that confirms the transfollicular route of absorption *in vivo* in humans.

2. Absorption across the skin

Although passage through the stratum corneum is the rate limiting step for the percutaneous absorption of most substances, there are other barriers that a drug has to pass through in order to reach the blood circulation (for systemic effects) or the appropriate site of action within the skin. It is considered that there are three compartments; the drug vehicle / delivery device; the stratum corneum and the viable epidermis. The drug has to partition from its vehicle or device in the case of a transdermal delivery system into the stratum corneum, it then has to cross the stratum corneum to enter the aqueous environment of the epidermis (Washington

et al, 1989). The hydrophilic epidermis can act as a barrier to hydrophobic drugs which are unable to partition into it. From the epidermis and upper dermis a drug enters a cutaneous blood vessel for systemic access. Within each compartment the compound may diffuse down its concentration gradient, bind to specific components or be metabolized.

A lot of work has been performed on modelling this process to enable the prediction and understanding of the passage of hydrophobic, hydrophilic and intermediate drugs through the skin. Most models assume that drugs move through the skin according to Fick's law of diffusion. This is unlikely to be a true assumption *in vivo* because an extremely long time is usually needed to reach steady state due to the impermeability of the stratum corneum.

The form of Fick's law that is often quoted is:

$$J = \frac{D \times k_1}{e} \Delta C = K_p \Delta C$$

J = the flux (quantity absorbed per unit area per unit time)

D = diffusion constant of the substance in the stratum corneum

k_1 = partition coefficient of the substance between stratum corneum and vehicle

e = thickness of the stratum corneum

ΔC = concentration difference across the membrane

K_p = permeability constant (Friedmann 1986)

Thus, as interactions exist between the drug, its vehicle and the skin it can be seen that partitioning behaviour is highly important. The main characteristics of a permeant that influence its transfer rate are its partition coefficient between its vehicle and the stratum corneum, and the

diffusion constant (diffusion coefficient) in the stratum corneum. Multiplication of these two values gives a permeability coefficient for that particular permeant.

Although useful as a starting point for the prediction of drug absorption, Fick's law does not apply to the calculation of dermal drug levels *in vivo* as cutaneous metabolism of drug and its clearance by blood flow is not accounted for.

Epidermal Reservoirs

Following topical application, some drugs appear to be stored in the skin and released following removal of the source. This 'epidermal reservoir' or depot was originally noted by Malkinson and Ferguson who observed that topically applied hydrocortisone was excreted for up to 7 days after application (Malkinson *et al*, 1955). The reservoir was investigated in detail by Vickers who applied small quantities of fluorinated steroids to the surface of the skin and occluded the area with plastic film (Vickers 1963). After 16 hours the film was removed and as expected, vasoconstriction was seen. This faded within 10 to 16 hours following thorough washing of the skin. Re-occlusion of the skin up to 14 days after the initial steroid application caused the vasoconstriction to reappear. He also showed that the reservoir was in the stratum corneum as it could be removed by stripping with adhesive tape. The presence of an intact and normal stratum corneum is essential for the establishment of the reservoir, and factors such as increasing humidity and temperature that enhance percutaneous absorption will also increase the size of the epidermal reservoir (Vickers 1963). Although potent steroids such as fluocinolone may be retained in sufficient quantities to influence epidermal cell division (Munro 1969), the therapeutic significance of the epidermal reservoir is questionable.

Factors affecting percutaneous absorption

Factors affecting percutaneous absorption can be broadly divided into those of the permeant and those of the skin.

1. Features of the permeant affecting percutaneous absorption

The characteristics of the topically applied drug affecting its percutaneous absorption are; the physicochemical properties of the drug; the vehicle it is applied in and the concentration of drug in the vehicle.

a. Drug characteristics

The percutaneous absorption of a drug is largely determined by its physicochemical properties: Polar molecules are not as well absorbed as non-polar molecules; the lower the melting point of a solid the more easily it is absorbed; organic liquids are better absorbed than solids; small molecules penetrate more easily than large molecules. However it is the relative hydrophilicity / hydrophobicity of a drug that is the most important property concerning skin penetration (Barry 1991). A strongly hydrophobic drug will partition from its vehicle into the stratum corneum and will move easily by diffusion across the stratum corneum. However, it may not partition into the epidermis due to its low solubility in water, and hence there will be a large concentration difference at this tissue interface. Conversely, a strongly hydrophilic drug may only partition very weakly into the stratum corneum and will not diffuse easily through it despite a high concentration of drug at the vehicle / stratum corneum interface. A drug that possesses both hydrophilic and lipophilic properties will be able to partition from its vehicle to the stratum corneum and then into the epidermis for clearance by the blood (Guy *et al*, 1985a). Such molecules have good solubility in both oils and water. There is reasonably good

correlation between skin permeability and how well a compound partitions between octanol (oil) and water (McKone *et al*, 1992; Sartorelli *et al*, 1998). This is given by logP (log (octanol / water partition coefficient)). Compounds with a logP of about 2 are absorbed the best across the skin and so are most suitable for transdermal application.

b. Vehicle

The vehicle can affect drug absorption through hydration of the stratum corneum or by the inclusion of materials that will enhance the absorption of the drug – 'penetration enhancers'.

i. Vehicle – hydration

In general, increased hydration of the stratum corneum improves permeability to both polar and non-polar drugs. This is a widely utilized principle of dermatological therapy – occlusive vehicles such as fats and oils trap transepidermal moisture and reduce water loss. Similarly, the use of occlusive patches and films promotes drug absorption by enhanced skin hydration. This was first documented by Garb in 1960 (Garb 1960). *In vivo* studies examining the enhanced penetration of substances with augmented skin hydration have reported increases of up to 9 fold (Surber 1995).

The normal water content of the stratum corneum is 5 to 15%. This can be increased up to a total of 50% by occlusion (Blank *et al*, 1964a). Histologically, occlusion causes swelling of the corneocytes and uptake of water into intercellular lipid domains. Hence there is alteration of both the lipid and protein constituents of the stratum corneum (Goodman *et al*, 1989). It is thought that stratum corneum hydration alters the stratum corneum-viable epidermis partitioning step such that the partition coefficient of the penetrant between these two tissues is reduced.

This facilitates transfer of the penetrant from the stratum corneum to the epidermis and so enhances percutaneous absorption. In theory the relative effect of this should become greater with higher lipophilicity - this is used to promote topical steroid absorption in severe or resistant skin diseases (Guy *et al*, 1985a). However occlusion does not necessarily always increase skin absorption and in the case of hydrophilic compounds may impede permeation (Bucks *et al*, 1989). Occlusion can also lead to skin irritation which is a major problem in the design of transdermal delivery systems such as skin patches.

ii. Vehicle – penetration enhancers (accelerants or absorption promotors)

Penetration enhancers are materials that partition into and interact with the stratum corneum constituents thereby decreasing the barrier to drug diffusion (Marjukka *et al*, 1999). They are frequently added to topical drug formulations to enhance drug absorption, particularly when percutaneous absorption of the drug of concern is low due to its physicochemical properties. The action of penetration enhancers has been studied by Goodman and Barry using differential scanning calorimetry (Goodman *et al*, 1989). This is a technique that examines the thermal behaviour of the stratum corneum *in vitro* with reference to lipid and protein activity. The modification of heat transitions by the different penetration enhancers is investigated through identification of their mode of action on the stratum corneum in terms of lipid or protein alteration.

There is great diversity in the types of chemical that can act as accelerants, however they all act (via several different mechanisms) on the stratum corneum lipid, with or without additional affects on protein. A fundamental drawback of penetration enhancers is that as they work by altering the structure of the stratum corneum they can provoke an irritant reaction. This

is of particular consequence when the enhancer is a constituent of a topically applied dermatological therapy for a condition where the skin is already damaged or irritated.

The four penetration enhancers that have been investigated as part of this thesis are outlined below.

Dimethyl sulphoxide (DMSO)

DMSO is a colourless, odourless liquid that has been used as a skin penetration enhancer since the 1960's for a wide range of drugs including steroids, antibiotics and salicylates (Williams *et al*, 1992). It is used as a vehicle for idoxuridine in the treatment of herpes simplex infections and has activity against herpes simplex virus (Williams *et al*, 1992). DMSO has a concentration dependent site of action. At high concentrations (>60%), it affects skin lipid fluidity and alters keratin structure by competing with water for protein binding sites (Goodman *et al*, 1989). This disruption of the stratum corneum structure leads to an increase in drug penetration. High concentrations of DMSO are needed to promote steady state drug penetration, however they are irritant to the skin. Lower concentrations of DMSO will have less of an effect on stratum corneum lipid and only affect water-binding sites on protein. DMSO does not possess potent enhancer activity at low concentrations. A further indirect effect of DMSO on skin permeability arises from its solvent power for most drugs leading to higher concentration of drug dissolved. As DMSO may be present in both the lipid and protein parts of the membrane it may enhance the partitioning of polar and relatively non-polar drugs into the skin (Goodman *et al*, 1989). A number of other enhancers, namely, dimethylformamide (DMF), N-methyl-2-pyrrolidone (NMP) and 2-pyrrolidone (2P) act in the same way as DMSO (Barry 1987). The initial optimism for this group of solvents for accentuated drug absorption has been reduced

because of skin irritancy and toxicity at the high concentrations needed, and the side effect of odor caused by their metabolites (Barry 1989).

Sodium Lauryl (Dodecyl) Sulphate (SLS)

SLS is a powerful anionic surfactant that acts on stratum corneum protein (Goffin *et al*, 1999). It denatures and uncoils keratin alpha helices opening up the protein controlled polar pathway. Its effect is concentration dependent with concentrations as low as 0.1% having a marked effect on protein structure as detected by differential scanning calorimetry (Barry 1987). It may also affect lipid structure by disrupting the ability of lipid chains to pack together. The overall effect is increased hydration of the stratum corneum with a more porous structure that enables freer diffusion of drugs. Like DMSO, at high concentrations SLS is a powerful irritant (Washington *et al*, 1989; Williams *et al*, 1992).

Propylene Glycol

Propylene glycol is an alcohol that is widely used in dermatological formulations. It hydrates the keratin of the stratum corneum and occupies hydrogen binding sites which reduces drug/tissue binding – this will promote drug partitioning through the stratum corneum. It has less of an effect on fully hydrated tissue. In addition, propylene glycol is a powerful solvent, and like DMSO can act by promoting drug partitioning into the skin yielding higher fluxes (Barry 1987). Propylene glycol is often combined with azone to act as a 'co-enhancer'. Azone acts on the lipid barrier but has no effect on protein. Propylene glycol will help azone to penetrate the stratum corneum barrier; propylene glycol molecules may enter the hydrophilic regions between lipid molecules, azone being more soluble in propylene glycol than water may then be able to

partition more easily into the intercellular lipid layer. Such a combination of enhancers that affects both lipid and protein domains is extremely potent and hence the combination of azone and propylene glycol is one of the most efficient of currently used penetration enhancers (Washington *et al*, 1989).

Ethanol

Ethanol is the most popular alcohol that is used as a skin penetration enhancer. It is widely used in topical and cosmetic preparations as a solvent and / or penetration enhancer. At high concentrations however (>70%), ethanol can reduce drug penetration – this has been seen with both salicylate and nitroglycerin (Berner *et al*, 1989; Kurihara-Bergstrom *et al*, 1990). Ethanol is thought to work as a penetration enhancer by extracting stratum corneum lipids and forming pores in the stratum corneum (Ghanem *et al*, 1987).

Recent work has concentrated on alternative methods of enhancing the absorption of otherwise poorly absorbed molecules. One technique has been to conjugate well absorbed amino acids with the drug of choice. Some success has been seen with the absorption of an arginine-cyclosporin conjugate in a mouse/human skin model (Rothbard *et al*, 2000).

A second widely used technique is for the drug to be applied topically as a well absorbed prodrug that itself has no therapeutic activity. The prodrug undergoes change within the skin to form the active substance. Some of the topically applied steroids utilize this method; the acetonides of triamcinalone, fluocinolone and fluclorolone and the valerate ester of betamethasone all have higher absorption coefficients than the parent drug. It is not known if these steroid esters exert significant intrinsic activities in their derivatized forms or actually require bioconversion (Barry 1991).

c. Drug Concentration

The amount of drug absorbed per unit surface area per time interval increases as the concentration of the drug in the vehicle is increased (Wester *et al*, 1993a). However for some compounds, such as phenol, increasing concentration of drug produces significant reduction in absorption rates. This is due to a caustic effect on the skin by the compound thereby producing an artificial barrier (Macht 1938). Most commercially produced topical formulations are saturated with drug to facilitate partitioning of the drug from its vehicle at the vehicle / stratum corneum interface. The release of drug will be favoured by the selection of vehicles which have a low affinity for the drug or in which the drug is least soluble (Blank *et al*, 1964b).

Features of the skin affecting percutaneous absorption

Characteristics of the skin that may influence percutaneous absorption are; the anatomical site; age; gender; race; temperature; local blood flow and the presence of skin disease.

a. Anatomical site

Anatomical site demonstrates significant differences in barrier function on account of differences in the thickness and lipid concentration of the stratum corneum (Berti *et al*, 1995). Barrier function is greatest in heavily keratinized areas such as the palm and plantar skin (Wester *et al*, 1991), the abundance of sweat glands in these areas does not seem to have an effect in enhancing percutaneous absorption. In other areas barrier function can be ranked as back > arm > chest > thigh > abdomen > forehead, with the forehead being three times more permeable to benzoic acid than the back (Rougier *et al*, 1986).

b. Age

Skin condition and structure varies with age (Washington *et al*, 1989) however the skin barrier is only impaired in preterm infants. Histological evidence suggests that epidermal development is complete *in utero* at 34 weeks gestational age. Infants born prematurely have increased levels of transepidermal water loss, increased transcutaneous heat loss and experience difficulty in maintaining homeostasis (Cartlidge 2000). Neonates born at 23 to 25 weeks gestation need at least four weeks for development of a fully functional stratum corneum. Neonates born between 25 and 32 weeks need from 2 to 4 weeks (Kalia *et al*, 1998). There is disagreement as to the integrity of the skin barrier of older people. It has been found that in old age the stratum corneum thickens and is less hydrated, increasing its barrier function. Larger surface areas of topical drug delivery are needed to obtain equal plasma drug concentrations as compared with a young population (Berti *et al*, 1995). However it is also thought that aged epidermis displays altered drug permeability, increased susceptibility to irritant contact dermatitis, and often severe xerosis, which suggests compromise of the aged epidermal barrier (Ghadially *et al*, 1995). Further work is needed to clarify these conflicting schools of thought.

c. Gender

There are no reported studies that have identified a difference in percutaneous absorption between the sexes. Baseline transepidermal water loss is the same as is susceptibility to skin damage by irritants (Pinnagoda *et al*, 1990).

d. Race

Most of the work investigating the barrier function of the skin has been in Caucasians, however racial differences in skin permeability have been demonstrated. Black skin has a higher number of cell layers in the stratum corneum compared with Asian or Caucasian skin (Berardesca *et al*, 1988), although the overall stratum corneum thickness is the same (Weigand *et al*, 1974; Weigand *et al*, 1980). Transepidermal water loss is higher in black skin and this may be explained on the basis of a thermoregulatory mechanism (Wilson *et al*, 1988). In general, black skin is considered to be less permeable than Caucasian and this has been demonstrated with the absorption of methyl nicotinate as measured by Laser Doppler velocimetry (Kompaore *et al*, 1993b; Kompaore *et al*, 1993a). However there are a number of studies that dispute differences in skin permeability between races (Vanakoski *et al*, 1998).

The size of corneocytes does not differ between racial groups although corneocyte adhesion is increased in black skin. The stratum corneum lipid content differs between races which could account for permeability differences (Corcuff *et al*, 1991; Vanakoski *et al*, 1996)

e. Temperature and blood flow

Temperature affects drug penetration by two mechanisms; firstly, it alters the physiology of the skin including sweating and cutaneous blood flow, and secondly it can alter the physicochemical diffusion rates of a drug. An increase in cutaneous blood flow will increase drug clearance from the dermis, this may be a desired effect in the case of topically applied drugs with a systemic site of action, but will be disadvantageous for those drugs whose activity is within the skin. Clinical studies on increasing body temperature by use of a sauna or physical exercise have shown significantly increased transdermal delivery of nicotine, nitro-glycerine, clonidine and insulin

(Vanakoski *et al*, 1998). The plasma level of methylsalicylate applied topically was also found to be significantly increased in healthy subjects either exercising, resting at 40°C, or both, the latter increasing the absorption three fold (Danon *et al*, 1986). The increased absorption was assumed to be due to vasodilatation clearing absorbed drug and increasing the concentration gradient of drug absorbed across the skin. Alternative routes for drug absorption such as the diffusion of penetrant down actively secreting sweat glands was not considered.

It is well recognized that drug absorption is enhanced when cutaneous blood flow is increased and that this is due to maintenance of a concentration gradient of drug across the skin (Mukhtar *et al*, 1992). Hence conditions of exercise, fever, or disorders of the autonomic nervous system will have effects on blood flow and therefore drug absorption. The converse may also be true, transdermal nicotine absorption was reduced when there was concomitant intravenous administration of deuterium-labeled nicotine causing vasoconstriction. Plasma concentrations of percutaneously absorbed nicotine rose less rapidly, reached a lower peak and peaked at a later time. This was considered to be due to the intravenous nicotine causing vasconstriction of the dermal blood vessels, thereby limiting percutaneous absorption. However, care should be taken in the interpretation of this study as the intravenous nicotine may have had effects on the transcutaneous nicotine diffusion gradient (Benowitz *et al*, 1992).

f. Disease

Skin disease almost always involves structural changes of the stratum corneum which alters the barrier and allows increased penetration of substances. Even when the skin layer is unbroken, inflamed or irritated skin has higher permeability. Diseases that give rise to thickening of the stratum corneum such as psoriasis and ichthyosis also cause increased permeability (Frost *et al*,

1968; Scheuplein 1976), this is due to changes in the structural integrity of the stratum corneum and loss of barrier function (Carr *et al*, 1968). As well as skin diseases, damage from ultraviolet light (sunburn) and irritants increase permeability by barrier damage (Washington *et al*, 1989).

Methods for studying percutaneous absorption

The study of percutaneous absorption is essential to the field of dermatotoxicity in which compounds pose a threat to human life, and to dermatopharmacology for which drugs need to be delivered into and through the skin to treat disease both locally and systemically (transdermal delivery). There are four approaches to the study of percutaneous absorption; predictive mathematical models; animal studies; human *in vitro* work and human *in vivo* work. Each approach does provide relevant information for drug design, development and formulation, however, for topically applied drugs it can be argued that human *in vivo* work provides the most accurate and significant data.

1. Predictive Mathematical Models

The ultimate aim of a mathematical model for percutaneous absorption is to provide a simple but effective means of estimating drug levels in the skin, blood or urine after topical administration. Approaches to this are varied, but in general relate solute structure and physicochemical characteristics with transport through the skin. Invariably Fick's laws of diffusion form the basis of the model. The more detailed models break down the skin into a series of membranes each possessing a different partition coefficient for the solute of interest. Drug metabolism rates, protein binding and the effect of blood flow on clearing absorbed drug may be included in the formula. The foundation for such models is typically obtained from *in*

vitro studies. For those models that have been tested with *in vivo* data there is often good agreement between the predictive and experimental results (Guy *et al*, 1985b; Guy *et al*, 1985c).

2. Animal Studies

The most accurate way to determine the penetration potential of a compound in man is do the actual study *in vivo* in humans. Many compounds are potentially too toxic to test *in vivo* in man and so have been tested on animals. Animal skin differs from human skin in its stratum corneum lipid / protein constituents and structure, and the density and type of skin appendages. It is misleading to draw firm conclusions about penetration of a drug using animal studies alone. Studies on animals *in vivo* demonstrate that the pig and rhesus monkey (shaved skin) are the most suitable models for comparison of drug penetration rates in man (Wester *et al*, 1989). Percutaneous absorption in rats, rabbits, mice and guinea pigs is greater than human skin and therefore cannot be used as a valid predictor for penetration in man.

In vitro studies using animal skin in diffusion cells also conclude (not surprisingly) that the skin of common laboratory animals (rat, mouse, rabbit) is more permeable than human skin. In addition, skin from the pig and monkey more generally approximates the permeability of human skin (Marzulli *et al*, 1969). It is debatable as to whether or not animal studies give a valuable contribution to the study of percutaneous absorption in humans for the development of drugs, however in the field of dermatotoxicity they do have a role.

3. Human Studies

a. in vitro.

Virtually all human *in vitro* work involves examining the diffusion of substances through excised skin in diffusion chambers. It is a relatively easy and rapid procedure once set up and is used to screen potential drugs for topical administration. For highly toxic compounds *in vitro* methodology may be the only means of obtaining percutaneous data with human skin. The advantage of *in vitro* procedures is that absolute control of the environment is obtained. This allows the examination of the importance of individual factors in determining percutaneous absorption. The main disadvantages are that any active processes present *in vivo* are ignored. This includes the effect of cutaneous blood flow, the effect of lymph drainage, active transport mechanisms in the epidermis and metabolism of drugs in the skin - it cannot be assumed that excised skin will retain full metabolic activity (Wester *et al*, 1993b). Another major unknown factor is the skin surface bacterial population and what role it might play in percutaneous absorption.

The most common way of investigating percutaneous absorption in a diffusion chamber is by applying radioactive compound to one side of the skin and then assaying for radioactivity in the collection vessel on the other side. The skin can be wholly intact or separated into epidermis or dermis. Results from such experiments can pave the way for further *in vivo* work and this is an approach that has been adopted by many workers (Marzulli *et al*, 1969). Care should be taken that the skin obtained is not damaged or diseased as this will affect its barrier function. Similarly storage conditions such as freezing can affect the integrity of skin (Bronaugh 1989). It is better to use fresh samples as with animal *in vitro* studies but this is usually not possible. Intersubject and site variability exists for *in vivo* work and the same is seen with *in vitro* work. Often the skin

available has come from surgical procedures such as abdominoplasties and mastectomies and so is limited in its anatomical site. It is preferable to use a wide range of skin sites and multiple human sources for validation of results obtained.

Human Studies

b. in vivo

There are a number of different approaches to studying drug absorption *in vivo* in man. The most commonly used are outlined below.

i. Indirect Radioactivity Method.

This classic method was first described by Feldmann & Maibach and has been used extensively to investigate the percutaneous absorption of different substances (Feldmann *et al*, 1969; Feldmann *et al*, 1970; Feldmann *et al*, 1974; Schaefer *et al*, 1978). The compound of interest is labelled with ^{14}C or tritium and applied topically. The total amount of radioactivity excreted in urine, or urine plus faeces is measured over the next few days. A correction for incomplete elimination, or excretion by a route not assayed (CO_2, sweat) is made by determining the amount of radioactivity excreted after intravenous administration. Percutaneous absorption is determined by the following equation:

$$\% \text{ percutaneous absorption} = \frac{\text{total radioactivity after topical administration}}{\text{total radioactivity after parenteral administration}} \times 100$$

This method does not allow discrimination between parent drug and metabolites which severely limits its kinetic interpretation. It is also time consuming, expensive and unsuitable for routine studies.

A variation on this method is to measure plasma radioactivity (Wester *et al*, 1978) however there are the same limitations of inability to differentiate parent molecule from metabolite, and expense and time involved.

Measurement of plasma or urine levels of drug that is not radioactively labeled is often difficult because the concentrations involved following topical administration are low and commonly below assay sensitivity. This may become a more useful technique in the future with the advent of more sensitive assay systems.

ii. Biological/Pharmacological Response

This method is limited to compounds that elicit responses within the skin that can be measured easily and accurately. It provides information on whether or not a substance capable of causing a physiological reaction is able to penetrate the skin, and if it does penetrate, the time taken for the reaction to develop. Quantitation of the actual amount of drug penetrating is not as accurate as *in vitro* methods, however comparative estimates of the penetration of a substance can be made under different environmental conditions (such as humidity and temperature) and anatomical sites. Comparison of activity between drugs of the same family can also be made. This has been done with topical steroids and measurement of cutaneous vasoconstriction (McKenzie A W *et al*, 1962) and topically applied nicotine with measurement of erythema (Albery *et al*, 1979).

iii. Skin stripping

The chemical of interest is applied to the skin for about 30 minutes. Serial tape strips remove the cells of the upper layers of the stratum corneum, and the tape strips are analysed for drug content. This will provide information on the penetration depth / absorption of the drug and its partitioning at the vehicle / stratum corneum interface. Using linear extrapolation its percutaneous absorption over the following hours and days can be predicted. Although this method is simple, it is proven to be an accurate measurement of percutaneous absorption; Rougier *et al* found a linear correlation ($r=0.97$, $p< 0.001$) between the amount of radiolabelled drug excreted over 4 days following topical administration and the measurement of radioactive drug on cellophane strips removed 30 minutes after drug application. The system was tested with caffeine, benzoic acid, benzoic acid sodium salt and acetylsalicylic acid with close correlation of the results obtained (Rougier *et al*, 1986; Rougier *et al*, 1987). The disadvantage of this method is that some lipid soluble drugs such as steroids, may partition into the stratum corneum but not diffuse deeper into the epidermis – this is the effect of the 'epidermal' or 'stratum corneum' reservoir. It has been demonstrated that certain steroids remain for up to 14 days in this reservoir (Vickers 1963), therefore prediction of epidermal or systemic drug levels will be inaccurate. Similarly, if the degree of stratum corneum / epidermis partitioning is low then drug diffusion into the epidermis and dermis will be low; tape stripping studies will give a falsely high result of systemic levels of drug. A further source for inaccuracy is that the quantity of stratum corneum cells removed by tape stripping is not linearly proportional to the number of tape strips. This will vary according to operator technique. Intersubject differences and anatomical site are not sources for inaccuracy of the system but rather will provide additional information on percutaneous absorption within these variables.

Skin tape stripping is also used to disrupt the stratum corneum barrier to investigate its effect on drug absorption (Benfeldt 1999). This is one of the techniques which has been investigated as part of this thesis (see chapter 5) to quantify the effect of barrier disruption as measured by transepidermal water loss on drug absorption.

iv. Skin Biopsy

A classical method to measure drug levels in the skin is via a punch biopsy under local anaesthesia. Drug that has been administered topically or systemically can be studied. Following fixation of the skin biopsy, it can be divided into epidermis, dermis and subcutaneous tissue with the amount of drug measured in each (Surber 1995). A concentration gradient for topically administered drug can then be obtained. The disadvantages of this technique are that it is invasive, leaves permanent scars and only provides information about drug levels within the skin at a single time point. Serial biopsies would provide greater pharmacokinetic data although subject tolerance of multiple invasive biopsies may be low.

v. Skin Suction Blisters

The production of suction blisters in the skin has mainly been used to investigate drug levels in the dermis following systemic dosing although it has been applied to transdermally absorbed substances (Surber *et al*, 1993). It is a technique whereby a vacuum is created at the skin surface that causes the epidermis to lift away from the dermis and form a subepidermal blister. The blister fluid corresponds to the extracellular fluid of the dermis. It is technically difficult to use this method to investigate percutaneous absorption as there is high potential for contamination of the blister fluid by topically applied drug. Even if drug is removed from the

skin surface prior to blister formation, drug present in the stratum corneum reservoir can artefactually enter the blister fluid resulting in inaccurate data.

vi. Cutaneous Microdialysis

Cutaneous microdialysis is a technique that enables the continuous sampling of the dermal extracellular fluid *in vivo*. It is a superior method for measuring percutaneous absorption and provides pharmacokinetic data that is directly relevant for those drugs whose site of action is within the skin. Microdialysis is the main technique used in this thesis for the investigation of antiviral drug absorption in man, and so will be described in some detail:

Cutaneous Microdialysis

Microdialysis was introduced in 1972 for use in experimental brain research in monkeys (Hsiao *et al*, 1990) and was further developed by Ungerstedt for neuro-pharmacological studies in rats (Ungerstedt 1991). It was adapted and applied to the study of substances in the dermal interstitial fluid by Lönnroth in the latter half of the 1980's (Lonnroth *et al*, 1990). It is a versatile technique that has been modified and used in many tissues and species including adipose tissue, skeletal muscle, heart and brain (Graumlich *et al*, 2000; Muller *et al*, 1995a; Stich *et al*, 2000; Wu *et al*, 2000).

Principles of microdialysis

The basic principle of microdialysis is to mimic a small blood vessel situated in the tissue of interest. The microdialysis fibre consists of a semipermeable membrane which is perfused with a physiological solution. Substances will diffuse passively in and out of the membrane with no

overall gain or loss of solvent volume. Compounds present in the medium surrounding the fibre at higher concentrations than in the perfusate will diffuse into the dialysis membrane. Conversely compounds at a higher concentration in the perfusate will diffuse into the extracellular fluid. Diffusion of substances across the membrane is according to Ficks law. By keeping the flow rate low (between 1 and 10μl/min), ultrafiltration does not occur because pressure within the fibre is kept at a minimum. Sampling of the tissue of interest can continue over many hours or even days, with minimal tissue trauma.

Cutaneous microdialysis - recovery and loss

In cutaneous microdialysis the fibres are situated in the dermis at a depth of between 0.6 and 0.8mm. The concentration of a given compound or drug in the dialysate will reflect the concentration of drug in the dermal extracellular fluid (ECF), however, it will not equal the ECF concentration. The constant flow of perfusate through the fibre does not allow equilibration of the concentration of drug on either side of the membrane (Groth 1996). Hence the dialysate will always have a lower concentration of drug. Relative recovery is defined as the ratio between the concentration in the dialysate (C_d) and the concentration in the extracellular fluid immediately surrounding the probe (C_{ecf}).

$$\text{Recovery} = \frac{C_d}{C_{ecf}}$$

Factors affecting relative recovery

Several factors affect the dialysis of substances from the extracellular space. They can be broadly defined as those pertaining to the microdialysis equipment, the substance of interest and the tissue where sampling takes place.

1. Microdialysis equipment

   Microdialysis fibres can be manufactured in a range of different pore sizes. As they are produced in the laboratory from artificial 'kidneys' used in renal dialysis, the semipermeable membrane used can allow the transfer of substances of different sizes, with the most permeable membranes enabling movement of molecules up to 3000kDa in size. The pore size of the membrane determines which substances are allowed to pass across the membrane as expressed by the molecular weight cutoff. In this thesis a 2kDa molecular cutoff has been used for all experiments. In fact the average pore size of the membrane will be considerably less than 2kDa – as this value represents the largest holes in the membrane. Using a membrane with a low molecular cutoff prevents large molecules such as proteins entering the dialysate and so the collected sample is relatively 'clean' and suitable for analysis. There are three different types of probe : the microdialysis probe *per se* which consists of a single straight fibre attached to in- and outlet tubes which are also straight and arranged in series;, the loop probe – where the tubes are positioned in parallel, and the concentric probe. In all experiments described in this thesis the basic straight dialysis tube is used. The choice of fibre material does not significantly affect relative recovery *in vivo* (Hsiao *et al,* 1990).

   Perfusate flow rate should be low to remove as little as possible of the extracellular fluid – this will minimize interference with the normal physiology of the

tissue and prevents ultrafiltration. Low flow rates also increase recovery as there is more time for equilibration of the substance across the membrane (Ungerstedt 1991). However, the flow rate may be dictated by the assay as sample volume can be critical for the analytical procedure. The length of membrane exposed to extracellular fluid and across which sampling can occur will affect relative recovery. Efficiency of dialysis increases as the surface area available for the transfer of substances increases. This includes increasing length of the membrane (Groth 1996).

The perfusion fluid should be sterile, free of bubbles and particles (which can interfere with flow and dialysis efficiency), and identical in composition and tonicity to the dermal extracellular fluid. If the extracellular fluid contains substances that are not present in the perfusate then those substances will diffuse through the membrane and constitute part of the microdialysate - as is the principle of microdialysis. Ringer's solution and phosphate buffered saline are the two solutions most commonly used as perfusate in cutaneous microdialysis.

2. Dialysed compound

The ideal molecule for recovery by microdialysis is one that has a low molecular weight, is not charged, does not bind to plasma proteins and is water soluble. Very lipophilic drugs are not easily sampled by microdialysis as they do not partition into the aqueous perfusate and may adhere to the plastic tubing and vials used (Groth *et al*, 1997). A study investigating the percutaneous absorption of the lipophilic corticosteroid betamethasone by microdialysis found that no drug was detected in the aqueous dialysate. When repeated in rats using systemically administered steroid it was only at extremely

high doses that steroid could be detected (Benfeldt 1999; Groth *et al*, 1997). Similarly the hormone oestradiol has been declared 'non-dialysable' on account of its lipophilicity (Muller *et al*, 1995b). We have encountered similar problems with attempts to investigate the percutaneous absorption of dithranol – even *in vitro* using high concentrations of ethanol or albumin in Ringers as the perfusate, it was not possible to detect dithranol in the dialysate by HPLC assay (sensitivity 0.5μg / ml).

In vitro work has investigated the use of a lipid emulsion (parenteral nutrition lipid) as the perfusate instead of Ringers' solution. Recovery of alkylparabens was up to 390 times greater with the lipid perfusate (than Ringer's solution) with the greatest increase in recovery seen with the most lipophilic compounds (Kurosaki *et al*, 1998). This has not yet been extended to *in vivo* investigation.

The use of 2kDa fibres precludes the dialysis of proteins and also of substances that are heavily protein bound. It was not possible to dialyse fusidic acid (with 2kDa fibres) on account of its high degree of protein binding (Benfeldt *et al*, 1998; Kurosaki *et al*, 1998). With the current use of fibres with large pore sizes (3000kDa), dialysis of protein is possible, and is currently used to investigate the mediators of the inflammatory response. The use of such fibres to investigate the percutaneous absorption of drugs that demonstrate high protein binding may create assay complications in the separation of drug from protein.

3. The tissue

Recovery of a particular compound is different depending on the tissue (Stahle 1991). Blood flow and metabolism can affect the concentration of a drug in a tissue such

that the concentration available for dialysis varies between tissues. The actual relative recovery by microdialysis in terms of dialysing the available concentration may be the same. Tissues also vary in the time taken to recover from the trauma of needle insertion. Finally, in cutaneous microdialysis, recovery may be affected by fibre depth within the dermis (Andersson *et al*, 1996).

Advantages of Microdialysis as a technique for measuring dermal drug levels

In the present study microdialysis has been the technique of choice to measure dermal levels of the antiviral agents penciclovir and aciclovir. Its advantages over the extensive list of other techniques for investigating percutaneous absorption can be summarized as:

1. It samples the extracellular fluid, as distinct from sampling the whole tissue collected by biopsy or blister fluid collected by ultrafiltration.
2. It allows continuous sampling over many hours and therefore provides a pharmacokinetic profile for the drug of interest.
3. It can be used to sample topically applied or systemically administered drug within the dermis. Alternatively it can sample endogenous substances.
4. As long as there is a 90 minute delay between fibre insertion and the start of substance sampling, trauma from fibre insertion does not affect the dialysis process (Anderson *et al*, 1994). There is no increased oedema or inflammatory cell infiltrate around the fibre that will affect sampling (Krogstad *et al*, 1996; Ault *et al*, 1994; Lonnroth *et al*, 1990)
5. It is pain free, relatively non-invasive and does not cause permanent scarring.

Therefore it was decided that cutaneous microdialysis was the most appropriate technique to investigate factors surrounding the percutaneous absorption of penciclovir and aciclovir as examples of hydrophilic drugs that would not be expected to demonstrate good penetrative properties.

Other techniques used have been the measurement of transepidermal water loss (TEWL), scanning laser Doppler imaging (LDI) and dermal ultrasound.

Other Investigative Techniques Used in Thesis

1. Transepidermal Water Loss

The measurement of transepidermal water loss is a non-invasive method of assessing stratum corneum integrity and barrier function of the skin (Maibach *et al*, 1984). Transepidermal water loss increases with skin damage or disease and accordingly the penetration of topically applied substances will increase. The skin's thermoregulatory mechanisms will also affect TEWL with higher levels seen with sweating. Cutaneous vasoconstriction or vasodilation does not affect TEWL (Pinnagoda *et al*, 1990). Anatomical site affects TEWL and accordingly percutaneous absorption varies. Lotte *et al* found a correlation of up to 0.73 between TEWL and the absorption of radiolabelled benzoic acid sodium salt, acetyl salicylic acid and caffeine at 4 different anatomical sites (arm, forehead, abdomen and post auricular). The order of cutaneous permeability and increasing TEWL was: arm ≤ abdomen < postauricular < forehead, with the forehead skin being 2 to 3 times more permeable than that of the arm or abdomen (Lotte *et al*, 1987).

Transepidermal water loss is affected by circadian rhythm and is higher in the morning than the afternoon. The measurement of TEWL is affected by temperature and air movement.

We have used transepidermal water loss as a measurement of barrier integrity and to investigate sweat gland activity following pharmacological stimulation.

2. Scanning laser Doppler imaging

Skin blood flow can be imaged and measured with scanning laser Doppler imaging (LDI) (Moore Instruments Ltd, Axminster, UK). This measures blood flux up to a 1mm depth in the skin. A red, low-power laser beam scans across the skin surface. The haemoglobin of red cells reflects the laser beam. If the red cells are stationary (as in arterial occlusion) then the laser is reflected to the detector without any change in wavelength. If the cells are moving then the wavelength of the reflected laser beam is altered, the degree of alteration being related to the speed of red cell movement (the Doppler principle). The reflected laser beam signal is then processed to give a laser Doppler flux output that can be analysed to give arbitary units of vascular perfusion. This technique is useful to quantify changes in blood flux in an individual.

3. Dermal ultrasound

Ultrasound utilizes the principle that sound waves travel at different rates through tissues of different densities. If a beam of ultrasound is directed at the skin it will be reflected when it reaches an interface between two tissues and is detected as an echo. The time interval between the original pulse of ultrasound and detection of the echo combined with knowledge of the speed of sound through tissues (for human tissue this is 1580m/s) is the basis for the generation of ultrasound images (Serup 1992). The Dermascan C (Cortex Technology, Denmark) is a high

resolution ultrasound unit that uses a wavelength of 20 MHz. This is the best frequency for a compromise between scanning depth (3mm) and resolution. It is not possible to accurately measure epidermal thickness with a wavelength of 20 MHz – a higher frequency would be needed, although the trade off would be a reduced depth of ultrasound penetration in the skin. Nor it is possible to differentiate particular structures within the skin such as blood vessels and sweat glands. Ultrasound can be used to measure skin thickness, size of benign and malignant tumours (Fornage *et al*, 1994; Harland *et al*, 1993), certain areas of oedema or inflammation, or as in the current study, fibre depth.

Scans are made using the A mode which is unidirectional. It plots the magnitude of echo against the time of the returning echo. The Dermascan converts this time into a distance measurement. Hence the A mode is suitable for measuring skin thickness and *in vivo* distance measurement between interfaces. B mode scanning produces a 2D image of a cross section through the skin. The ultrasound beam points in many directions to produce a cross section through the skin that is 22.4mm deep (x axis) and 22.4mm wide (y axis). This image is made up of 224 A scans – with a preset inter-slice difference of 0.1mm. Water is used as the transmission medium in the scanner head and is held in place by a disposable plastic film membrane. Both the water and membrane allow the ultrasound signal to pass with minimal attenuation. A water-based gel is used as a coupling medium between the scanner head and skin.

Compounds of interest – penciclovir, famciclovir and aciclovir

Penciclovir and aciclovir are nucleoside analogues with activity against herpes simplex I and II and varicella zoster virus.

Penciclovir

Penciclovir (PCV) is produced commercially as a 1% topical cream (Vectavir) for the treatment of labial herpes simplex. As penciclovir is very poorly absorbed through the gut, it is administered orally as the prodrug famciclovir (Pinnagoda *et al*, 1990; Pue *et al*, 1993). Famciclovir is the diacetyl 6-deoxy derivative of PCV. Conversion of famciclovir to PCV is initiated in the gut with the removal of one ester group and completed in the liver with removal of the second ester group and oxidation of the purine ring (Crumpacker 1996). There is high bioavailability of penciclovir (77%) after oral administration of famciclovir at a range of doses. The volume of distribution of PCV is 1.5l/kg – this is double that of body water and so it shows extensive tissue distribution. Protein binding of PCV is relatively low (<20%).

PCV is actively taken up into herpes virus infected cells where it is phosphorylated by viral thymidine kinase and host enzymes to the active form penciclovir triphosphate. Penciclovir triphosphate competes with the nucleotide deoxyguanosine triphosphate for viral DNA polymerase and so inhibits viral replication by chain termination (Vere Hodge *et al*, 1993). PCV is considered to be an excellent anti-herpes treatment because of its high selectivity for virally infected cells, long half life within infected cells (up to 20 hours) and high bioavailability following oral administration as famciclovir.

The IC50 for virally infected cells measured in vitro is 0.4 μg/ml in Herpes Simplex I and 1.5μg/ml in Herpes Simplex II (Crumpacker 1996). Latent herpes simplex I virus most

commonly resides in the trigeminal ganglion (Scott *et al*, 1997). Once activated there is virus replication resulting in epidermal destruction and the development of a 'cold sore'. The site of action of PCV is therefore at the basal epidermis and also deeper at the trigeminal ganglion. Topically applied PCV needs to cross an intact stratum corneum and epidermis during the prodromal period of virus activation.

PCV is a hydrophilic molecule with a water solubility of 1.7mg/ml (20°C,pH 7). It has a molecular weight of 253.3 and is negatively charged. These physicochemical properties would suggest that it may be very poorly absorbed through the skin by the conventional intracellular pathway. Indeed it is poorly absorbed through the gut mucosa which also favours the absorption of non polar, lipophilic molecules, hence the prodrug famciclovir for oral administration.

PCV was chosen as the drug of interest to study as its site of action is within the skin. The natural history of HSV I infection producing ulceration of the skin lends itself to the measurement of penciclovir absorption with varying degrees of barrier impairment as an imitation of the clinical situation. PCV is produced commercially for topical or systemic administration so can be used in comparable pharmacokinetic studies of dermal drug levels with the two different methods of administration.

Aciclovir

Aciclovir (ACV) was the first specific antiviral drug to become widely used against herpes viruses, and has a similar action against virally infected cells as penciclovir (PCV). It is considered to be less effective with a shorter half life and lower bioavailability than PCV when given systemically (Vere Hodge *et al*, 1993). It has a similar structure to PCV although is a smaller molecule with a molecular weight of 225. It is more water-soluble (2.5mg/ml) and has a

lower degree of protein binding (15.4%) (Dollery 1999a). Like PCV, ACV is commercially produced as a topical or oral formulation. The sodium salt of ACV is also available for intravenous use.

The absorption of ACV was investigated in two studies described here (chapters 4 & 5) for direct comparison with PCV, and to see if the pharmacokinetic profile of PCV absorption could be extended to a class effect.

Chapter 2

Materials and Methods

Subjects

112 healthy volunteers were recruited (53 male, 59 female), aged 19 to 65, mean age 27. None of the volunteers were taking any medication. All subjects gave written informed consent. The study was approved by The South and West Research Ethics Committee (ref 265/98).

Drugs

Penciclovir (1% Vectavir cream, SmithKline Beecham Pharmaceuticals, UK), aciclovir (5% Zovirax cream, Glaxo Wellcome Pharmaceuticals, UK), noradrenaline (1mg/ml Levophed, Sanofi Winthrop, UK), lidocaine / prilocaine (5% EMLA cream, Astra Pharmaceuticals Ltd, UK), Ringer's intravenous solution (Baxter Healthcare Ltd, UK), Famciclovir (250mg tablets, SmithKline Beecham Pharmaceuticals, UK), pilocarpine nitrate (0.5%, Mandeville Medicines, Stoke Mandeville Hospital, UK), aqua gel lubricating jelly (Adams Healthcare, UK), glyceryl trinitrate solution (5mg/25ml, David Bull Laboratories, UK) white soft paraffin (Adams Healthcare, UK) were obtained from the hospital pharmacy.

Microdialysis fibre

Microdialysis fibres were manufactured in the laboratory. Fibres were obtained from an kidney dialysis capsule (Gambro model GFE 18, Gambro Dialysaten AG, Germany). Individual linear fibres of 2kDa molecular cut off, 216 μm outer diameter, 8 μm wall thickness were glued (cyanoacrylate (Loctite Superglue®)) to a 10cm length of tubing internal diameter 0.58mm,

external diameter 1.02mm (Portex Ltd., UK). A 0.1 mm diameter, 5 cm long stainless steel wire (Goodfellow Cambridge Ltd, UK) was inserted into each fibre as a support to increase robustness. The fibres were sterilized with ethylene oxide prior to use.

Generalized Microdialysis Method

Volunteers were asked to refrain from drinking caffeine-containing drinks (caffeine has weak vasodilator activity) on the day of the study and to avoid applying any moisturizing creams or other topical agents to the study arm 24 hours prior to the study. All studies were conducted in the clinical room of the Dermatopharmacology Unit with the volunteer lying supine throughout. Room temperature was 22 ° C (± 2° C). The volar surface of the non-dominant forearm was anaesthetized with EMLA cream under occlusion for 90 minutes. Following removal of the EMLA (figure 2.1), up to six 23 gauge needles (0.6mm x 30mm, Terumo Europe N.V, 3001 Leuven, Belgium) were introduced into the dermis parallel to the epidermal surface for a length of 25mm with at least 20mm between adjacent needles (figure 2.2). Microdialysis fibres were passed through each needle and the needle removed leaving the fibres in the dermis (figure 2.3). Fibre sequence was randomized for each study as it has been found that transepidermal water loss is slightly higher at the wrist, which may affect percutaneous absorption (Panisset *et al*, 1992). The fibres were connected via flexible tubing (length 200cm, volume 1.5ml; Alaris Medical Systems, UK) to 10ml syringes (Terumo Europe N.V, 3001 Leuven, Belgium) situated in P3000 syringe pumps (IVAC Medical Systems, Hants, UK) and perfused with the appropriate solution per experiment at a rate of 5μl/min. Dialysate was collected hourly for up to 5 hours and immediately frozen at -20°C.

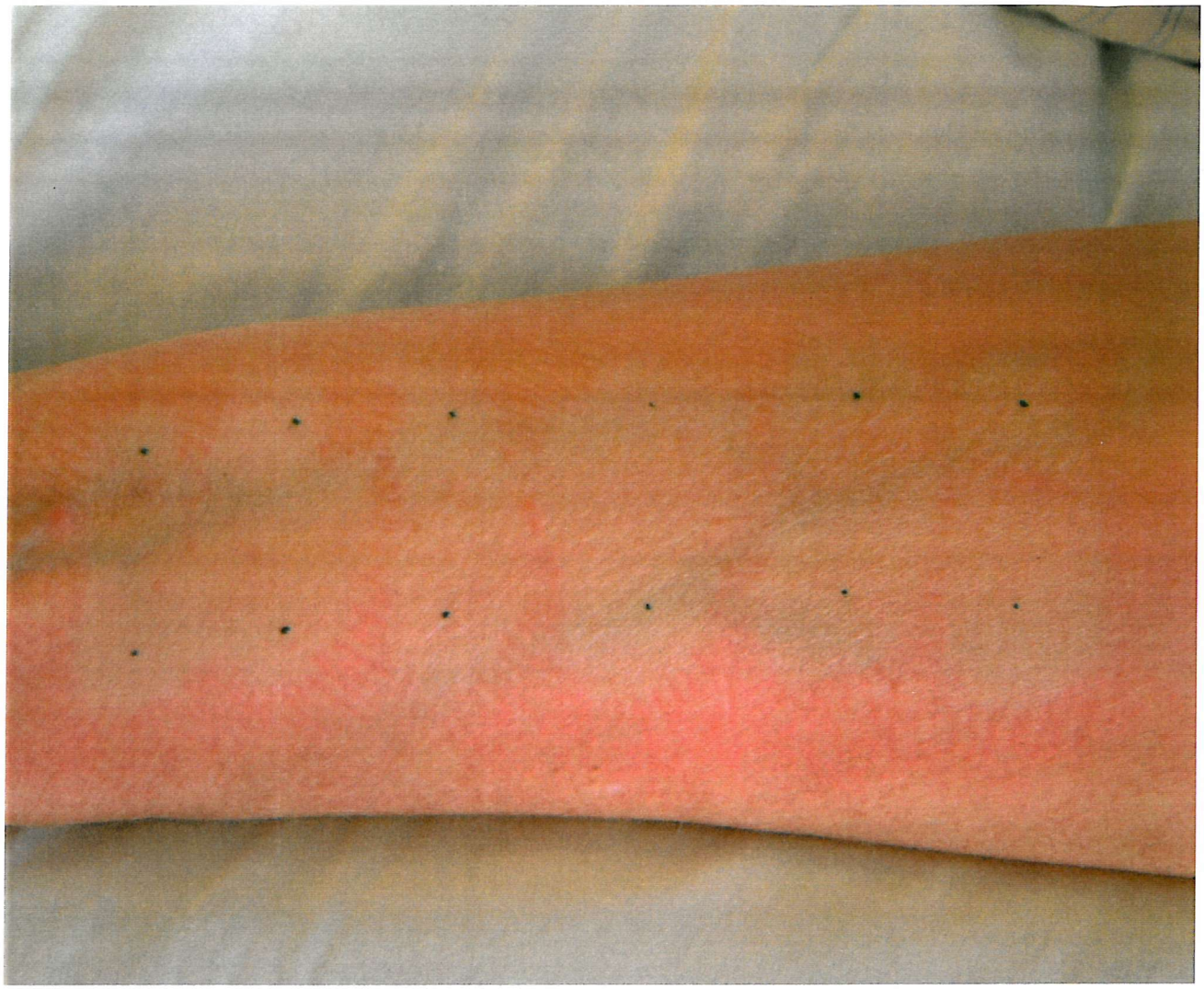


Figure 2.1 Photograph of the volar surface of a subject's forearm after removal of EMLA cream that had been applied to the arm under occlusion for 90 minutes. The vasoconstrictive action of EMLA is clearly seen. The pen dots on the arm act as a guide for needle insertion to ensure that each needle is passed 2.5cm through the dermis.

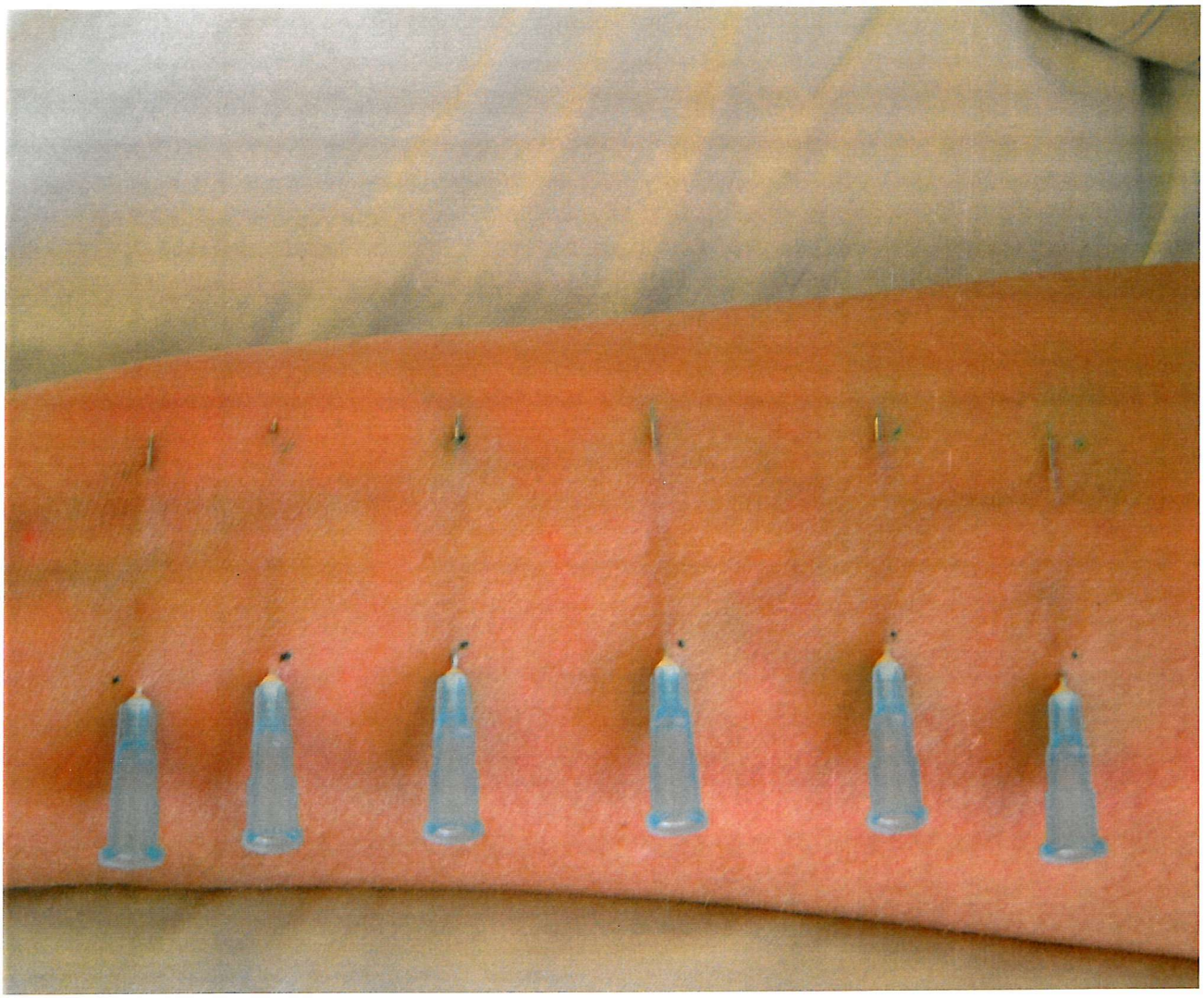


Figure 2.2 Photograph of six 23 gauge needles that have been inserted through the skin under EMLA anaesthesia.

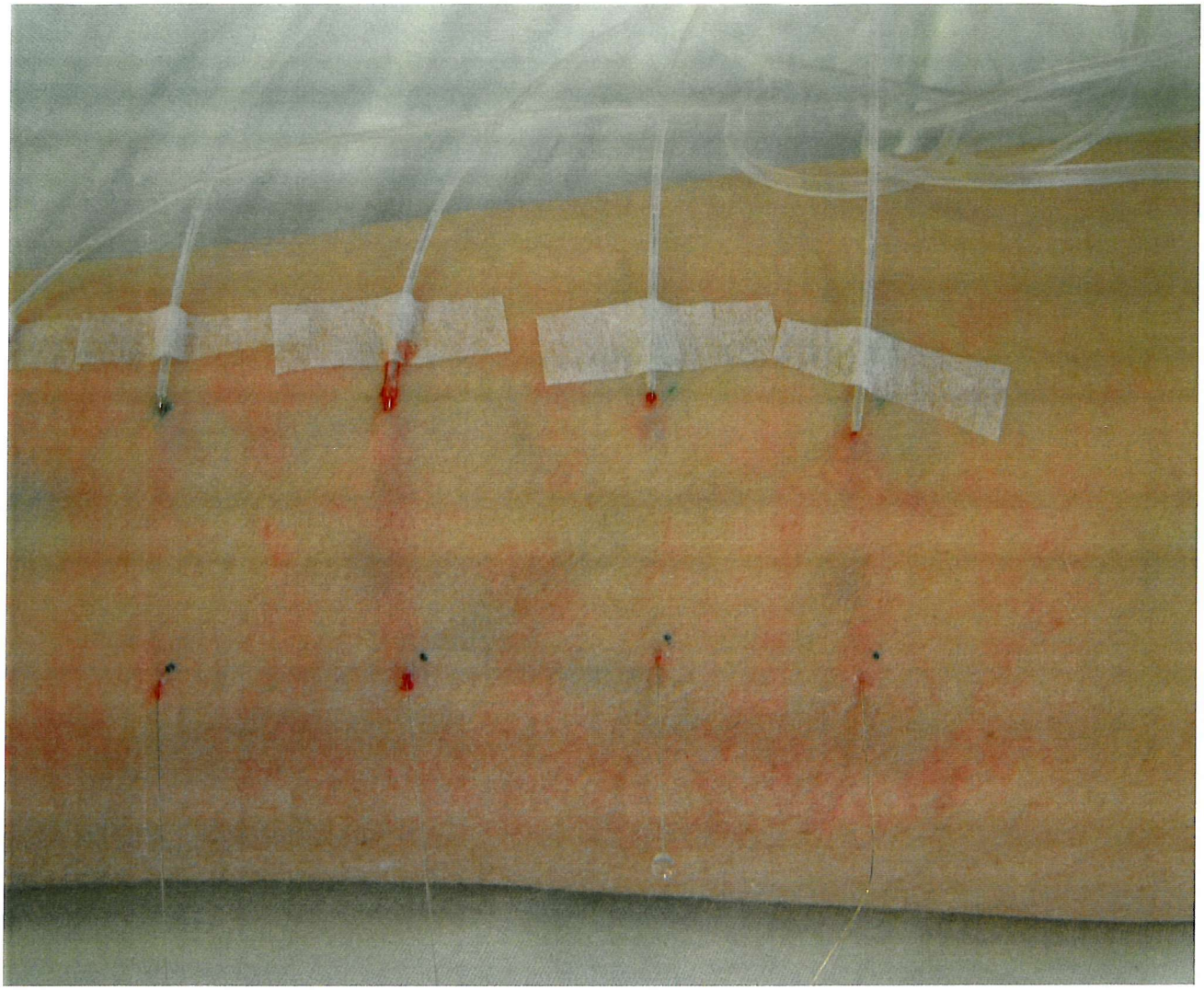


Figure 2.3 Photograph of 4 microdialysis fibres that have been inserted into the volar surface of the forearm using the 23 gauge needles as an introducer. The needles are then removed leaving the fibres in place.

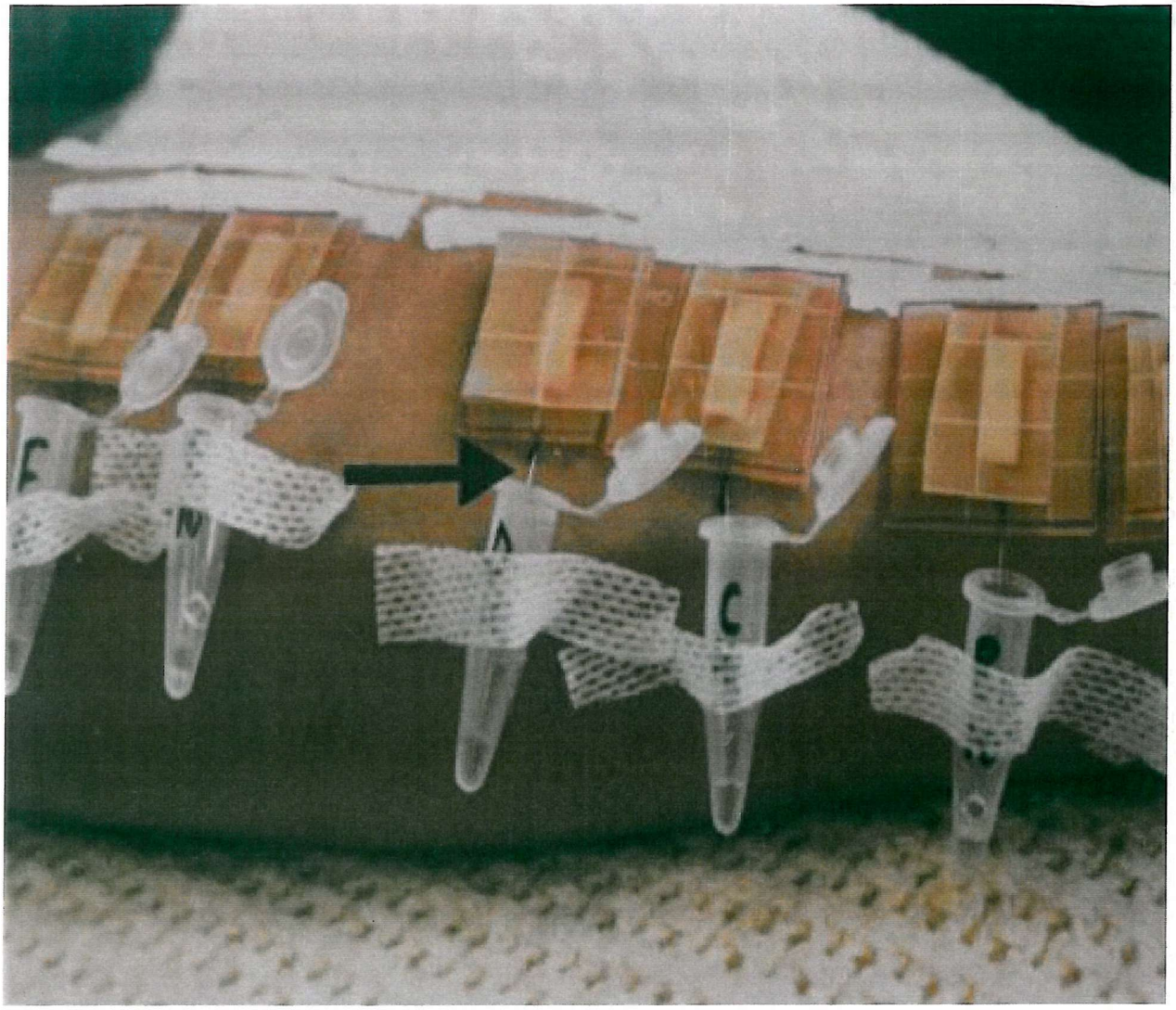


Figure 2.4. Photograph of 5 microdialysis fibres that have been inserted into the volar surface of the forearm. Penciclovir cream in a drug well has been applied to the skin overlying each fibre. The black arrow is pointing to a microdialysis fibre as it emerges from its exit point in the skin. The dialysate is collected in labeled eppendorf tubes.

Sample Analysis

1. Dialysate analysis

The collected dialysates were analyzed within 72 hours by isocratic high performance liquid chromatography (HPLC). To each PCV sample, 1000ng of ACV internal standard was added and the sample vortexed. Samples were evaporated under a stream of nitrogen gas at 65°C. The residue was reconstituted in 100μl of mobile phase and vortexed again. PCV standards from 0.5ng to 1000ng in duplicate were treated in the same way. Mobile phase was 2% methanol and 98% double distilled water with the pH reduced to 4.3 using glacial acetic acid. 90μl aliquots were injected into the HPLC. A 250mm x 4.6mm C8 Prodigy column (5 micron particle size (Phenomenex, UK)) was used and kept at a temperature of 27°C. Both ACV and PCV were detected at a wavelength of 254nm and peak heights were used for quantification. Retention time was 16 minutes for PCV and 10.5 minutes for ACV. The limit of quantification was 0.05ng.

For ACV analysis, 1000ng of PCV was added as an internal standard. The extraction was as for penciclovir, however the mobile phase was 3% methanol and 97% double distilled water containing octanesulphonic acid at pH 4.3. Using the same column and UV wavelength, ACV retention time was 6 minutes and PCV 10 minutes. The limit of quantification was 0.5ng. A different mobile phase was used for aciclovir because of the presence of several small interfering peaks at the 10.5 retention time with the penciclovir assay. These would have confused the identification of low quantities of ACV. They did not affect the identification of ACV (1000ng) when used as an internal standard for the penciclovir assay. The Millennium software package (Waters, UK) was used to analyse HPLC traces.

2. Blood sample analysis

The solid phase extraction and assay of serum samples for PCV was taken from that described by Fowles and Pierce (Fowles *et al*, 1989). 500µl aliquots of plasma were spiked with 1000ng of aciclovir internal standard. 520µl of 16% trichloroacetic acid was added and the solution centrifuged at 3000rpm for 5 minutes. Cationic exchange columns (SCX 3ml) were primed with 1ml of methanol and 1ml of 1M disodium hydrogen phosphate buffer. The supernatant from centrifugation was added to the columns and washed with a further 1ml of disodium hydrogen phosphate buffer. Analytes were eluted with 1ml 0.25M methanol-potassium phosphate buffer (20:80% v/v). Samples were evaporated under a stream of nitrogen gas at 65°C and reconstituted in mobile phase as described in the penciclovir assay method above.

A standard curve was run with each set of study samples. Standards were 0ng, 0.5ng, 1ng, 2ng, 10ng, 100ng, 1000ng and were run in duplicate. There was always a very close correlation between amount of standard and the ratio of standard to internal standard, with $r^2 \approx 0.99$. (See appendix for examples of standard curves for PCV and ACV).

Transepidermal Water Loss

Transepidermal water loss was measured using a Tewameter TM 210® (Courage & Khazaka UK). This consists of a probe weighing 25g that contains a cylindrical open chamber measuring system. Measurements were taken according to 'The Guidelines for TEWL Measurement'; with the subject at rest in a closed room with no possibility of air circulation (such as doors opening, draught from open windows, movement of other persons within the room) affecting the results, and an ambient room temperature of 20°C to prevent thermal sweating (Pinnagoda *et al*, 1990). Care was taken that the subject was not lying in direct sunlight and that each subject was aware that they had to lie very still whilst readings were being taken. Following a 15 minute self-calibration phase, measurements were taken by gently resting the probe head on the area of skin of interest - this positions the two sensor units of the Tewameter at 2 and 4mm from the skin surface. Care was taken to not press the probe head too firmly against the skin as this can falsely increase the reading. Continuous measurements were taken for 90 seconds and the mean of the last 20 seconds of recording calculated. At three monthly intervals (suggested by manufacturer guidelines) the Tewameter was calibrated using an *in vitro* constant water evaporation device (manufacturer supplied) consisting of three known concentrations of sodium chloride. Calibration adjustments needed were always only very slight suggesting high accuracy of the Tewameter between these times.

Laser doppler perfusion imaging

Skin blood flow was imaged and measured with scanning laser Doppler imaging (LDI) (Moore Instruments Ltd, Axminster, UK). Scans 0.5 x 1.5cm were taken with the laser beam 30 cm from the skin surface. Data was analysed using Moor SLDI per 3 software.

Dermal Ultrasound

High frequency ultrasound (20Mhz) was used to measure fibre depth. Using the 2D B mode the fibre was followed along its length to ensure that its depth within the dermis was at the same level. Three measurements of fibre depth were taken using the A mode and the average calculated.

Statistics

All data are presented as mean values ± standard error of the mean (SEM). The statistical significance was calculated using one-way analysis of variance and paired student t-test. Data was considered significant if $p<0.05$. For pharmacokinetic analysis of drug absorption, area under the curve was calculated using the trapezoid rule:

$$AUC = \frac{\text{the sum of } (t_2 - t_1)(C_{p1} + C_{p2}) \text{ etc.}}{2}$$

Where t_1 and t_2 are the first two time points and C_{p1} and C_{p2} are the corresponding plasma / microdialysate concentration of drug.

Other statistical tests used were correlation coefficient and linear regression. The Microsoft Excel statistics package was used.

Chapter 3

Method Validation

Introduction

A series of experiments were carried out both *in vitro* and *in vivo* to validate microdialysis as a means of accurately sampling antiviral drug. Each step of the technique was examined to ensure that future results obtained would not be due to errors in basic experimental technique and that absorption of the antiviral drug under investigation was maximized.

This set of investigations were:

1. Assess pump accuracy
2. Assess *in vitro* fibre efficiency
3. Assess *in vivo* fibre efficiency
4. Measurement of the mean amount of drug in a drug well
5. Drug absorption with and without local vasoconstriction
6. The effect of local anaesthesia (EMLA) prior to microdialysis fibre insertion on penciclovir absorption
7. The optimal length of fibre in the dermis available for microdialysis

1. Pump flow rate accuracy

Six IVAC pumps with 10ml Terumo syringes were used for all microdialysis studies – both *in vivo* and *in vitro*. Flow rate was 0.3ml h^{-1} (5µl min^{-1}).

Individual pump accuracy was investigated *in vitro*. Perfusate (distilled water) from each pump (flow rate 0.3ml h^{-1}) was collected hourly in plastic eppendorfs over 3 ½ hours (first collection 30 minutes), and the volume measured. Evaporation of water from open eppendorfs was taken into account by filling 6 further eppendorfs with 400µl of water and calculating the loss of water after one hour.

Results

Mean percentage loss of water per eppendorf by evaporation in one hour was 4.3% by volume. Pump accuracy was lowest in the first 30 minutes of collection, with all pumps overperfusing fluid (1.9% to 54.7% inaccuracy (mean 31.2%)). Pump accuracy improved after the initial 30 minutes to give a mean percentage volume of water per hour of 92.34% that expected (range 86.4 to 98.5%) (0.28ml/h).

Measurement of fibre efficiency.

Fibre efficiency was measured both *in vitro* and *in vivo*:

2. Measurement *in vitro* of efficiency of dialysis

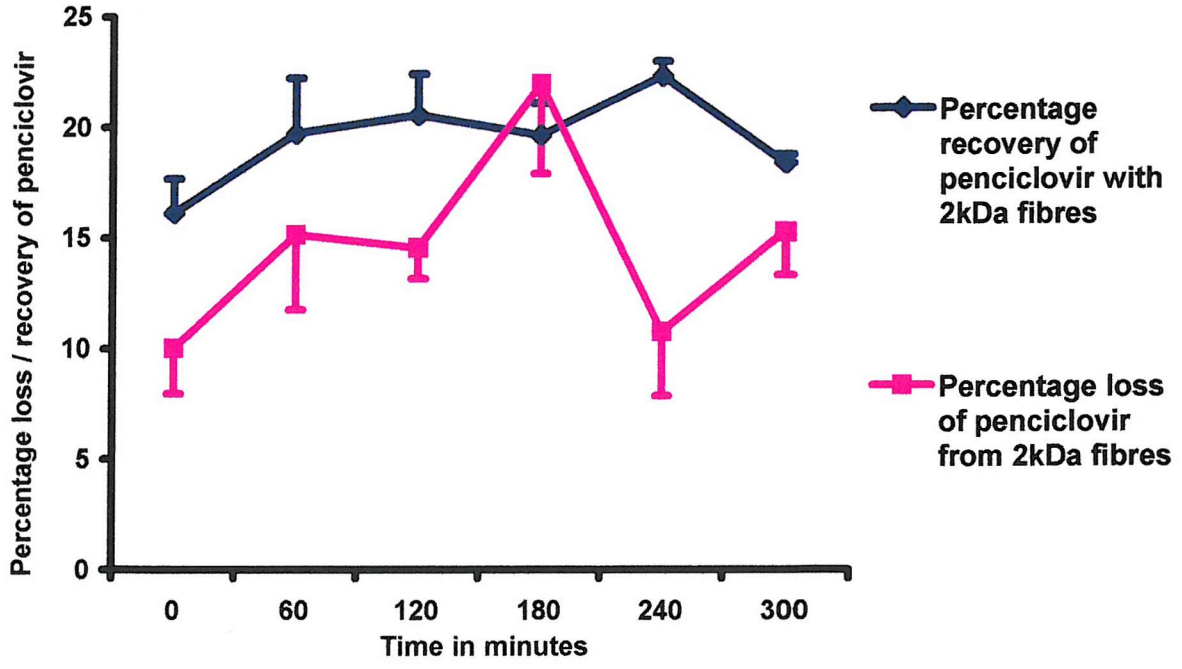
Fibre efficiency for recovery or loss of drug was determined *in vitro* to ensure that the fibres used would give reproducible results. The efficiency of drug recovery for 2kDa molecular cutoff fibres was determined by immersing the fibres in a Petri dish containing a 50µg ml^{-1} solution of

PCV. The fibres were perfused with Ringer's solution at a rate of 5μl/minute and dialysate collected hourly for 5 hours (5 fibres). Conversely loss from the fibres was obtained by immersing the fibres in a Petri dish of Ringer's solution and perfusing the fibres with a PCV solution of 50μg ml^{-1} (4 fibres). Dialysate was collected hourly for 5 hours.

Results

Under these conditions the 2kDa fibres had a relative recovery for PCV of 19.4 ± 0.8% (SEM), and relative loss of 15.7 ± 1.3% (SEM) (difference not significant) (Figure 3.1).

Figure 3.1 Percentage recovery and loss of penciclovir from 2Kda fibres *in vitro.* Error bars are standard error of the mean



3. Measurement *in vivo* of loss of penciclovir from perfusate to extracellular space.

In order to assess that there is movement of PCV between microdialysate and the extracellular fluid, six fibres were inserted into the volar surface of the forearm of one subject. Penciclovir sodium was dissolved in Ringer's solution at a concentration of 15μg ml^{-1} and perfused through the fibres at a rate of 5μl min^{-1} (0.3ml h^{-1}). The pumps were run for 30 minutes to allow flow rates to equilibrate prior to the first hourly collection. Dialysate was collected hourly for 5 hours and analysed for PCV content. This was compared with the concentration of PCV in the perfusate to obtain a measure of PCV loss *in vivo* and hence an indirect measurement of PCV recovery.

This method is based on the assumption that there is equal diffusion of drug from the perfusate within the microdialysis fibre to the extracellular fluid and *vice versa* (Stahle, 1991).

Results

The mean volume of dialysate collected per hour was 0.265ml (SEM 0.005). Taking into account fluid loss by evaporation (4.3%), the mean volume of dialysate collected hourly was 0.28ml h^{-1}. The mean percentage loss of PCV from the perfusate to the extracellular fluid was 24% (SEM 2.2%). For each fibre the loss of PCV per hour was very similar in 4 fibres with steady state maintained at approximately a loss of 26% (figure 3.2). The other fibres had similar losses of PCV for the initial 90 or 150 minutes of the study, and then the percentage loss of PCV to the extracellular fluid was reduced. Fibre depth did affect loss of PCV (figure 3.3); the correlation coefficient for the percentage loss of PCV per hour and fibre depth was 0.79. The more superficially positioned fibres demonstrated a reduced loss of PCV to the extracellular fluid than the more deeply positioned fibres.

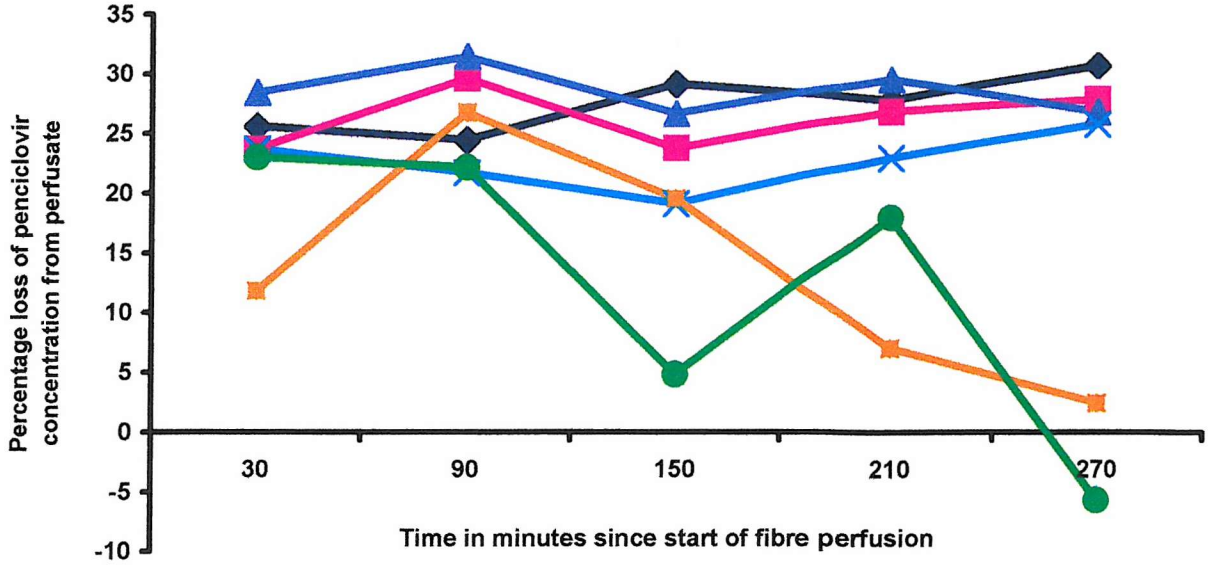


Figure 3.2 The percentage loss of penciclovir from six 2kDa microdialysis fibres to the extracellular fluid *in vivo* in one subject. Mean percentage loss of penciclovir per fibre per hour was 24%. The two fibres that are not consistent in penciclovir loss (green and orange lines) are those which were sited more superficially in the dermis.

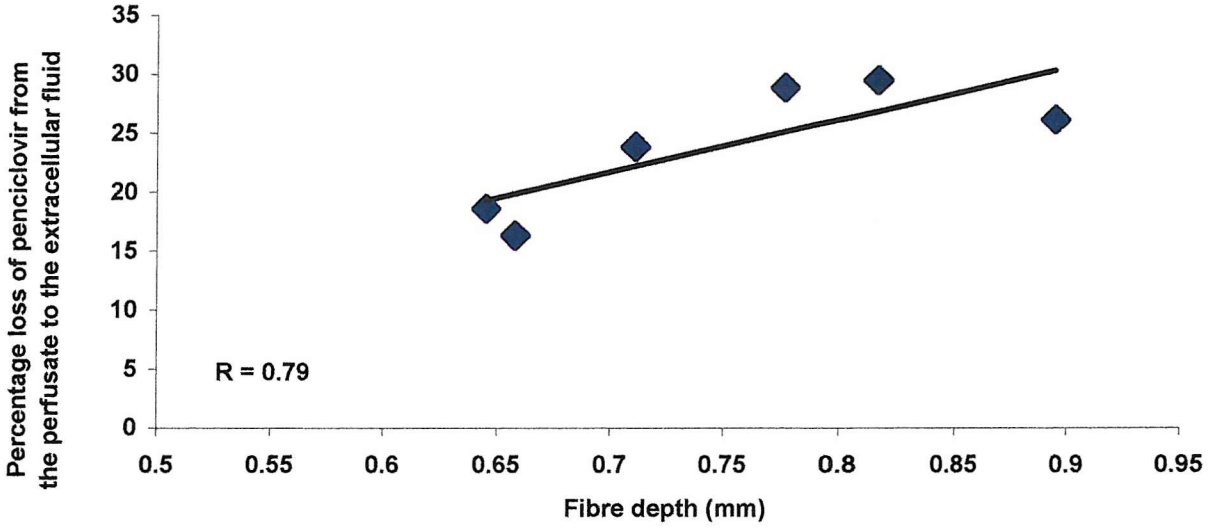


Figure 3.3 Fibre depth and the percentage loss of penciclovir per hour from the perfusate to the dermal extracellular fluid in one subject.

4. Measurement of amount of drug in skin surface drug well

For all percutaneous absorption studies, the antiviral drug of interest was applied to the skin in a drug well. Each drug well was made by cutting out a 2cm x 2cm square piece of Comfeel® ulcer dressing, with a rectangular area (dimensions 5mm x 15mm x 2mm) cut out of the center of the square. Comfeel® ulcer dressing is 2 mm thick and has an adhesive coated surface by which it is attached to the skin. Following application of the dressing to the forearm, the central cavity was filled with antiviral drug – usually as the commercially produced Vectavir (1% penciclovir cream) or Zovirax (5% Aciclovir cream). The drug well was then occluded with a lid 2cm x 2cm – also made of Comfeel® dressing. The mean amount of drug present in each drug well (and potentially absorbable through the skin) was calculated by filling six drug wells (weight predetermined) with penciclovir or aciclovir cream (Vectavir / Zovirax). Any excess was removed and the combination of drug well and cream weighed.

Results

The mean amount of antiviral cream per well was 87.1mg (SEM 3.8mg). Vectavir has a penciclovir concentration of 1% whereas Zovirax contains 5% aciclovir. The total amount of drug potentially absorbable from each drug well was 870μg for penciclovir and 4350μg for aciclovir.

5. Penciclovir absorption through normal skin with and without local vasoconstriction

As PCV is a hydrophilic drug it may be expected to be poorly absorbed through the stratum corneum. PCV absorption through the gut mucosa is very low which is why penciclovir is given orally as the more lipid soluble pro-drug famciclovir (Alrabiah *et al*, 1996). Recovery of

substances by microdialysis can be maximized by shutting down local blood flow. This reduces the clearance of drug from the dermis and so provides a higher dermal concentration of drug to diffuse through the microdialysis membrane.

The effect of local vasoconstriction on recovery of PCV was investigated in 4 subjects. Each had 2 fibres perfused with Ringer's solution, and 2 with 5×10^{-4} % noradrenaline in Ringer's solution (8 fibres per variable). As before, flow rate was 5μl min^{-1}. Following a ninety minute recovery period, PCV cream was applied topically and dialysate collected hourly for 5 hours.

Results

5×10^{-4} % noradrenaline in Ringer's solution caused vasoconstriction in the skin visible up to 2mm either side of the fibre as it traversed the dermis parallel to the skin surface. Without noradrenaline induced vasoconstriction recovery of PCV was extremely low with PCV detected in dialysate from only 3 out of the 8 fibres. The mean area under the curve (AUC) for the eight fibres was 0.75ng $ml^{-1}h^{-1}_{(0\text{-}5)}$ (SEM 0.5). Recovery of PCV in the presence of vasoconstriction was significantly higher ($p<0.05$ two tailed t test), with a mean AUC of 8.4ng $ml^{-1}h^{-1}_{(0\text{-}5)}$ (SEM 3.5).

6. The effect of EMLA cream on percutaneous absorption

In all microdialysis studies *in vivo*, topical EMLA cream (2.5% lidocaine and 2.5% prilocaine) was applied under occlusion to the volar surface of the forearm for 90 minutes before the study started. It is recommended by the manufacturers of EMLA cream (Astra Pharmaceuticals Ltd), that, following removal of the cream, the skin is cleaned with alcohol. Alcohol is likely to affect

percutaneous absorption as it alters the stratum corneum barrier through lipid reorganization. This property is utilized in many topical formulations where ethanol is added to the vehicle as a penetration enhancer (Berner *et al*, 1989; Williams *et al*, 1992). Therefore in all studies the EMLA was removed by gently wiping the skin first with dry, then damp and then dry again tissue paper. This method could leave a film of cream on the skin that is not obvious visually but which might affect percutaneous absorption. In addition, application of EMLA might alter percutaneous absorption by the cream (oil in water vehicle) causing increased tissue hydration. Lidocaine and prilocaine cause a biphasic vascular response; an initial vasoconstriction that is maximal after 90 minutes of application, and vasodilatation seen with prolonged application (>3 hours) (Bjerring *et al*, 1989). This will affect tissue clearance of drug by dermal blood flow. In view of these three ways in which EMLA cream can potentially influence the movement of molecules through the skin, it was essential to measure the effect of EMLA anaesthesia on antiviral drug absorption.

In three subjects (2 female), two microdialysis fibres were inserted under EMLA anaesthesia, and two without anaesthesia. 5×10^{-4} % noradrenaline in Ringer's solution was perfused through the fibres at a rate of 5μl min^{-1}. A ninety-minute recovery period was allowed for resolution of fibre insertion hyperaemia and for stabilization of pump flow rates. After the recovery period, penciclovir cream was applied in drug wells over each fibre and dialysate collected hourly for 3 hours.

Results

Noradrenaline was added to the perfusate to cause local vasoconstriction in the area immediately surrounding each fibre. This will minimize drug clearance (by cutaneous blood flow) from the

dermis, and maximize drug recovery by microdialysis. Penciclovir recovery both in the presence and absence of topical anaesthesia was extremely low (figure 3.4). Mean AUC of PCV dialysed over 3 hours with EMLA anaesthesia was 1ng $ml^{-1}h^{-1}{}_{(0-3)}$ (SEM 0.5), without EMLA the mean AUC was 0.9ng $ml^{-1}h^{-1}{}_{(0-3)}$ (SEM 0.2). This difference was not significant.

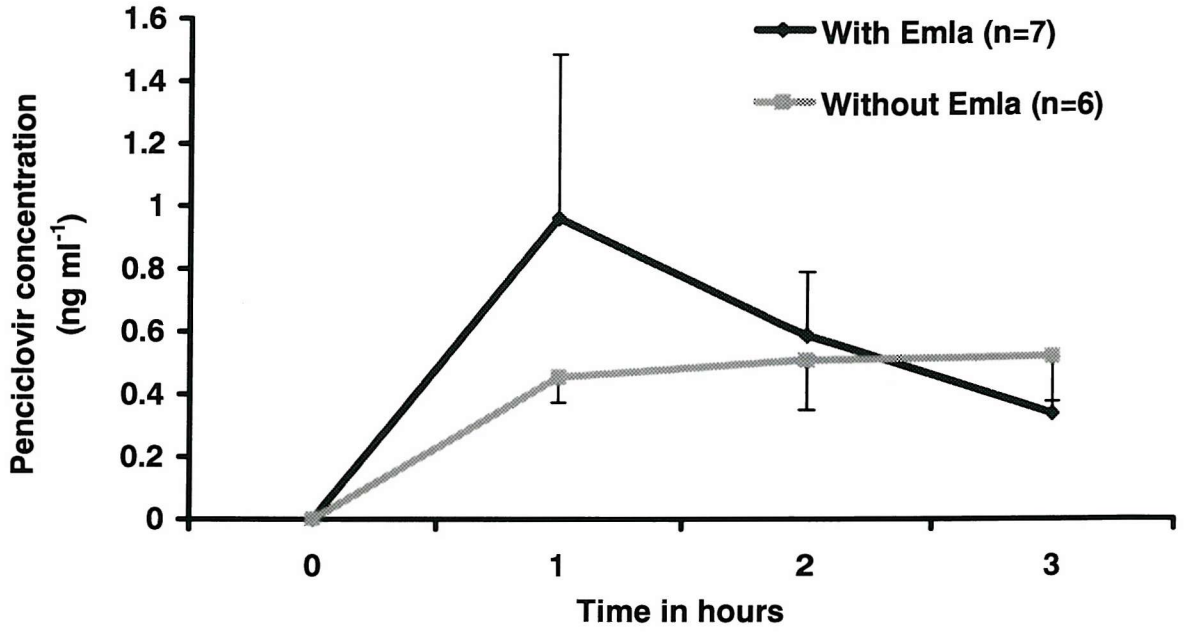


Figure 3.4 The effect of EMLA cream on penciclovir recovery in three subjects (n = number of fibres). Error bars are SEM. The absorption of penciclovir with and without EMLA was very similar.

7. Length of membrane accessible to microdialysis

Microdialysis fibres were inserted in all studies using 3cm long 23 gauge needles. Therefore the length of membrane inserted in the dermis accessible for microdialysis can be up to 3cm. In order to assess the optimum length of fibre for dialysis, in one subject three fibres were inserted for 2cm through the dermis and a further three fibres for 3cm.

As previously, the perfusate was 5 x 10^{-4} % noradrenaline in Ringer's solution at a flow rate of 5μl min^{-1}. PCV was applied topically in drug wells after 90 minutes recovery and dialysate collected hourly for 5 hours.

Results

Use of the full 3cm needle length for fibre insertion did result in slightly more tissue trauma as the skin abutted the plastic needle hub. There was no statistically significant difference in the concentration of PCV collected from the 2cm or 3cm membrane lengths; AUC for PCV collected from the 2cm fibres was 10ng $ml^{-1}h^{-1}$ $_{(0-5)}$ (SEM 3.25), and for the 3cm fibres 16.5ng $ml^{-1}h^{-1}$ $_{(0-5)}$ (SEM 6.7).

Discussion

The IVAC pumps used required 30 minutes to stabilize to a constant flow rate. Once equilibrium had been reached, pump accuracy was 92.3%. However, after 90 minutes pump accuracy was 96.15% (range 90.64% to 99.96%). Therefore at a set flow rate of 5μl min^{-1} the pumps would deliver 4.81μl min^{-1}. The inter-pump difference in efficiency was 9.32% which was considered acceptable.

Studies *in vivo* using laser Doppler perfusion imaging have shown that skin perfusion levels have returned to near baseline at 60 minutes post insertion of microdialysis fibres (Anderson *et al*, 1994). In view of these two factors both necessitating a recovery period, it was decided to allow 90 minutes between fibre insertion and the application of drug.

The 2 kDa microdialysis fibres yielded reproducible results for both *in vitro* PCV recovery and loss studies. This is extremely important for interpretation of any further microdialysis work. As *in vivo* recovery may differ from *in vitro* recovery it is important to complete fibre validation work for the substance of interest *in vivo*. Without knowing the absolute concentration of drug in the extracellular fluid of the dermis it is not possible to measure an accurate *in vivo* recovery for PCV. Hence the method was used of measuring PCV loss to the extracellular fluid as an indirect approach for measuring recovery.

A low flow rate of 5μl min^{-1} was used to allow the Ringer's solution to equilibrate with the dermal extracellular fluid. It is of interest that the volume of fluid collected per hour is the same both *in vivo* and *vitro* (0.28ml h^{-1}). Therefore, the volume exchange of fluid between the perfusate and petri dish solution *in vitro* is the same as that *in vivo* between the dialysate and dermal extracellular fluid. As a physiological solution (Ringer's) has been used in all microdialysis studies, it is anticipated that the movement of drug into the microdialysis perfusate will be by osmosis (diffusion along a concentration gradient), with free movement of solute both in and out of the membrane.

The mean percentage loss of PCV *in vivo* was greater than *in vitro* (24% versus 15.7%), however there was no significant difference between fibre recovery *in vitro* and fibre loss *in vivo* (19.4% versus 24%). Fibre depth affected the loss of PCV, with the most superficial fibres having the lowest degree of loss of drug. Despite the range of fibre depths being narrow

(0.645mm to 0.895mm), there is a correlation coefficient of 0.79 between percentage loss of PCV per hour and fibre depth. This difference is likely to be due to the position of the dermal capillaries which will clear PCV from the area immediately next to the fibres thus setting up a higher concentration gradient which will enhance drug loss from the fibres. In this subject the dermal capillaries may be situated below 0.7mm thus accounting for the very similar loss of drug with time of the four deeper fibres; the two more superficial fibres causing relatively greater saturation of the dermis surrounding the fibres, resulting in a reduction in the loss of PCV with time.

Although *in vitro* study of fibres is vital to ensure that relative recovery will be consistent for any number of manufactured fibres, they do not give an absolute value for the percentage of drug present in the interstitial space that will be sampled *in vivo.* If an absolute sampling percentage was known, then by regression, the absolute concentration of drug present in the extracellular space could be calculated. Here we have seen a proportionately greater amount of PCV lost from the perfusate to the surrounding fluid/tissue in fibres implanted *in vivo* as compared with the *in vitro* studies. This may be due to blood flow clearing absorbed drug and also due to some of the PCV binding to protein. Both these factors will inhibit the movement of PCV through the 2kDa fibre pores *in vivo.*

It is interesting that the *in vitro* PCV recovery and loss (19.4%, 15.7%) and *in vivo* PCV loss percentages (24%) are not dissimilar and therefore it is probable that PCV recovery *in vivo* will be in the region of about 20%. However without a definite figure for PCV recovery it means that it will not be possible to characterize PCV absorption under the different circumstances described in this thesis in terms of 'dermal concentrations' but rather in terms of

'concentration dialysed across the fibre' or 'concentration recovered' which will enable direct comparisons to be made under the different conditions.

To obtain meaningful data about the percutaneous absorption of antiviral agents, cutaneous blood flow needs to be minimized by the addition of noradrenaline to the perfusate; when the effect of noradrenaline-induced vasoconstriction on recovery of penciclovir by microdialysis was examined, with local vasoconstriction there was an increase in PCV detection of 11.5 fold. As expected, with normal physiological blood flow, the clearance of absorbed PCV was high enough that recovery of drug by microdialysis was below the assay threshold at all time points in 5 out of the 8 fibres inserted. As ACV has a similar structure and water solubility to PCV, it would be expected to be absorbed (and cleared) to a comparable degree. Therefore, it was essential to conduct all further *in vivo* studies with a noradrenaline perfusate.

EMLA anaesthesia does not significantly affect the percutaneous absorption of PCV. In three subjects, the mean area under the curve of PCV recovered over three hours was very similar both with and without topical anaesthesia. This is contrary to what may be anticipated: It is known that application of a cream under occlusion will hydrate the stratum corneum (Bucks *et al*, 1989). Increased skin hydration enhances percutaneous absorption (Katz *et al*, 1971). In addition, when applied for 90 minutes both lidocaine (lignocaine) and prilocaine cause localized vasoconstriction which reduces drug clearance. It is likely that during the 90 minute recovery period, the effects of increased hydration and cutaneous vasoconstriction will have worn off sufficiently to no longer affect drug absorption and clearance significantly. Further studies (see chapter 10), have demonstrated that following the removal of an aqueous gel from the skin, transepidermal water loss as a measurement of barrier integrity is high but falls to normal levels within 30 minutes. Hence the effect of increased stratum corneum hydration following

application of a cream under occlusion is temporary. In conclusion, insertion of fibres under topical EMLA anaesthesia does not affect drug recovery rates by microdialysis – provided a ninety minute recovery period is allowed. Using anaesthesia ensures that the insertion of microdialysis fibres into the skin is pain-free which is vital for volunteer tolerance of the technique and future recruitment.

In the presence of noradrenaline-induced local vasoconstriction, a 3cm length of membrane for microdialysis resulted in a higher amount of PCV recovered than the 2cm dialysis length. This is as expected as there is a higher surface area of membrane available for diffusion of solute. However the difference was not significant and very low concentrations of PCV were dialysed. As insertion of the 3cm length requires the use of the full length of the needle with the possibility of slight tissue damage it was decided to use 2.5cm as the optimal fibre length in the dermis for dialysis. This allows a clearance of 0.5cm on either side between the fibre entry and exit points and the contents of the drug well. There is also 2 to 3 mm between the external edge of the drug well and fibre entry and exit. By avoiding contact with the drug well there is a much lower chance of fibre and dialysate contamination with the drug under investigation.

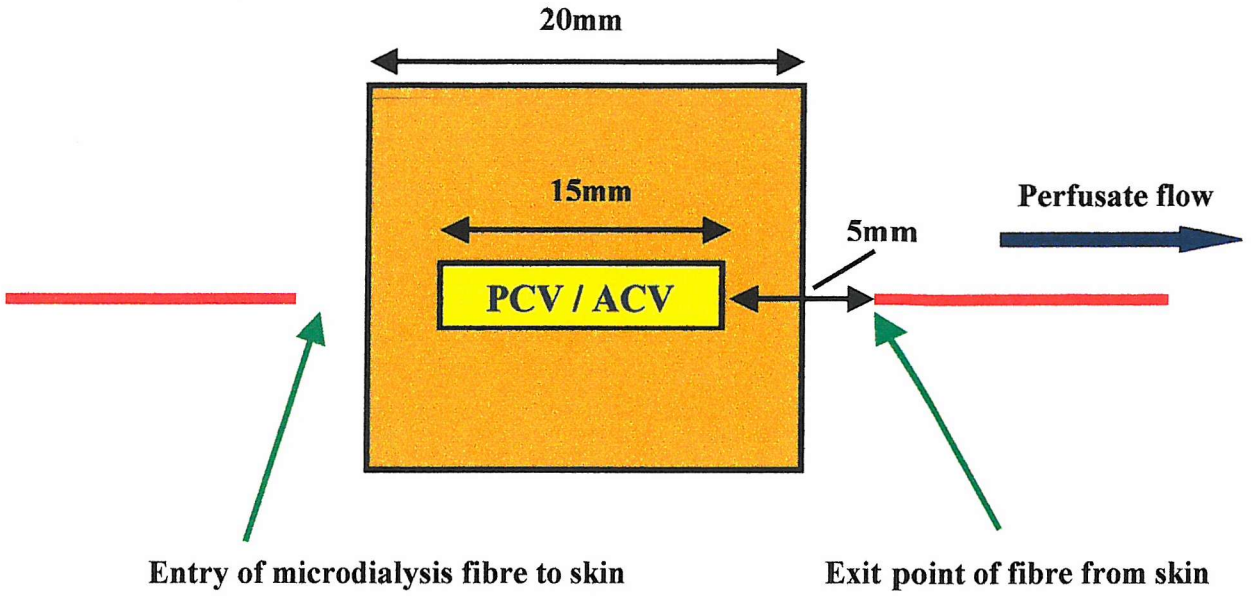


Figure 3.5. Diagram of the dimensions of the drug well (orange square) demonstrating the 5mm distance between topically applied PCV or ACV in the drug well (yellow) and the microdialysis fibre skin entry and exit points. The microdialysis fibre is depicted by the red line and extends through the dermis for 25mm.

Here several of the key aspects in the technique of cutaneous microdialysis have been tested to ensure consistency of equipment performance and to assess and validate the proposed protocol. In summary, it has been found that at a flow rate of 5μl minute^{-1}, 2kDa fibres with indwelling steel wire will collect in the region of 20% of available penciclovir from the dermis *in vivo*. EMLA cream can be used for fibre insertion without affecting drug absorption and a 90 minute recovery period allows the microdialysis technique to settle down prior to the start of the

study. Noradrenaline-induced vasoconstriction will enhance drug detection which is essential for the study of a poorly absorbed drug such as PCV. A 2.5 cm intradermal length of membrane gives an adequate dialysis surface area without causing excess damage to the skin, and the Comfeel® drug wells hold a consistent weight of drug resulting in similar concentrations of drug (within the same formulation) available for absorption studies.

Chapter 4

The percutaneous absorption of hydrophilic drugs through normal skin

Introduction

In this chapter the experiments carried out to investigate the absorption of hydrophilic drugs through normal skin and the effect of vehicle are described. Absorption of PCV from its commercial form Vectavir was extremely low in 4 subjects (section 5, chapter 3). Without noradrenaline-induced vasoconstriction PCV was below the detection threshold in most fibres. In order to test the validity of this initial observation the absorption of PCV through normal skin was examined in a considerably higher number of subjects. The degree of inter-subject variation was looked at, including the effects of age and sex on percutaneous absorption. The absorption of ACV through normal skin was examined in a further 8 subjects for comparison with PCV. In addition the effect of the mechanical pertubation of rubbing in Vectavir was assessed.

Having established a class effect for the absorption of anti-herpes topical therapy, the role of vehicle was examined and the penetration enhancers dimethyl sulphoxide, propylene glycol, sodium lauryl sulphate and ethanol assessed for their ability to promote PCV absorption. As hydration of the skin can increase permeation of drugs, white soft paraffin was rubbed in to normal skin and PCV absorption measured.

1. Absorption of penciclovir through normal skin

The absorption of PCV through normal skin was examined in 29 subjects (14 male), 45 fibres in total were inserted. In all subjects the perfusate was Ringer's solution containing 5×10^{-4} % noradrenaline. At the end of the ninety minute period for pump stabilisation , 1% penciclovir cream (Vectavir) was applied to the skin in drug wells. Dialysate was collected hourly for 5

hours. At the end of the study, individual fibre depth in the dermis was measured using ultrasound.

Results

Recovery of PCV by microdialysis was followed over time: Concentrations rose to reach a steady state at 120 minutes with 2.8ng ml^{-1} (± 0.5) (SEM) collected per hour (figure 4.1). The steady state persisted for the rest of the 5 hour experiment. The mean highest concentration of drug collected in one hour (C_{max}) was 8.5 ± 2.3 ng ml^{-1}, collected at mean time 175 ± 15 minutes (T_{max}). The mean area under the curve was 14.55 ± 3.3 ng ml^{-1} $h_{(0-5)}$.

The 29 subjects were aged between 20 and 65 (mean age 28 ± 2). There was no relationship between subject age and PCV recovered (correlation coefficient was –0.047). Mean area under the curve of PCV collected in male subjects was 15.67 ng ml^{-1} $h_{(0-5)}$, and in females 12 ng ml^{-1} $h_{(0-5)}$, this difference was not significant (ANOVA).

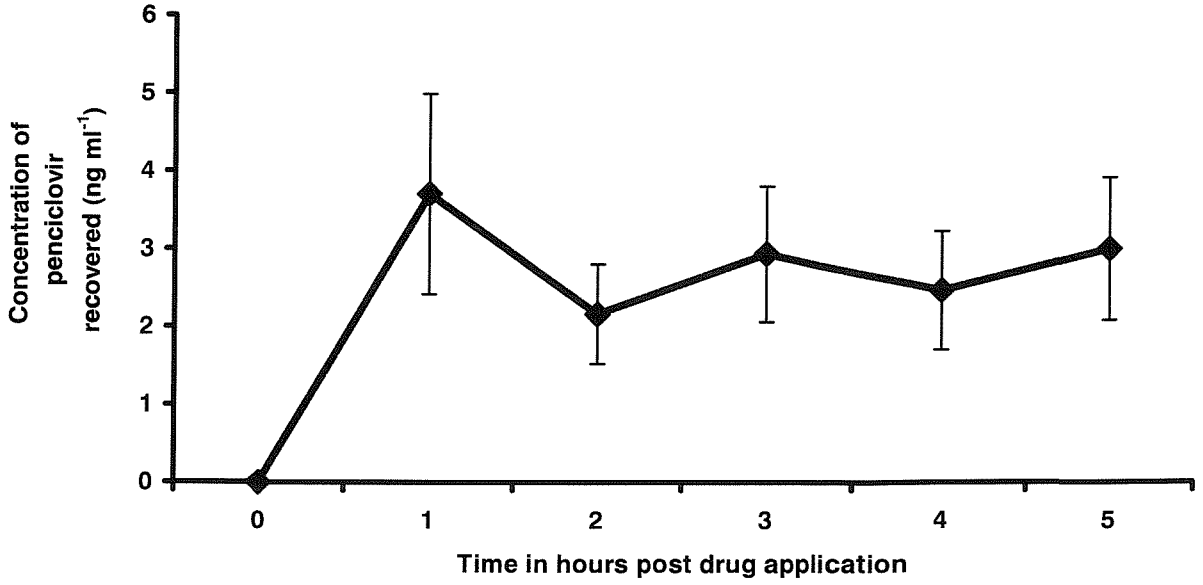


Figure 4.1 Recovery of penciclovir through normal skin with local vasoconstriction in 29 subjects. Error bars are SEM.

2. Absorption of aciclovir through normal skin

The absorption of ACV through normal skin was investigated in 8 subjects (6 male), with the insertion of 16 fibres in total. Experimental protocol was as for PCV with the exception that 5% aciclovir cream (Zovirax) was applied to the skin in drug wells.

Results

The recovery of ACV over 5 hours was approximately double that of PCV; mean area under the curve (AUC) was 33 ± 10.5 ng ml^{-1} $h_{(0-5)}$ and C_{max} was 19.5 ± 6 ng ml^{-1} (Figure 4.2, Table 4.1) Although the average time at which peak concentration was collected (T_{max}) was 180 ± 23 minutes, this showed great inter-subject variation (range 60 to 300 minutes). There was also a moderate degree of intra-subject variation with T_{max} occurring at the same time point for both fibres in only 3 out of the 8 subjects. Steady state was reached at 180 minutes, with 5.5 ± 1.7ng ml^{-1} collected per hour. The 8 subjects were aged from 21 to 47 (mean 27). As with PCV absorption there was no relationship between subject age and ACV absorption (correlation coefficient 0.03).

	PENCICLOVIR (Vectavir)	**ACICLOVIR (Zovirax)**
Concentration of cream applied topically	1%	5%
T_{max} (± SEM)	175 ± 15 minutes	180 ± 23 minutes
C_{max} (± SEM)	8.5 ± 2.3 ng ml^{-1}	19.5 ± 6 ng ml^{-1}
Area under the curve	14.55 ± 3.3 ng ml^{-1} $h_{(0-5)}$	33 ± 10.5 ng ml^{-1} $h_{(0-5)}$
Time steady state reached	120 minutes	180 minutes
Concentration at steady state (± SEM)	2.8ng $ml^{-1}h$ (0.5)	5.5ng $ml^{-1}h$ (1.7)

Table 4.1. Summary of the pharmacokinetics of penciclovir and aciclovir recovery by microdialysis (with local vasoconstriction) following absorption through normal skin.

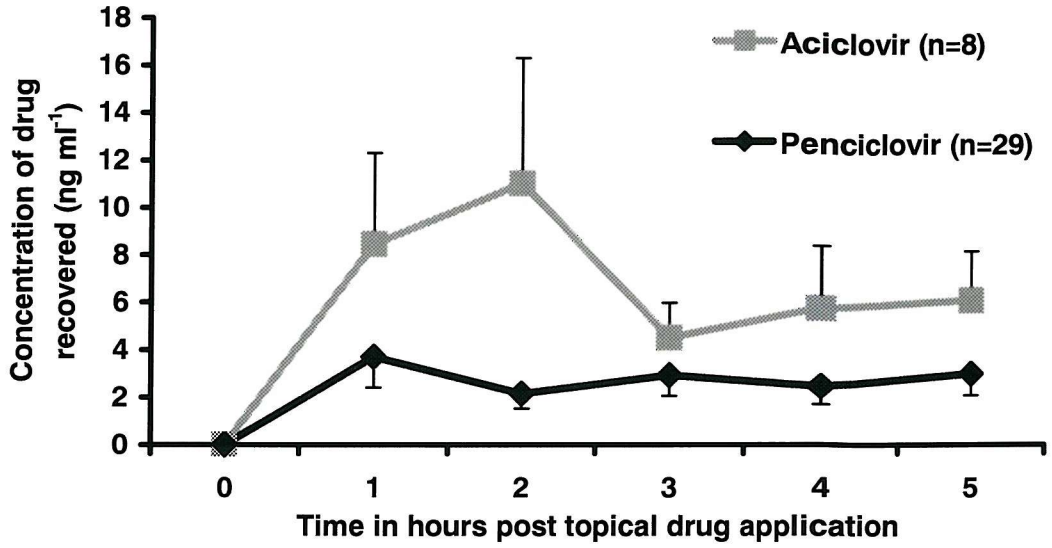


Figure 4.2. The recovery by microdialysis of penciclovir and aciclovir following absorption through normal skin, in the presence of localized vasoconstriction. Error bars are SEM.

3. The effect of rubbing in penciclovir

In one subject the effect of rubbing Vectavir into normal skin was examined. Following the insertion of 6 fibres, drug wells (with internal dimensions of 1.5 x 1.5cm) were applied to the skin. In four of the wells 50mg of Vectavir was rubbed into the skin (using a finger tip) until it disappeared. Two of these wells were then refilled with Vectavir and the top occluded, whilst the other two were just occluded with a drug well lid. The two remaining fibres acted as controls with Vectavir applied to normal skin (with no rubbing in) contained within drug wells. As before, to maximize PCV recovery, cutaneous blood flow was restricted by the addition of noradrenaline ($5x10^{-4}$%) to the Ringer's solution perfusate.

Results

Rubbing in PCV did not increase its absorption. There was no significant difference between the three different conditions surrounding PCV absorption. The mean AUC of penciclovir collected by microdialysis per pair of fibres was:

Penciclovir rubbed in and drug well filled	5.1 ng ml^{-1} $h_{(0-5)}$
Penciclovir rubbed in and skin occluded	6.2 ng ml^{-1} $h_{(0-5)}$
Control fibre	8.6 ng ml^{-1} $h_{(0-5)}$

4. The role of vehicle on penciclovir absorption

Having established that in its commercially produced form (Vectavir), PCV absorption through the skin was extremely low, a series of experiments were performed to assess if absorption could be promoted by the addition of penetration enhancers. As the addition of substances to a commercially prepared formulation may affect the drug's solubility, polarity and ease with which it can partition from the vehicle, the penetration enhancers were tested by adding them to a solution of PCV in aqua gel to produce overall a 1% suspension of PCV. The absorption of PCV when applied in an aqua gel vehicle was assessed prior to penetration enhancer studies.

a. Absorption of penciclovir through normal skin when applied topically as 1% penciclovir in aqua gel.

In 6 subjects, sodium penciclovir salt was added to aqua gel to make a 1% suspension. In the presence of local vasoconstriction, the absorption of PCV was measured over 5 hours in 10 fibres.

Results

The mean AUC of PCV dialysed was 13.8 ± 3.7 ng $ml^{-1}h_{(0-5)}$. This is very similar to the mean AUC of PCV absorbed from Vectavir (14.55 ± 3.3 ng ml^{-1} $h_{(0-5)}$). Therefore a simple aqua gel can be considered just as effective a vehicle for PCV as the exipients of Vectavir and is a suitable medium to which to add penetration enhancers to assess their activity on enhancing the absorption of PCV. (Full details of ingredients in Vectavir and Zovirax in appendix).

b. Dimethyl sulphoxide (DMSO)

Before adding DMSO to Vectavir or a suspension of PCV in aqua gel it was necessary to assess the irritancy of different concentrations of DMSO on the skin:

Three concentrations of DMSO in aqua gel (30%, 50% and 70%) were applied to the volar surface of the forearm in a drug well and the drug well occluded. After 30 minutes the drug well was removed and the skin was examined.

There was erythema and discomfort experienced at the 70% DMSO site, the other two concentrations did not cause any skin irritation. Leaving the 50% and 30% concentrations on the skin for up to 5 hours also did not cause any irritation. Application of 100% DMSO cause marked skin irritation and burning within one minute.

Following these preliminary studies up to a 50% concentration of DMSO was used for microdialysis PCV penetration experiments:

In one subject, following the insertion of 6 microdialysis fibres, the absorption of 1% penciclovir from 50% and 20% DMSO in aqua gel (with 1% PCV in aqua gel as a control) was examined over 5 hours. In a second subject, 5% and 20% concentrations of DMSO were added

to Vectavir and dialysis carried out for 5 hours. Both studies were with a noradrenaline perfusate to minimize the clearance of absorbed drug.

Results

There was no significant difference in the concentration of PCV dialysed from 20% and 50% concentrations of DMSO as compared with 1% PCV in aqua gel alone. The highest concentration of DMSO (50%) resulted in the least amount of PCV collected (mean AUC (1ng ml^{-1} $h_{(0-5)}$). The mean AUC for 20% DMSO was 1.6ng ml^{-1} $h_{(0-5)}$ and for 1% PCV in aqua gel the AUC was 2.6ng ml^{-1} $h_{(0-5)}$.

Adding DMSO to Vectavir resulted in similarly low concentrations of PCV recovery: 20% DMSO AUC 2.6ng ml^{-1} $h_{(0-5)}$; 5% DMSO AUC 1.2ng ml^{-1} $h_{(0-5)}$ and the Vectavir control AUC was 3.4ng ml^{-1} $h_{(0-5)}$.

None of the concentrations of DMSO used caused skin irritation.

c. Sodium lauryl sulphate (SLS)

Sodium lauryl sulphate is an ingredient of Zovirax but is not present in Vectavir. As the absorption (recovery) of Zovirax is approximately double that of Vectavir, the effect of SLS on PCV absorption was examined: Sodium lauryl sulphate was added to a suspension of PCV in aqua gel in one subject. Following the insertion of 6 microdialysis fibres, 10% SLS and 2% SLS with 1% PCV in aqua gel was applied topically in drug wells over 4 fibres. 1% PCV in aqua gel was the control in the two remaining fibres.

Prior to this study, irritancy tests of direct application of SLS were carried out - with a 20% suspension not causing any adverse reactions to the skin.

Results

The addition of sodium lauryl sulphate to a 1% suspension of PCV in aqua gel inhibited rather than promoted PCV absorption. AUC for 10% SLS was 4.5ng $ml^{-1}h_{(0-5)}$, for 2% SLS it was 3.9ng $ml^{-1}h_{(0-5)}$ and for the control of PCV in aqua gel AUC was 20.4ng $ml^{-1}h_{(0-5)}$. On removal of the drug wells at the end of the study there was no evidence of skin irritation.

d. Propylene glycol (PG)

The effect of propylene glycol as a penetration enhancer was examined in one subject. Penciclovir in aqua gel was mixed with PG to produce a 25% and a 50% final propylene glycol concentration. Each mixture was applied over two fibres, with 1% PCV in aqua gel as the control. As before, dialysate was collected hourly for 5 hours for penciclovir analysis.

Results

Propylene glycol did not enhance penciclovir absorption, the concentrations of PCV recovered by dialysis were:

50% propylene glycol, AUC 0.9ng ml^{-1} $h_{(0-5)}$

25% propylene glycol, AUC 2.8ng ml^{-1} $h_{(0-5)}$

1% PCV in aqua gel, AUC 4.9ng ml^{-1} $h_{(0-5)}$

e. Ethanol

In a further subject the absorption of penciclovir in a 30% ethanol and aqua gel vehicle was examined. Over 5 hours the AUC was 2.3ng $ml^{-1}h_{(0-5)}$.

5. The effect of hydration on penciclovir absorption

The role of increased skin hydration on PCV absorption was investigated in three subjects. Although the occluded drug wells will increase tissue hydration, the effect of rubbing white soft paraffin into the skin to cause maximal hydration was assessed.

Following the insertion of 6 fibres per subject, 50mg of white soft paraffin was rubbed into the area of skin over two fibres. The area was then occluded with a 2cm by 2cm patch of Comfeel® dressing to prevent the skin from drying out. After 30 minutes Vectavir was applied to the skin overlying each fibre in an occluded drug well and dialysate collected hourly for five hours. As in all previously described studies, 5×10^{-4} % noradrenaline was added to the Ringer's solution perfusate.

Results

Hydrating the skin with white soft paraffin reduced PCV absorption: Over 5 hours the mean AUC was 3.9 ± 1.5 ng $ml^{-1}h_{(0-5)}$ (SEM) for skin hydrated by white soft paraffin and 18 ± 7.5ng $ml^{-1}h_{(0-5)}$ in the control fibres. This difference was not significant (ANOVA).

Discussion

The absorption of both PCV and ACV through normal skin when applied as their commercially produced topical formulations is extremely low. Both formulations are supersaturated with drug and in the case of ACV the surfactant sodium lauryl sulphate is added to the preparation as a penetration enhancer. Despite these measures, the high water solubility of both PCV and ACV retards their partitioning from their vehicle into the stratum corneum.

Compounds which are absorbed best across the skin have good solubility in both oils and water. Both ACV and PCV possess high water solubility but have very low solubility in oil. This is reflected in their logP (log (octanol water partition coefficient)); compounds that are well absorbed across the skin have a logP of about 2, the value for penciclovir is –2.12 and aciclovir – 1.8 (calculation using software; Advanced Chemistry Development, Inc., Toronto. Personal communication with Professor J. Hadgraft). Glucose which is also hydrophilic and absorbed poorly across the skin has a logP of minus 1.01. Therefore it is of no surprise that both ACV and PCV have poor penetrative powers when applied topically to normal skin. This is reflected in the extremely low concentration of both ACV and PCV dialysed by cutaneous microdialysis.

Over the 5 hour collection period, ACV absorption was approximately double that of PCV (both commercial topical formulations), however on examination of the standard error of the mean of drug levels collected at each time point, it is seen that there is considerable overlap between the levels of ACV and PCV. Therefore there is no apparent difference in the absorption of either drug through normal skin.

There was no correlation between subject age and drug absorption. This is as would be expected as differences in skin permeability are only seen at both extremes of age - here we have used subjects aged from 20 to 65. The absorption of PCV through different anatomical sites has not been examined and all studies have been carried out on the volar surface of the forearm. PCV is not a good probe to investigate absorption through different anatomical sites as its absorption is so low. Only a 2 to 3 fold difference has been reported for absorption of substances applied topically to the arm, abdomen, post auricular area and forehead (Rougier *et al*, 1987), if PCV was used to compare absorption at different sites there is a danger that the observed differences would be too small to be of significance.

The patient information leaflet for Vectavir recommends that cream be applied "at approximately two hourly intervals during waking hours". It does not suggest that the cream should be rubbed in to the skin for maximum benefit. Applying a cream and then rubbing it into the skin might be expected to enhance absorption of a drug. However as seen with PCV, its permeability coefficient is so low that rubbing it into the skin has not improved it's partitioning between the stratum corneum and epidermis. As the cream was rubbed in until it disappeared it must be concluded that the PCV is sitting in the upper levels of the stratum corneum and during the five hour duration of the experiment was unable to diffuse through the lipid pathway of the stratum corneum in appreciable amounts to enter the aqueous environment of the epidermis and dermis and be dialysed.

Adding substances to commercially produced formulations would not be expected to enhance drug penetration as the delicate balance between drug concentration within the formulation and its ability to partition from vehicle to stratum corneum has been tampered with. Most topically applied drugs have been formulated following a series of *in vitro* skin diffusion studies, animal work and the *in vivo* measurement of urine and plasma levels of drug, thus would be expected to have optimized permeation. It is of interest that 1% PCV in aqua gel is absorbed to the same extent as PCV from Vectavir. However Vectavir is more aesthetically pleasing to apply and is less sticky than aqua gel.

The addition of DMSO to an aqua gel formulation or to Vectavir did not improve the absorption of PCV. This is because the concentration of DMSO used was too low to affect stratum corneum lipid fluidity or keratin structure. Concentrations in excess of 60% are needed, and this would have caused skin irritancy. At the concentrations of DMSO used (5%, 20% and 50%), its main effect would be to increase the amount of PCV in solution. As the concentration

of PCV recovered is less than that from plain PCV in aqua gel or from Vectavir, the addition of low concentration DMSO is having an inhibitory effect on PCV absorption – possibly by increasing the affinity of PCV for its vehicle thereby reducing its ability to partition into the stratum corneum.

Sodium lauryl sulphate also did not enhance PCV absorption. Concentrations as low as 0.1% of SLS affect protein structure as detected by differential scanning calorimetry, however with the two much higher concentrations of SLS used in this study, absorption of PCV is actually reduced. This is contrary to what would be expected however exemplifies how the partition coefficient of a substance from its vehicle is altered by the addition of other substances. The alcohols propylene glycol and ethanol also did not enhance PCV absorption.

Although in a small number of experiments it has not been possible to enhance the absorption of PCV, the ease with which such studies can be carried out has been demonstrated. Microdialysis is sampling the target tissue for drug concentration and so real data is obtained concerning a drug's penetrative properties as opposed to indirect or *in vitro* studies. Here the use of DMSO, SLS, propylene glycol and ethanol can all be dismissed as likely candidates to significantly increase the absorption of PCV.

The commercial topical form of PCV is formulated in a vehicle of white soft paraffin, liquid paraffin, propylene glycol, cetostearyl alcohol and cetomacragol 1000 in purified water. This vehicle promotes PCV absorption to the same extent as a simple water-based gel. The addition of penetration enhancers to vehicles can (at best) only enhance drug penetration through the skin by up to 9 times, therefore it is likely that modification of the structure of PCV is needed in order to increase its permeation – as has been done with the oral formulation of famciclovir to enhance its absorption through the gastrointestinal tract.

A lipophilic vehicle such as white soft paraffin prevents water loss from the stratum corneum and can cause full hydration. This should cause a marked increase in skin permeability. Here we have seen that white soft paraffin has reduced PCV absorption. This may be due to the white soft paraffin causing a physical barrier to drug absorption although it was fully rubbed in to the skin. There have been reports of hydration reducing the absorption of hydrophilic compounds as has occurred here (Bucks *et al*, 1989).

The very poor penetrative properties of PCV mean that it is an ideal probe to investigate some of the factors surrounding percutaneous absorption. As absorption through normal skin with physiological blood flow is below assay threshold, measures that affect absorption can be quantified by the increase in PCV levels. Such measures include modifications of the stratum corneum barrier or blood flow. These experiments are described in the following chapters.

Chapter 5

The stratum corneum barrier

Introduction

The stratum corneum provides the main barrier to percutaneous absorption. Barrier function is not uniform throughout the stratum corneum but increases with depth (Blank *et al*, 1964a). The measurement of transepidermal water loss (TEWL) is a recognized indication of barrier function (with the proviso that eccrine sweating is at a basal rate). This chapter describes tape stripping as a method of barrier impairment. With successive strips removing further layers of the stratum corneum, the measurement of TEWL can be compared with barrier damage in terms of number of tape-strips. Absorption of PCV through skin tape-stripped to varying degrees can be measured to see if there is a relationship between barrier impairment and concentration of drug absorbed. As with the absorption of drug through normal skin (chapter 4), the absorption of ACV through skin with a physically modified barrier was investigated for confirmation of a class effect.

1. The measurement of transepidermal water loss in tape stripped skin.

The skin barrier was disrupted by tape stripping in 8 subjects (age range 21 to 65). This involved application of cellophane adhesive tape (Niceday, Guilbert, UK) with firm pressure to an area of skin 3 x 1cm. As the tape was pulled off the skin it brought with it part of the stratum corneum. The relationship between number of tape strips and disruption of the permeability barrier was examined by measurement of transepidermal water loss; TEWL measurements were taken every 5 strips until no further increase in TEWL occurred (glistening of the skin surface).

Results

The mean TEWL of intact forearm skin (volar surface) in 8 subjects was 10 ± 0.8 g m^{-2} h^{-1} (SEM). Glistening of the skin surface was seen between 40 and 45 strips for each subject. Subject age did not affect TEWL of unaffected skin nor was there a relationship between the area under the curve of TEWL with 0 to 40 strips and subject age (correlation coefficient –0.32). Sequential tape strips increased TEWL such that there was a very close correlation ($r = 0.998$) between the $\log_{10}$ of TEWL and number of tape strips (figure 5.1) up to 40 strips. When both axes are linear (figure 5.2) the curve of TEWL with successive number of tape strips assumed a sigmoid shape with a straight line response from 25 to 35 strips ($R=0.999$).

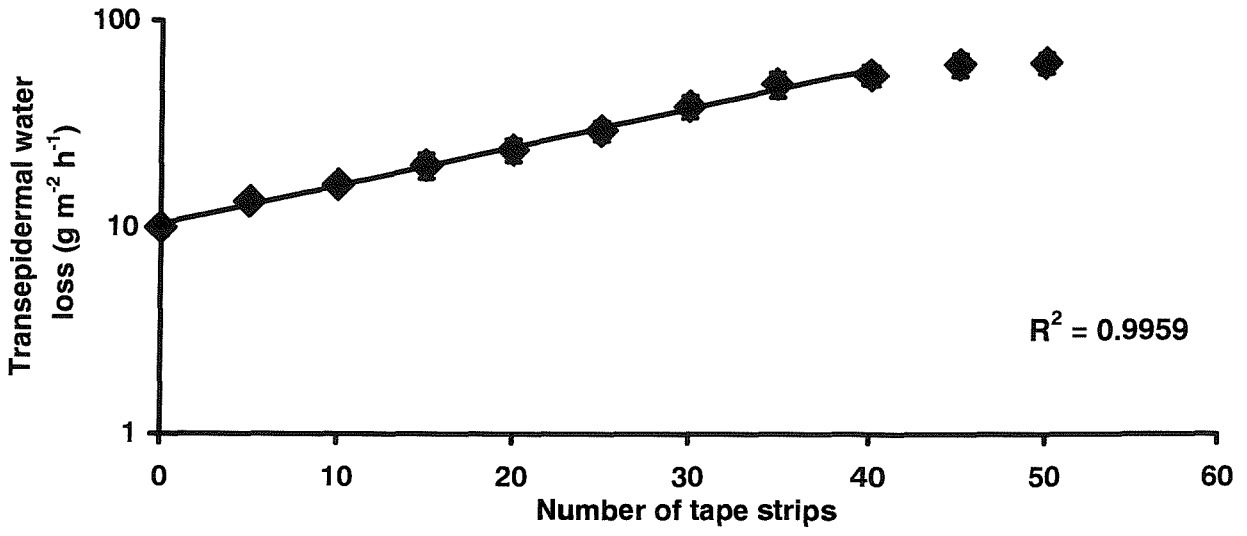


Figure 5.1 The log10 TEWL as a result of sequential tape strips in 8 subjects. Error bars are standard error of the mean.

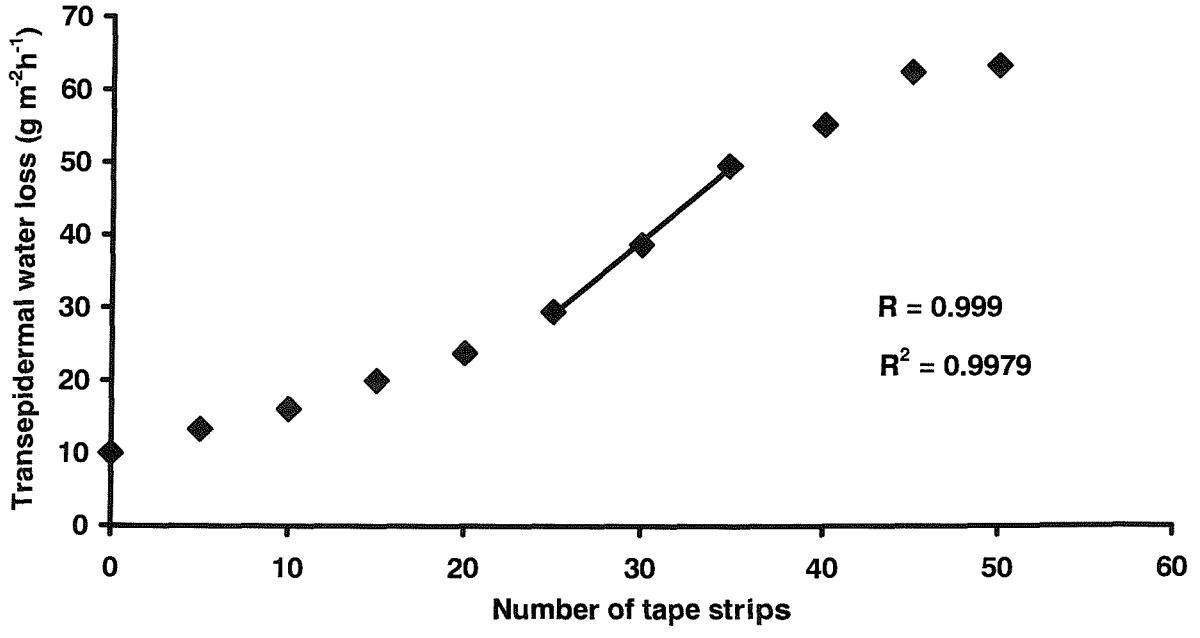


Figure 5.2 Transepidermal water loss as a result of sequential tape stripping in 8 subjects. Linear axes.

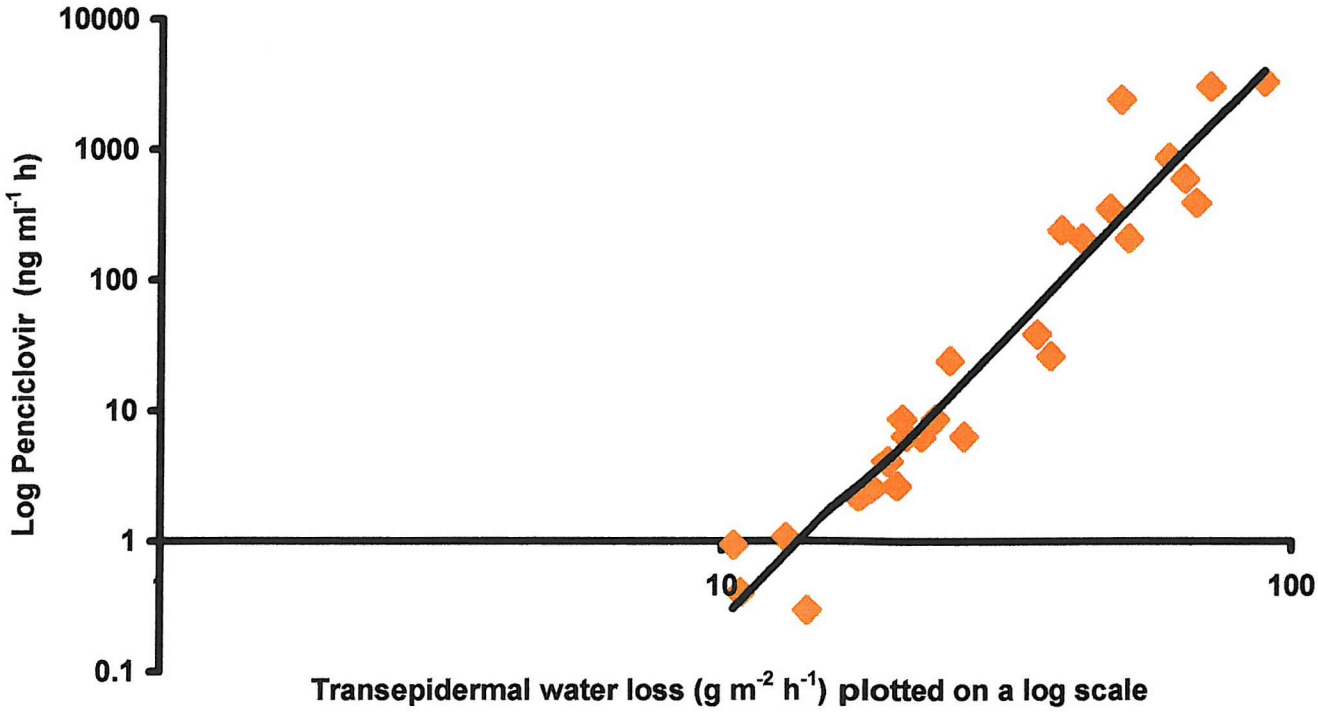
2. The absorption of penciclovir through skin tape-stripped to different degrees.

Drug absorption was measured through epidermis with different degrees of barrier disruption. In 5 subjects, following insertion of microdialysis fibres, the skin overlying each of the 6 fibres was stripped between 0 and 50 times. TEWL measurements were taken before PCV (Vectavir) was applied in a drug well. Cutaneous blood flow was shut down using noradrenaline so that observed differences would be due to change in barrier function rather than trauma-induced vasodilatation (caused by the skin stripping) affecting dermal drug levels.

Results

TEWL prior to applying topical PCV ranged from 10.5 (barrier intact) to 88.5 g m^{-2} h^{-1} (glistening skin). The concentration of PCV recovered increased in proportion to the barrier disruption reflected by TEWL. When plotted with linear axes (figure 5.4) the curve appeared exponential. When TEWL was expressed on a log scale, the log of PCV recovered was directly proportional ($R = 0.963$, $r^2 = 0.9283$) (Figure 5.3). The use of linear axes makes it easier to appreciate that only low concentrations of penciclovir are absorbed with moderate barrier impairment and that quite severe barrier disruption is needed to permit penciclovir absorption to a therapeutic level (500ng ml^{-1}).

Figure 5.3 Concentration of penciclovir (log_{10} axis) recovered per hour with different degrees of barrier impairment as measured by TEWL (log_{10} axis) in 5 subjects (30 fibres).



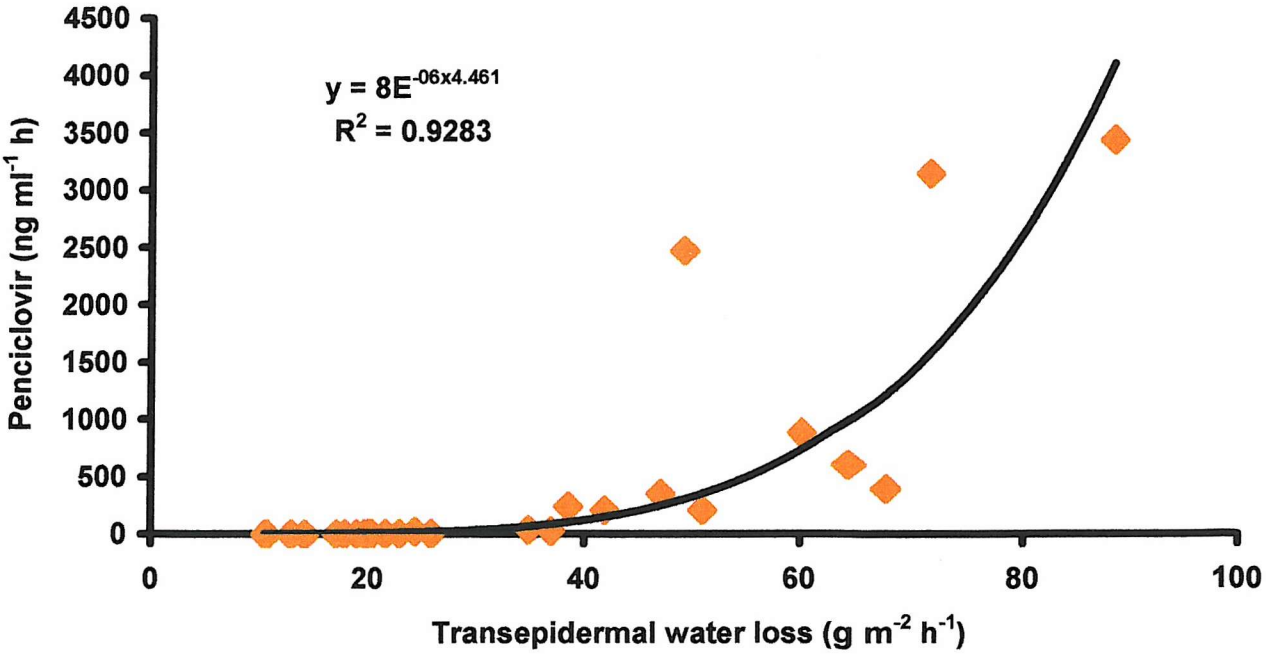


Figure 5.4 Concentration of penciclovir recovered per hour with different degrees of barrier impairment as measured by TEWL, linear axes. Power trend line.

3. Penciclovir and aciclovir absorption with complete disruption of the stratum corneum.

To investigate the overall contribution of the stratum corneum in preventing drug absorption, studies were carried out in the presence of complete barrier disruption. In 12 subjects the skin overlying two microdialysis fibres was tape-stripped to glistening. Two other fibres per subject acted as controls. The absorption of topically applied penciclovir (Vectavir) was investigated in 7 subjects, and of aciclovir (Zovirax) in 5. In order to investigate barrier function alone, cutaneous blood flow was shut down with the addition of noradrenaline to the perfusate.

Results

The concentration of PCV and ACV collected over time in the presence of vasoconstriction in skin tape-stripped to glistening (mean number of tape strips was 45) was at almost maximal levels one hour after drug application (figure 5.5). The kinetic profile of absorption for both drugs was very similar (Table 5.1).

	PENCICLOVIR (Vectavir)	**ACICLOVIR (Zovirax)**
C_{max} (± SEM)	5781 ± 1317 ng ml^{-1}	3901 ± 1498 ng ml^{-1}
T_{max} (± SEM)	222 ± 18 minutes	240 ± 18 minutes
AUC	17489 ± 3779 ng $ml^{-1}h_{(0-5)}$	12207 ± 4994 ng $ml^{-1}h_{(0-5)}$

Table 5.1. The pharmacokinetic profile of PCV and ACV recovery by microdialysis from skin tape-stripped to glistening.

Complete abrogation of the stratum corneum barrier by tape stripping increased PCV percutaneous penetration by 1300 times and ACV absorption 440 times as compared with normal, barrier intact, skin.

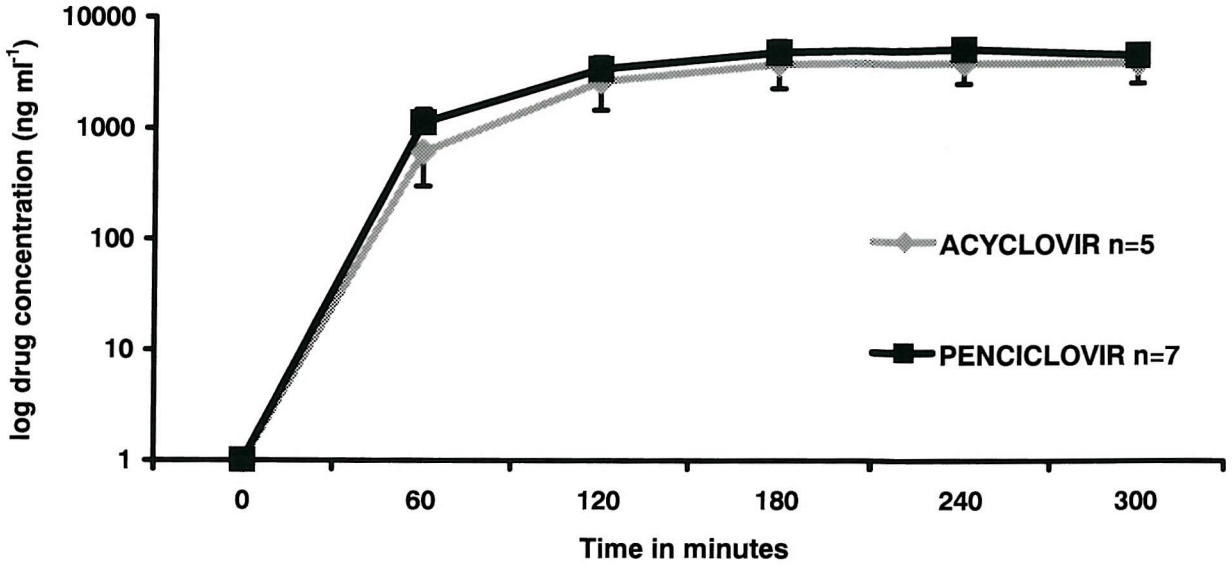


Figure 5.5 Penciclovir and Aciclovir recovery over 5 hours with complete disruption of the stratum corneum and local vasoconstriction. Error bars are SEM.

Discussion

There was a very close relationship between the log of transepidermal water loss and number of tape strips (figure 5.1). This close correlation was seen from 0 to 40 strips – when glistening of the stratum corneum was seen indicating complete disruption. When the rate of TEWL was plotted on a linear axis the curve assumed a sigmoid shape. This confirms that the lower layers of the stratum corneum provide the greatest barrier function and that the barrier is not uniform throughout the layers of the stratum corneum. All layers, even the most superficial, do contribute to barrier function as there is an increase in TEWL seen with just 5 tape strips, however the greatest barrier is present in the layers of stratum corneum that are removed with 25 to 35 strips.

Studies that carry out tape stripping and then weigh the strips to calculate the number of cells removed have found that more skin is removed in the outer strips (Trebilcock *et al*, 1994). The cells of the outer stratum corneum are loosely attached to one another and thus contribute very little to the barrier. The lipid concentration of the stratum corneum increases with advancing depth of the stratum corneum, and it is known that corneocyte adhesion is greater with the lower levels. Therefore, as we have confirmed here, the main barrier to absorption is present in the lower levels of the stratum corneum.

When we investigated this with the absorption of PCV we found that there was a very close correlation between barrier disruption as measured by TEWL and PCV absorption per hour (mean over 5 hours) ($r = 0.963$, figure 5.3). At lower levels of TEWL (less than 40), PCV absorption remained extremely low. This can be seen in figure 5.4 where linear axes are used for both TEWL and AUC of PCV collected. From figure 5.2 it can be seen that about 30 tape strips are needed to disrupt the barrier and increase TEWL to 40 g m^{-2} h^{-1}. Hence a significant degree of damage to the barrier must occur before PCV is absorbed to a level approaching therapeutic concentration (500ng ml^{-1}).

There was no correlation between the number of tape strips and PCV absorption. The degree of barrier impairment per tape strip is subject dependent, operator dependent and also affected by the brand of cellophane adhesive tape used. Here the operator and brand of tape is kept the same, however it is still possible for different degrees of pressure to be applied to the tape prior to its removal which will affect the number of cells of the stratum corneum removed. The main source of variability is the subject (King *et al*, 1979; Weigand *et al*, 1974): People vary in the thickness of their stratum corneum and the degree of inter-corneocyte adhesion, therefore tape stripping within a population will result in different degrees of barrier impairment.

A previously reported study has used cutaneous microdialysis to examine the effect of barrier pertubation by tape-stripping on drug absorption (Benfeldt *et al*, 1999). Tape-stripping 20 times (resulting in a mean TEWL of 31 ± 22 g m^{-2} h^{-1} (SD)) enhanced the absorption of salicylic acid by a factor of 157. We found that tape stripping 20 times increased the TEWL to a similar degree; 24 ± 8 g m^{-2} h^{-1}(SD). However PCV absorption remained very low with only 20 tape strips.

This data has been presented with a line graph where the log of both axes has been used and also with linear axes to clarify the numbers involved. Together they demonstrate the exponential relationship that exists between barrier damage as measured by TEWL and penciclovir absorption. As the correlation is so strong the power trend line (figure 5.4) can be used to describe the relationship between the absorption of PCV and barrier disruption:

$$y = 8E^{-06x4.461}$$

Where y is the amount of penciclovir absorbed per hour

And x is the transepidermal water loss

As this equation has been derived in a situation in which cutaneous blood flow is at a minimum, and it is known that PCV metabolism in the skin is very low (Vere Hodge *et al*, 1993), it provides a real value of the stratum corneum barrier to PCV absorption.

There are no previous reports of drug absorption through skin that has been disrupted to varying degrees in the same subject. This method gives vital information concerning the penetrative properties of a molecule and should be considered an essential investigation in the development of any topically applied formulation.

Complete barrier disruption increased the absorption of PCV by 1300 times and ACV by 440 times. However, as with the absorption of both drugs through barrier intact (normal) skin, there is overlap of the standard error of the mean, which indicates that the difference in absorption between ACV and PCV through barrier deficient skin is not significant. These figures also represent the absolute maximal concentrations of ACV and PCV that can be recovered following topical application of the commercial forms of both creams.

Noradrenaline induced vasoconstriction was a part of all our studies investigating barrier function with microdialysis. By removing the variable of cutaneous blood flow and examining the absorption of a drug that is not metabolised by the skin, the true effect of the stratum corneum barrier can be investigated in isolation. The studies here confirm that it is the main barrier to hydrophilic drug absorption. The percutaneous pathway is most likely to be intercellular as it is only by removal of the stratum corneum that penciclovir and aciclovir can penetrate into the dermis. The high levels of drug seen with removal of the stratum corneum also demonstrate that these water soluble drugs are freely able to partition into the aqueous environment of the viable epidermis and dermis. This can be correlated to the clinical situation – in the presence of a herpetic cold sore, topically applied drug will reach its epidermal and dermal site of action.

Chapter 6

The effect of cutaneous blood flow on dermal drug levels

Having established the role of the barrier in determining drug absorption, it was necessary to investigate what happens to a drug once it has reached the dermis - the effect of cutaneous blood flow and the movement of drug within the dermis (chapter 7)

Introduction

The organization of cutaneous blood vessels

The blood vessels of the skin are organized to form three vascular plexuses (Rothman 1954): The arteries entering the skin form a deep plexus in the subcutaneous fat. This deep network gives branches to the various cutaneous appendages with the formation of two plexuses in the dermis; the superficial (subpapillary) vascular plexus in the upper dermis and deep vascular plexus in the lower dermis. The subpapillary plexus forms capillary loops which pass up through the dermal papillae, to lie at the dermo-epidermal junction. The capillaries do not enter the epidermis but reach within 150μm and 200μm of the outer surface of the skin (Washington *et al*, 1989). The superficial plexus and capillaries form the nutritional supply, providing oxygen and other vital nutrients to the viable cells of the epidermis. The hair follicles and sweat glands are mainly supplied by the lower dermal plexus.

The two horizontal networks of dermal blood vessels are connected through vertical channels and arterio-venous anastomoses. It is believed that blood flow through the upper dermis is controlled by the arteriovenous anastomoses which act as shunts to short circuit blood flow. This is an important part of temperature control - cutaneous blood flow can vary by up to 100 fold in response to environmental conditions.

Variations in flow of the microcirculation are the result of interactions between neural and endothelial control mechanisms. Neural control has traditionally been attributed to a balance between the parasympathetic nervous system causing vasodilatation mediated by acetylcholine, and sympathetic stimulation causing vasoconstriction via noradrenaline. However it is now established that the non-adrenergic non-cholinergic (NANC) nervous system plays a part through the release of a number of transmitters including substance P, vasoactive intestinal peptide (VIP), neuropeptide Y (NPY) and nitric oxide (Fahrenkrug, 1989; Malvin *et al*, 1990).

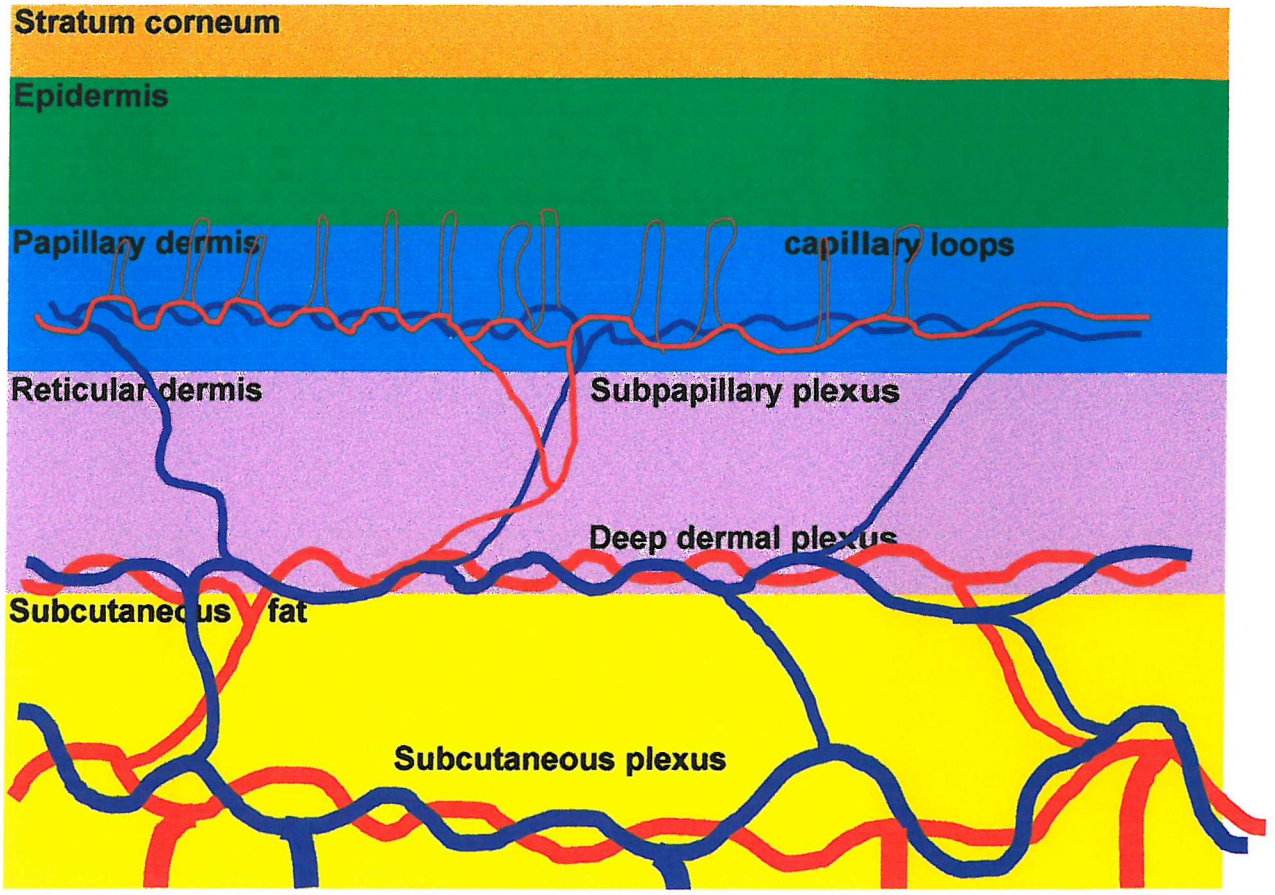


Figure 6.1. Schematic diagram of the three vascular plexuses of the skin. There are anastomosing arteries at three levels (depicted in red); at the subcutaneous fascia; at the junction of the dermis and cubcutaneous fascia, and sub papillary. Capillary loops that arise from the subpapillary plexus traverse the dermis to the dermo-epidermal junction. Veins are depicted in blue.

The effect of cutaneous blood flow on drug absorption

Absorption through the stratum corneum is primarily a passive process that is not affected by cutaneous blood flow. This is the rationale for the use of *in vitro* diffusion cells for human skin permeation studies with direct application of the results to the *in vivo* situation. However, cutaneous blood flow will affect the dermal concentration of topically absorbed drug and so affects absorption of drug across full thickness skin. In general, systemic drug absorption following topical application is enhanced when cutaneous blood flow is increased. This is assumed to be due to the generation of a concentration gradient of drug across the skin. This is potentially detrimental for those drugs whose site of action is within the skin. In skin disease or damage there is cutaneous vasodilatation cased by the release of inflammatory mediators – this will rapidly clear absorbed drug. The flux of drug through the skin will increase, but dermal levels will decrease as a result of heightened clearance by blood flow. An increase in blood flow will also increase the breakdown of certain susceptible compounds as higher levels of metabolically active enzymes are delivered to the dermis by the enhanced blood flow. The use of vasoconstrictors to retard drug clearance is widely used in medicine; noradrenaline is co-administered with lignocaine when injected subcutaneously to prolong local anaesthesia (and reduce bleeding).

Some drugs will affect their own absorption by direct action on the cutaneous vasculature. For example, topically applied steroids cause local vasoconstriction which will reduce their clearance and may retard systemic absorption. However as the application of topical steroids is usually for skin disease, the effect of prolonging the time that the steroid is kept within the skin is beneficial. For most drugs the rate limiting step of percutaneous absorption is diffusion through the stratum corneum. For those drugs that are highly lipophilic and diffuse

rapidly through the stratum corneum, cutaneous blood flow may limit absorption systemically as the drug is unable to partition from the lipophilic stratum corneum into the aqueous environment of the epidermis, dermis and vascular space. Such drugs may remain in the 'stratum corneum reservoir' with little diffusion deeper into the skin.

Siddiqui *et al* demonstrated the importance of dermal blood flow in clearing absorbed drug. The disappearance of 7 different steroids from the dermal surface (epidermis removed) of anaesthetized and sacrificed rats was examined. A 2 to 5 fold increase in absorption seen in the live rats was attributed to dermal blood flow (Siddiqui *et al*, 1989). Another method of measuring cutaneous blood flow is by the radioactive microsphere technique (Singh *et al*, 1993): Radioactive microspheres are injected intravenously into an anaesthetized animal. It is assumed that on the first pass through the circulation the microspheres become trapped in arterioles. Hence if the animal is then sacrificed and the tissues of interest (including dermis) examined, blood flow to individual tissues (reflected by levels of radioactivity) can be quantified. The use of this method together with comparisons of drug flux through dermis in anaesthetized and sacrificed rats gives a value for drug clearance by cutaneous blood flow.

There are no reported studies in man that give a figure for the effect of blood flow on dermal drug concentration.

Use of microdialysis to investigate the effect of cutaneous blood flow on drug clearance

An indirect method of measuring the effect of cutaneous blood flow on percutaneous absorption *in vivo* is by pharmacological manipulation of the vasculature whilst continuously measuring dermal drug concentration. By shutting down cutaneous blood flow through the use of noradrenaline, dermal drug levels can be measured and compared with drug levels in skin with

an unmodified blood supply. Noradrenaline acts on vascular smooth muscle via $alpha_1$-adreno-receptors causing contraction. By continuously delivering noradrenaline (via the microdialysis fibres) to the cutaneous blood vessels, sustained vasoconstriction is obtained.

As PCV is poorly absorbed through intact skin and is undetectable without noradrenaline-induced vasoconstriction, this study had to be conducted examining absorption through barrier-disrupted skin – to guarantee PCV absorption to sufficient levels for comparisons to be made.

The effect of cutaneous blood flow on the dermal concentration of penciclovir

1. Absorption through barrier impaired skin with and without noradrenaline induced vasoconstriction.

In 5 subjects, six microdialysis fibres were inserted; 2 fibres for each of the following conditions under examination:

1. Skin tape-stripped to glistening, perfusate plain Ringer's solution
2. Skin tape-stripped to glistening, perfusate 5×10^{-4} % noradrenaline in Ringer's solution
3. Unmodified skin, perfusate 5×10^{-4} % noradrenaline in Ringer's solution

Penciclovir was applied topically and dialysate collected hourly for 5 hours.

Results

The mean number of tape strips to cause visible glistening of the stratum corneum was 39 (± 2). Noradrenaline in the perfusate caused visible vasoconstriction extending 2mm either side along the length of the fibre within the skin. Concentrations of PCV recovered in dialysates after total disruption of the stratum corneum barrier were compared in the presence and absence of noradrenaline-induced vasoconstriction. Although there was considerable intersubject variation

in the amount of drug absorbed through barrier-disrupted skin with and without cutaneous vasoconstriction, the intrasubject effect of vasoconstriction on penciclovir absorption was consistent (figure 6.2). In the presence of noradrenaline-induced vasoconstriction the mean amount of penciclovir collected through tape stripped skin was 15.6 fold (SEM 4.8) greater than in the absence of vasoconstriction.

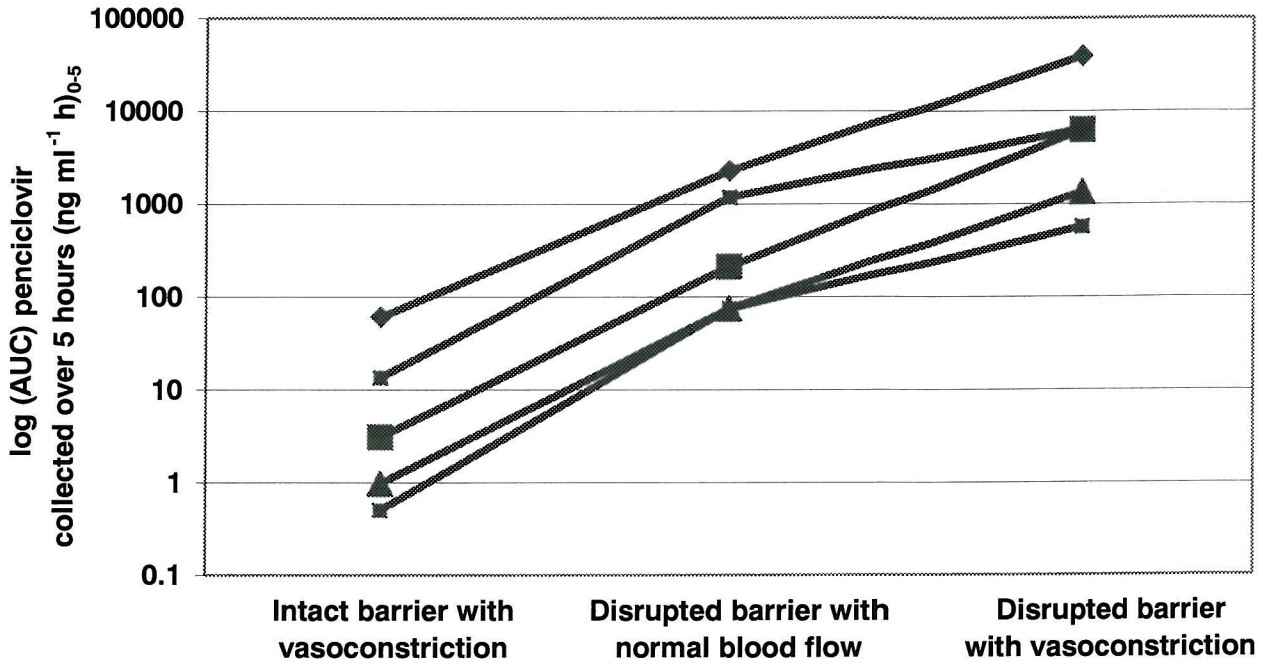


Figure 6.2 The role of cutaneous blood flow on penciclovir recovery in 5 subjects.

2. The effect of vasodilatation on penciclovir absorption

The role of blood flow on percutaneous absorption and dermal drug levels was assessed further in three subjects. Once again microdialysis fibres were used to both deliver the vasoactive pharmacological agents directly to the cutaneous blood vessels, and to sample percutaneously absorbed penciclovir. Six microdialysis fibres were inserted into the skin. In two fibres the perfusate was plain Ringer's solution, the second pair was perfused with 5×10^{-4} % noradrenaline in Ringer's solution and the third pair were perfused with 0.5mg/ml glyceryl trinitrate in Ringer's solution to induce vasodilation. Scanning laser Doppler images were taken of the 0.5cm x 1.5cm area over each fibre before PCV was applied topically in a drug well.

Results

The delivery of glyceryl trinitrate (GTN) did not cause any systemic side effects such as headache in the three subjects. Laser Doppler readings showed that there was significant vasodilatation in the GTN containing fibres and a reduction in blood flux in the noradrenaline containing fibres; mean values (in arbitary units) were:

Noradrenaline 77.15, Ringer's solution 109.7 and GTN 163.7.

Expressed as percentages where the baseline flux for fibres perfused with plain Ringer's solution is 100%, flux with GTN was 150% and flux with noradrenaline was 70% that of Ringer's solution alone.

The recovery of topically absorbed PCV in the presence of local vasoconstriction, vasodilatation and normal blood flow is shown in figure 6.3. PCV was below detection levels in three out of the 6 fibres with plain Ringer's solution as perfusate. Overall the AUC of penciclovir absorbed over 5 hours was only 0.95 (± 0.6) ng ml^{-1} h. With vasoconstriction the

AUC increased to 8.9 (± 4.6) ng ml^{-1} h and surprisingly, with vasodilatation, the AUC was greatest at 11.9 (± 5.3) ng ml^{-1} h. The difference in PCV absorption with vasoconstriction and vasodilatation was not significant (ANOVA) and there was considerable overlap of the standard error of the mean at each time point (figure 6.3). PCV absorption increased by about 9 times with vasoconstriction and 12 times with vasodilatation.

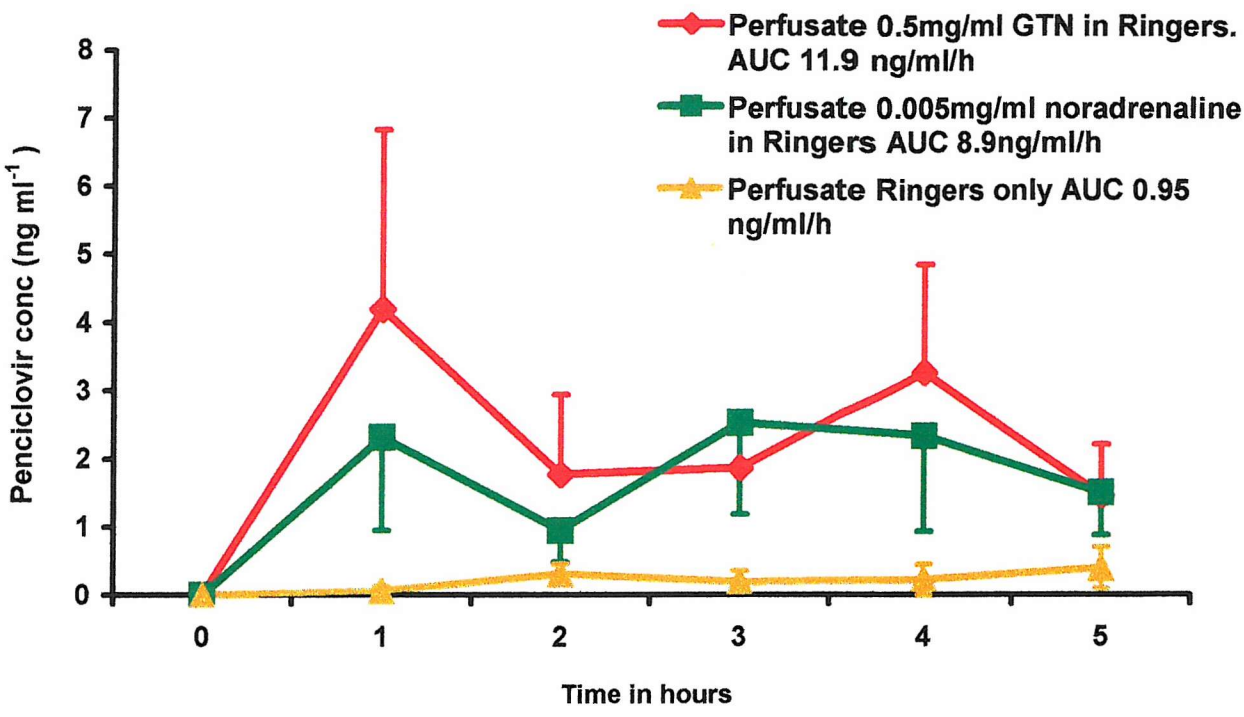


Figure 6.3 Penciclovir recovery per hour over 5 hours with local vasoconstriction, vasodilatation and physiological blood flow. N = 3. Error bars are SEM.

Discussion

The role of blood flow in determining tissue levels of drugs was examined in tape-stripped skin to ensure that measurable levels of penciclovir were present both with and without vasoconstriction induced by noradrenaline. We observed a consistent increase of about 15 fold in drug detected in the presence of vasoconstriction.

There was considerable intersubject variation in the concentration of drug recovered (figure 6.2). In each subject the skin was tape-stripped to glistening, however the degree of barrier impairment was not measured with transepidermal water loss (TEWL meter unavailable). It is likely that there were intersubject differences in the stratum corneum barrier effect and this is reflected by the levels of drug absorbed. The effect of shutting down blood flow is consistent among the 5 subjects. Although in this experiment there is a 15 fold difference in drug level with and without noradrenaline-induced vasoconstriction, it is not possible to state with certainty that cutaneous blood flow will wash away this amount of drug under normal circumstances. Trauma caused by tape stripping will result in local vasodilatation which might be expected to enhance drug removal, so it is likely that the real figure for drug clearance under 'normal conditions' is less than 15 fold. Cutaneous blood flow changes constantly according to environmental conditions and so will affect percutaneous absorption and dermal drug levels under normal circumstances to varying degrees.

On examination of PCV absorption through normal (barrier intact) skin, vasoconstriction increased PCV dialysis by 9 times (figure 6.3). The concentrations of penciclovir detected in the fibres perfused with plain Ringer's solution were extremely low – either at or below the threshold of assay sensitivity. This value (a 9 fold increase) may be a more realistic indication of the effect of cutaneous blood flow on clearing dermal drug levels as it has been derived without

trauma-induced vasodilatation. However, as it has been obtained at the very limit of assay sensitivity there is scope for a degree of inaccuracy. Therefore, it is likely that the real figure for drug clearance by cutaneous blood flow lies between the two estimates of 9 and 15 fold derived here.

It is surprising that PCV recovery was increased when vasodilatation was induced by the addition of glyceryl trinitrate (GTN) to the perfusate. It would be expected that vasodilatation should wash away drug and that the amounts detected ought to be less than those recovered with plain Ringer's solution (i.e nil). This suggests that GTN is affecting passage of drug through the stratum corneum. GTN is metabolized to nitric oxide which is a powerful vasodilator. Within the skin nitric oxide (NO) is endogenously produced by several cell types including endothelial cells, keratinocytes, Langerhans cells, melanocytes and fibroblasts. The list of functions of NO increases every year as more is discovered about this important molecule. It is known to be involved in the cutaneous inflammatory response to UV light and pathogens, modulation of neuronal activity within the skin and wound healing as well as vascular smooth muscle relaxation (Bruch-Gerharz *et al*, 1998). Studies on toad skin have suggested that NO, acting on cyclic guanosyl monophosphate, increases the transport of certain ions through the skin (Neumann *et al*, 1996). NO may play a part in percutaneous absorption and has increased PCV absorption and dermal levels despite greater drug clearance by the dilated cutaneous microcirculation.

The derivation of a value for drug clearance by blood flow is the important finding of these experiments and can be stated as being between 9 and 15 fold. This measure of drug clearance is a key value essential to the calculation of dermal drug concentrations at steady state which is highly relevant to the formulation of topically applied agents. It has not been obtained

in humans *in vivo* previously, and although obtained here using penciclovir, the value should be applicable to the clearance of other small hydrophilic molecules.

Chapter 7

Movement of drug molecules within the dermis

Introduction

The dermis is an aqueous environment. It consists of a supporting matrix or ground substance that contains proteins (collagen, elastin, fibrillin) and polysaccharides that coexist and interact to produce hygroscopic proteoglycan macromolecules, which strongly attract and retain water (Eady *et al*, 1998). The upper (papillary) dermis is a thin layer that interdigitates with the ridged underside of the epidermis. Collagen fibres are thinnest in this layer. The remaining nine tenths of the dermis is called the reticular dermis. Here collagen fibres become increasingly thicker with depth. It is within the reticular dermis that microdialysis fibres are inserted.

As PCV is a water soluble drug it would be expected to move freely within the dermis. To investigate this we used dermal ultrasound to measure fibre depth and correlated it with the total amount of PCV collected at that depth.

1. Dermal ultrasound – operator and equipment validation

Before using the Dermascan to measure fibre depth it was essential to ensure that consistent, repeatable results could be obtained.

Using 4 subjects (2 male and 2 female), an area of skin on the volar surface of the left and right forearms 17cm from the wrist was selected. The combined dermal and epidermal thickness of the selected area was measured on three separate occasions over a one week period, with three scans taken on each occasion. From each B mode 2 dimensional ultrasound scan obtained, three measurements of skin thickness were taken at predetermined fixed points on the scan. This gave a total of 27 measurements of skin thickness per arm per subject.

Results

Table 7.1 shows the mean skin thickness with 95% confidence intervals per arm per subject.

	Left arm	Right arm
Subject D (f)	0.987mm (0.020)	0.982mm (0.029)
Subject A (f)	1.07mm (0.031)	1.034mm (0.026)
Subject B (m)	1.309mm (0.027)	1.297mm (0.03)
Subject C (m)	1.372mm (0.033)	1.34mm (0.031)

Table 7.1. The skin thickness (right and left arms) of 4 subjects measured on three occasions (mean of 27 measurements on ultrasound, with 95% confidence intervals)

2. Penciclovir movement within the dermis

Penciclovir absorption through normal forearm skin was measured in 29 subjects (40 fibres) with noradrenaline ($5x10^{-4}$%) in the Ringer's solution perfusate. Several of the fibres were deliberately inserted more deeply or superficially than is normal practice to increase the range of data obtained. At the end of the 5-hour dialysis collection period, fibre depth was measured using dermal ultrasound (Dermascan C) as described previously (Materials and Methods section).

Results

A typical 2D image obtained from the Dermascan of a fibre lying in the skin is shown in figure 7.1. For the 40 fibres measured, mean depth was 0.68 ± 0.197mm (SD),(range 0.24 to 1.04). There was no relationship between fibre depth and the cumulative amount of PCV collected in the presence of local vasoconstriction, correlation coefficient was –0.03 (figure 7.2).

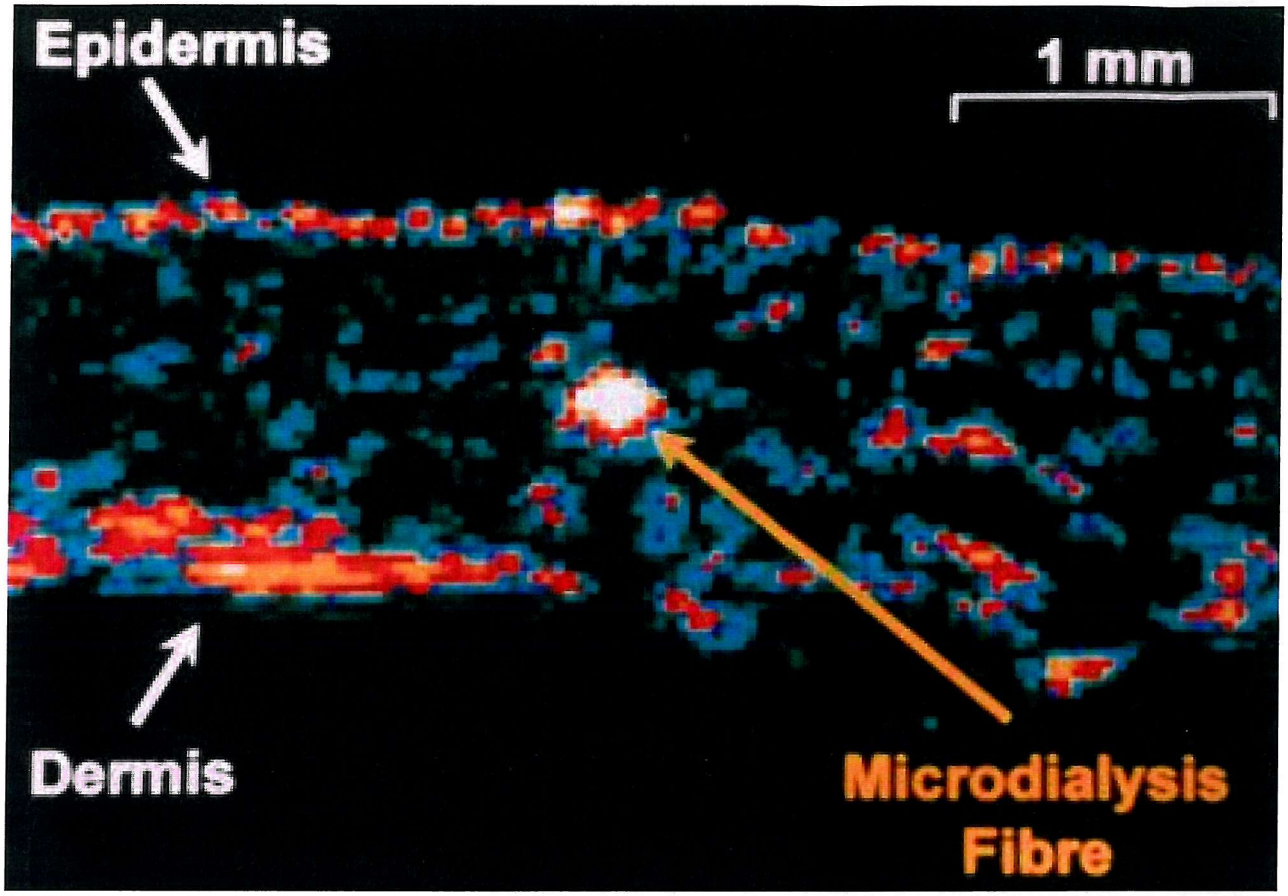


Figure 7.1 2D (B mode) ultrasound image of a cross section through the epidermis and dermis of normal skin. A microdialysis fibre is clearly seen as an area of high opacity and is lying at a depth of 0.8mm.

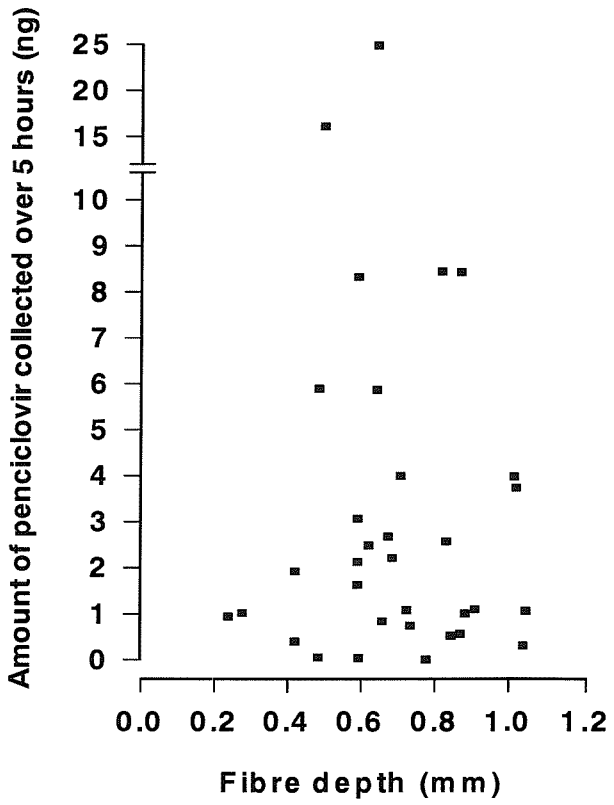


Figure 7.2 The cumulative amount of penciclovir recovered following absorption through normal skin in the presence of local vasoconstriction as a function of microdialysis fibre depth in the dermis. There was no relationship between the amount of penciclovir collected over 5 hours and fibre depth.

Discussion

The measurement of forearm skin thickness was essential to ensure that the Dermascan gave consistently reproducible results and that the operator's technique was accurate. The narrow 95% confidence intervals readings from each subject confirm that results obtained on different occasions can be compared. As anticipated, male skin was thicker than female skin and it is interesting that in all 4 subjects the left forearm skin was slightly thicker than the right. All subjects were right handed.

The second study examining fibre depth and drug absorption was performed with noradrenaline in the perfusate, so the effect of blood flow on absorbed drug level has been removed. In addition, PCV is not metabolized in the skin, so drug levels at different depths in the dermis are the result of free diffusion of drug. There was no relationship between fibre depth and PCV recovered. This suggests that PCV moves freely within the dermis – at least in the vertical plane. There was no drug concentration gradient or pooling of drug at the depths measured. It is not surprising that penciclovir appears to move freely within the dermis, it is a small water soluble molecule that would be expected to permeate throughout an aqueous environment.

It was not possible to measure drug concentration in the epidermis or stratum corneum where it might be expected that a concentration gradient would exist – the diameter of the microdialysis fibres used (216μm) is greater than epidermal depth. At the current time there are no microdialysis fibres with a small enough diameter to enable the measurement of drug movement through the epidermis *in vivo*.

Several studies have examined the relationship between fibre depth and percutaneous drug absorption: A correlation of 0.8 was found between fibre depth (1 to 9mm) and nicotine absorption in a group of 15 subjects who had fibres inserted into the umbilical dermis and subcutaneous fat (Muller *et al*, 1995b). There was no relationship between salicylic acid absorption and fibre depth (1 ± 0.31mm (SD)) in 15 subjects despite high levels of salicylic acid absorption (Benfeldt *et al*, 1999). Further work needs to be done to establish drug movement within the dermis as a function of individual drug physicochemical properties.

The role of lymph flow on penciclovir levels has not been investigated. The skin contains an extensive network of lymphatic vessels that form horizontally running superficial and deep plexuses within the dermis. The lymphatic system is predominantly responsible for the drainage of those substances from the tissue spaces that cannot directly return to the blood stream. Colloids, proteins, cells, inorganic material and microorganisms fall into this category (Mortimer 1998). Under normal circumstances PCV would be cleared by cutaneous blood flow, so it is unlikely that the lymphatic system affects the absorption and clearance of such a water-soluble drug.

Chapter 8

The oral absorption of penciclovir

Introduction

Penciclovir is orally administered as the inactive prodrug famciclovir which is metabolized by the intestinal wall and liver to the active form - penciclovir. Studies have shown that the bioavailability of penciclovir after famciclovir administration is 77% with low individual variability (Pue *et al*, 1993). Famciclovir is used for the treatment of acute severe herpes simplex, recurrent genital herpes and acute herpes zoster. Its sites of action are neuronal ganglia and the basal epidermal layer of the skin. Although the measurement of plasma and urine levels of penciclovir following famciclovir administration is well documented, we wanted to measure dermal levels. Microdialysis is the technique of choice as it allows continuous sampling and so a pharmacokinetic profile of drug level in the skin can be established.

We also investigated the effect of cutaneous blood flow on delivering penciclovir to the microdialysis probe by adding noradrenaline to the perfusate.

1. Dermal and plasma levels of penciclovir following oral administration.

Eight subjects (4 male, 4 female; mean age 26, range 19 to 45) took part in the study. Each had fasted for the previous 9 hours. Six microdialysis fibres were inserted and perfused with either plain Ringer's solution or 5×10^{-4} % noradrenaline in Ringer's solution (at least 2 fibres per variable per subject). After one hour of perfusion, a 10ml venous blood sample was taken from a 21G cannula that had been inserted into the antecubital fossa of the contralateral arm. The blood was stored in a plain tube at 4°C until the end of the study.

One hour after fibre insertion each subject took 250mg famciclovir orally with 100ml water. Dialysate and venous blood were collected hourly for five hours. Lunch was given at 3 hours into the study (2 hours after famciclovir ingestion) and consisted of a sandwich, crisps and water. At the end of the study, fibre depth was measured using ultrasound.

The blood was spun at 2000rpm for 15 minutes and the serum supernatant frozen at -20°C.

Results

1. Serum penciclovir concentration

Serum results were obtained for 6 volunteers (figure 8.1). The mean area under the curve of PCV in serum was 7947 ± 782 ng ml^{-1} $h_{(0\text{-}5)}$ (SEM). Maximum concentration during a single sample period (C_{max}) was 3027 ± 660 ng ml^{-1}. The time to C_{max} (T_{max}) was 60 minutes in 2 volunteers and 120 in the other 4 (samples were collected every 60minutes). Age and gender did not affect serum penciclovir concentration (ANOVA).

2. Dermal penciclovir concentration

In the absence of local vasoconstriction the mean AUC of PCV collected over 5 hours (27 fibres) was 378 ± 26 ng $ml^{-1}h$. Noradrenaline added to the perfusate reduced PCV recovery by a factor of 2.2. The mean AUC of PCV with vasoconstriction was 187 ± 15 ng $ml^{-1}h$ (figure 8.2). Comparison of PCV dialysis per subject with and without localized vasoconstriction was significant ($p<0.005$ ANOVA).

In the absence of noradrenaline the C_{max} (108 ng ml^{-1}) occurred at 120 minutes, although at 180 minutes was very similar at 107.2ng ml^{-1}. With noradrenaline-induced vasoconstriction

there was a similar concentration of PCV dialysed from 180 minutes to the end of the study, at a concentration of 54.7 to 56.3ng ml^{-1}. This established steady state was not seen when the perfusate was plain Ringer's solution. There was no relationship between fibre depth and PCV dialysed either with or without vasoconstriction.

The difference in PCV concentration from serum to dermis (taking into account the efficiency of dialysis fibres) when the perfusate was plain Ringer's solution was 4.4 ± 0.7 (SEM) fold.

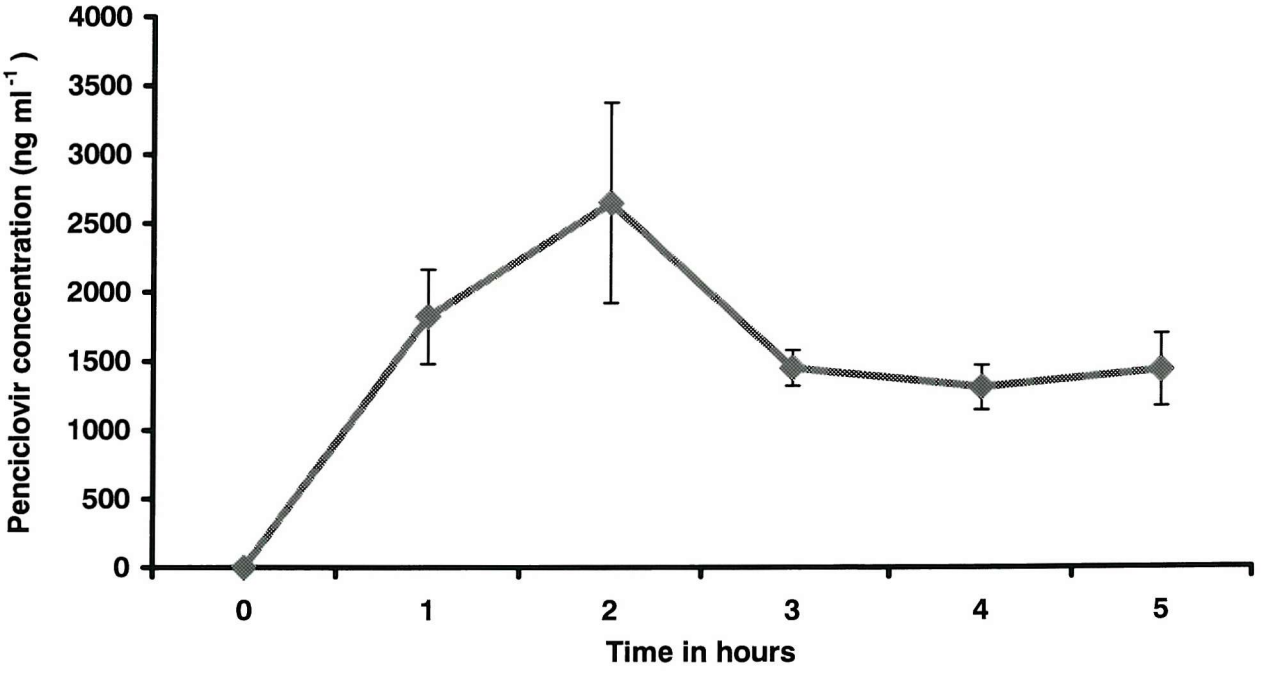


Figure 8.1 Serum concentration of penciclovir following 250mg of famciclovir at time 0, in 6 subjects. Error bars are SEM.

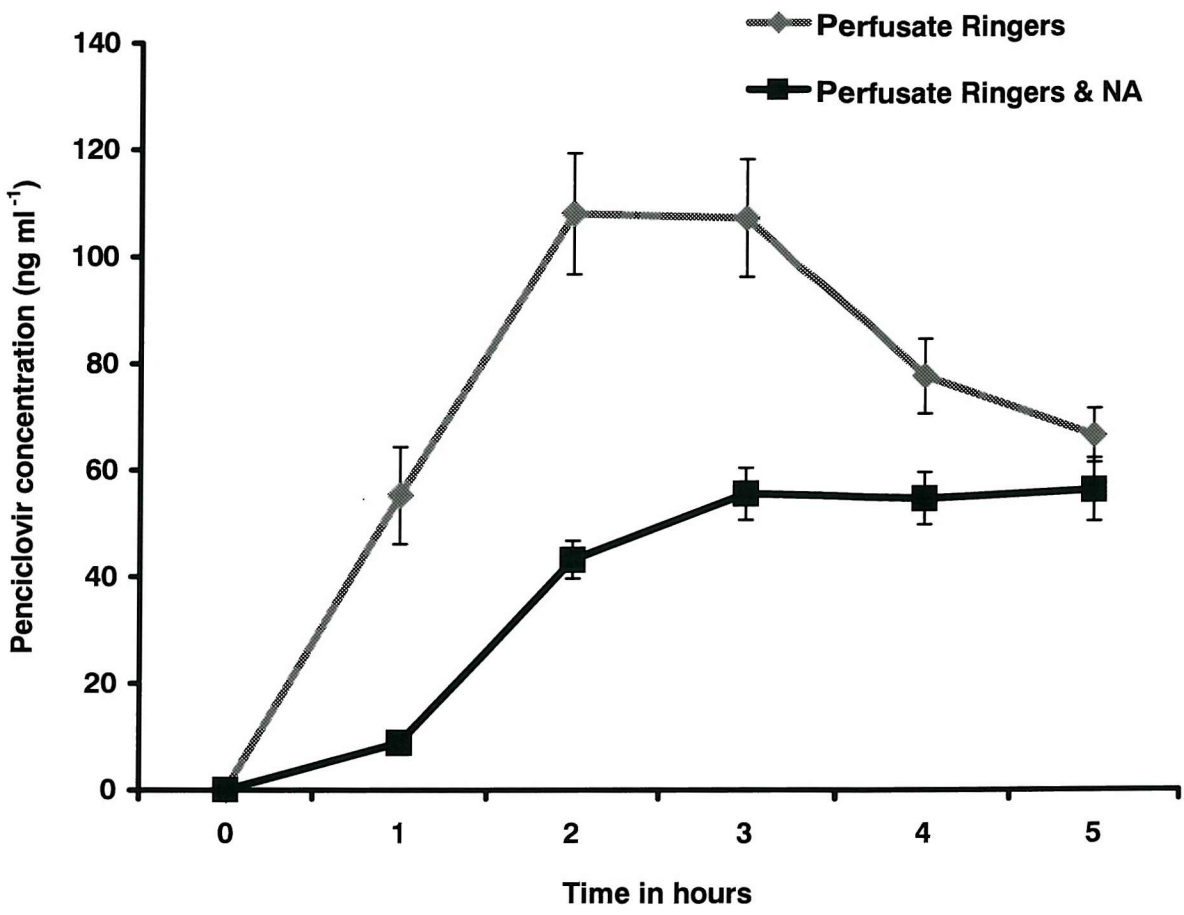


Figure 8.2. Concentration of penciclovir collected from the dermis by microdialysis with and without local vasoconstriction following the ingestion of 250mg famciclovir in 8 subjects. Error bars are SEM.

Discussion

The figures obtained here of serum pharmacokinetics for PCV taken orally are similar to those reported in published studies (Pratt *et al*, 1992); the oral administration of 250mg famciclovir in 20 male subjects gave a maximum concentration of 1590 ± 350 ng ml^{-1} (SD) at a T_{max} of 45 minutes (range 30 minutes to 186 minutes). The figure we have detected of 3027ng ml^{-1} with a SD of 1618ng ml^{-1} overlaps that reported above. Several studies have measured the time to maximum penciclovir concentration in plasma (T_{max}), quoting times of 90 minutes (Borg *et al*, 1999a) and 45 minutes (range 30 to 186 minutes) (Pratt *et al*, 1992). T_{max} obtained here of 100 minutes may be later as sampling of blood was only every hour. It is possible that if samples had been taken more frequently then a lower T_{max} would have been obtained. Indeed in two of the subjects the T_{max} was 60 minutes.

Our protocol of fasting subjects and feeding them 2 hours post famciclovir has not affected the pharmacokinetic parameters of penciclovir as compared with other studies using subjects who were fasted throughout the study. This makes endurance of the study more tolerable for the volunteers. It is known that the presence of food slows the absorption and conversion of famciclovir to penciclovir but it does not affect overall bioavailability or elimination. Therefore famciclovir can be taken without regard to meals.

If a 20% relative recovery is assumed for PCV dialysis (Ringer's solution only fibres) then the mean amount of PCV recovered per hour over the five hours following ingestion is approximately 415ng ml^{-1}. This value is just above the IC_{50} for Herpes Simplex Virus I (400ng ml^{-1}) but below the IC_{50} of Herpes Simplex Virus II (1500ng ml^{-1}) (Crumpacker 1996). It could be argued from this data that a higher dose of penciclovir is needed for efficacy against HSV II. The manufacturer's recommended dose of famciclovir for HSV II is 250mg three times per day.

At this dose famciclovir is clinically effective against HSV II. Therefore the difference between the IC_{50} and recovery of orally administered PCV may highlight inaccuracies in the use of microdialysis in sampling extracellular fluid; it may be that the plasma concentration of PCV measured is the effective concentration that is delivered to the ganglia where the herpes virus resides, or it may be that repeated dosing of drug increases the dermal extracellular fluid concentration to the IC_{50} concentration and so guarantees therapeutic efficacy. Further work needs to be done to examine dermal levels of drug with repeat dosing over a longer time period.

Localized vasoconstriction around the fibres reduced the recovery of PCV by approximately 2.2 fold. This value is lower than the 10 to 15 fold difference that was found to be the effect of blood flow on clearing percutaneously absorbed drug (chapter 6). The reason for this difference lies in the mode of delivery of PCV to the dermis; with systemic application of high concentrations of drug, the cutaneous blood flow actively delivers PCV to the dermis. Vasoconstriction surrounding a fibre means that the water-soluble PCV has to diffuse through the dermis from a distance of about 2mm to be dialysed (vasoconstriction is visible 2mm either side of the fibre as it courses through the dermis). With topically applied PCV the movement of drug through the skin is by passive diffusion. Much lower concentrations pass through the skin and the small amount that does reach the dermis is rapidly cleared by blood flow. The dermis is not saturated with drug nor is there constant active delivery of further drug, therefore the effect of blood flow on drug clearance is much greater. It is not accurate to use data obtained from systemically applied drug studies as a measure of drug clearance by blood flow. The drug is not being cleared but rather delivered to the dermis and the value of 2.2 obtained here is an indication of the balance between the degree of vasoconstriction that results from $5 \times 10^{-4}\%$

noradrenaline, and the ease with which PCV diffuses through an area of dermis that is not affected by the microcirculation delivering or clearing drug.

Steady state of PCV recovery was reached at 180 minutes with a noradrenaline and Ringer's solution perfusate. Steady state was not seen with a plain Ringer's solution perfusate, but rather a decline in PCV concentration from 180 minutes onwards. This suggests that those fibres that were surrounded by a cylindrical core of vasoconstriction (noradrenaline perfusate), the rate-limiting step for PCV recovery was movement of PCV by diffusion from areas of the dermis where PCV delivery was occurring, to the cylindrical vasoconstricted area surrounding each fibre where dialysis was occurring. The presence of such a rate-limiting step has allowed a steady state to be established. It would be compelling to continue the study for longer – up to 7 or 8 hours after famciclovir ingestion to see at what time point steady state is lost. It would be expected to coincide with the time that PCV dialysis in the Ringer's only fibres falls below 55ng ml^{-1} (the dialysis concentration of PCV at steady state in the noradrenaline fibres).

It is interesting that from 180 minutes onwards the PCV concentration in the Ringer's solution fibres starts to fall. This is a reflection of falling plasma drug concentration due to renal excretion.

Dermal concentrations of penciclovir are significantly higher following oral administration than topical application. The high water solubility of penciclovir precludes its oral or topical absorption hence the development of the prodrug famciclovir. The logical step from here would be to formulate a topical form of famciclovir, however this will only be active if converted to penciclovir by cutaneous enzymes capable of deacetylation and oxidation. A further study (which we have conducted in one subject) investigated the percutaneous absorption of famciclovir in an aqua gel vehicle applied topically to both normal and tape stripped skin.

Penciclovir was not detected in the dialysate using an assay that will only identify penciclovir (not famciclovir). This suggests that the skin is not capable of fully metabolizing famciclovir to the active drug penciclovir.

Chapter 9

The investigation of sweat gland pharmacology using microdialysis

Introduction

The anatomy and physiology of the human eccrine sweat gland

Human sweat glands have two major functions which are related in their anatomy and physiology. They allow body cooling by evaporation, and improve grip of the palms and soles at times of activity. The total number of eccrine sweat glands in a normal adult is estimated to be 2 million (Rothman 1953) and is the same in black people as in white people (Champion 1998). They make up only 1/10,000 of the skin surface as their mean diameter at the skin surface is 70μm. The average width of the sweat duct is between 5 and 14μm (Washington *et al*, 1989). The density of sweat glands varies anatomically with the greatest number per unit surface area being found on the palms and soles (440 cm^2), the volar surface of the forearm has 180 per cm^2 and the dorsal surface 175 cm^2. Sweat gland density is lowest on the back (67 cm^2) (Kuno 1934). They are distributed over the whole skin surface but not mucous membranes (Champion 1998).

Embryologically sweat glands are derived from a downgrowth of the surface epithelium. As they grow downwards they reach the subcutaneous fat which they are unable to fully penetrate and so assume a coiled configuration in the lower dermis or upper subcutis (Rothman 1953). The coiled portion, the secretory portion of the gland, is continuous with a straight duct that leads through the dermis to the spiral shaped intraepidermal sweat duct unit which opens onto the surface of the skin. The sweat gland opening is not related to hair follicles and there is no anastomosis between adjacent glands. No new sweat glands develop after birth.

The secretory coil is composed of two types of cell: large clear cells which are thought to be the main secretory cells and small dark cells whose function remains to be discovered. All the cells of the secretory coil, unlike those of the duct are attached to the basement membrane. Outside the basement membrane is an incomplete layer of myoepithelial cells whose function is possibly to help propel sweat out of the secretory fundus and up the duct. The myoepithelial cells contract forcefully in response to acetylcholine (although they are not directly stimulated by α or β adrenergic agents) and share many features of smooth muscle cells (Sato *et al*, 1979). However, their contribution to sweat expulsion is debatable – the myoepithelial cell rate of contraction is slow and maximum contraction in sweat glands induced by a high concentration of K^+ failed to pump out pre-formed sweat from the sweat gland lumen (Sato *et al*, 1979). Myoepithelial cells provide structural support to the secretory epithelium, this may be their main role.

The ductal portion of the eccrine sweat gland is divided into a proximal segment which is coiled and continuous with the secretory tubule and a straight segment which leads to the intraepidermal sweat duct unit. The function of the sweat duct is active reabsorption of sodium chloride and bicarbonate. This occurs predominantly in the proximal duct and results in the isotonic sweat produced by the secretory cells becoming hypotonic.

The secretory coil of the eccrine sweat gland has receptors for alpha-adrenergic, beta-adrenergic, and cholinergic agonists. Cholinergic stimulation produces maximal sweat secretion with a sigmoid shaped dose response curve (Sato 1983). Alpha or beta-adrenergic stimulation results in much lower sweat production; adrenergic sweat is only 10% as copious as cholinergic sweat (Sato 1973). Sweat production following cholinergic and alpha-adrenergic stimulation is dependent on calcium. Studies *in vitro* on isolated sweat glands in Ca^{2+} free medium resulted in

no sweat production (Sato 1983). It is thought that on alpha-adrenergic or cholinergic stimulation there is an increase in secretory cell membrane permeability to extracellular Ca^{2+}. The increased cytosolic [Ca^{2+}] stimulates chloride channels in the luminal membrane and potassium channels in the basolateral membrane, causing a net efflux of KCl from the cell. The decrease in intercellular K^+ and Cl^- provides a favourable chemical potential gradient for Na-K-2Cl cotransporters located in the basolateral membrane. The luminal negative potential attracts Na^+ into the gland lumen across a Na-conductive intercellular junction where it forms NaCl with the Cl^- already in the lumen (Sato 1995).

β-adrenergic stimulation is thought to rely on cAMP rather than Ca^{2+} as an intracellular mediator of sweating (Sato 1995). Cholinergic stimulation does not affect cAMP levels. As adrenergic stimulation of sweating is low it may be that it functions to regulate sweat gland function rather than direct stimulation of activity. Beta-adrenergic stimulation may affect protein and fatty acid synthesis by the sweat gland (Sato 1995). Hence, dual innervation would be instrumental in the synthesis and hypertrophy of the gland.

It is known that sweating can be enhanced by physical training. Fit individuals tend to have larger sweat glands that have increased secretion per unit volume of gland and have high cholinergic sensitivity (Buono *et al*, 1988; Sato *et al*, 1983). Hence the gland hypertrophy of fit individuals may be a result of high levels of adrenergic and cholinergic stimulation. In addition men generally have higher basal sweating rates than women (Fox *et al*, 1969) and sweat production decreases with age (Ellis *et al*, 1976).

Reabsorption of sodium and chloride occurs in the duct by an active transport mechanism (ATP dependent). These ions then leave the cells of the sweat duct via sodium and chloride channels. The presence of ductal acidification or bicarbonate absorption suggests that additional

transport processes such as Na-H exchangers or H pumps may also be involved (Sato 1995). β-adrenergic stimulation promotes NaCl reabsorption in the duct, however it is not known if α-adrenergic stimulation affects sweat tonicity.

The function and morphology of sweat glands varies between species, so it is unwise to draw conclusions from animal studies about the physiology and anatomy of human sweat glands (McEwan Jenkinson 1973). Therefore, most of the useful research into sweat gland pharmacology and physiology has been *in vitro* using human sweat ducts from excised skin and exposing them to different mediators in water baths. The human *in vivo* work typically involves a subdermal injection of mediator followed by measurement of the sweat gland response.

Microdialysis is a novel way of continuously delivering the pharmacological agent of choice to the skin to measure sweat gland function with time. We have used the cholinergic agonist pilocarpine, cholinergic antagonist atropine and adrenergic agonist noradrenaline to investigate sweat gland function. Each of these mediators are outlined below:

Pilocarpine

Pilocarpine is a direct acting muscarinic parasympathomimetic agent that is used as a miotic in the treatment of glaucoma, and in the diagnostic sweat test of cystic fibrosis. It is a tertiary amine with a molecular weight of 208 (so will freely move through the pores of a 2 kDa microdialysis membrane) and is produced commercially as either the hydrochloride or nitrate salt. Pilocarpine shows some selectivity in stimulating secretion from sweat, salivary, lacrimal and bronchial glands, with only weak effects on gastrointestinal smooth muscle and the heart (Rang *et al*, 1999). The cystic fibrosis sweat test utilizes a 0.5% solution of pilocarpine nitrate which is introduced into the skin by iontophoresis. At this concentration pilocarpine does not

cause systemic effects. The pilocarpine acts directly on the sweat glands as an acetylcholine mimetic to stimulate sweat production and the sweat produced is analyzed for its sodium chloride and potassium concentration. Pilocarpine nitrate is a hydrophilic drug (water solubility 1g in 4ml water), and so is suitable to be used as a microdialysis perfusate for the direct stimulation of sweat glands.

Atropine

Atropine is a muscarinic antagonist that has both peripheral and central effects. Like pilocarpine, it is a tertiary amine. Atropine is used in the treatment of gastrointestinal disorders, bradycardia and in ophthalmology as a cycloplegic and mydriatic. The sterile form for intravenous administration during cardiac arrythmias is atropine sulphate. This has a molecular weight of 695 and is very soluble in water (1g in 0.5ml) (Reynolds 1982a), so is suitable for use (at an appropriately low concentration) as a perfusate for microdialysis.

Noradrenaline

Noradrenaline is the transmitter substance released by postganglionic sympathetic neurons. It stimulates both α and β adrenergic receptors although it has higher selectivity for α receptors. Noradrenaline is a small molecule with a molecular weight of 337 (Reynolds 1982b) and is soluble in water (200mg ml^{-1}), so will easily move through the pores of the 2kDa microdialysis fibres. As was seen in the previous chapters, it is a potent vasoconstrictor through stimulation of α_1 receptors on blood vessels.

The stimulation of sweat glands *in vivo*

1. Pilocarpine:

Study a) Dose-response of pilocarpine-induced sweating in male and female subjects

i. Pilocarpine concentration less than 0.05%

Six microdialysis fibres were inserted into the forearm of four subjects (2 male and 2 female). Care was taken to ensure that the area of skin adjacent to the wrist was not involved as this area has a higher basal rate of transepidermal water loss (Panisset *et al*, 1992). Each of the fibres was perfused with an increasing concentration of pilocarpine nitrate by a factor of 10 - from 5×10^{-6} % to 5×10^{-2}%. One fibre in each subject was perfused with plain Ringer's solution. The perfusion rate for each fibre was 5μl minute^{-1}. The dialysate was collected in Eppendorf tubes to prevent its leakage onto the skin which might affect transepidermal water loss readings. The tubes were discarded at the end of the study.

Transepidermal water loss (TEWL) readings of the skin overlying each fibre were taken every 20 minutes. The TEWL of an area of skin on the contralateral forearm was also measured every 20 minutes to control for differences in TEWL over the study period not caused by sweating stimulation. Arterial blood pressure and pulse were monitored hourly to check that there was not significant systemic absorption of drug causing central effects.

Results

Erythema was seen along the length of fibre at a 0.05% concentration of pilocarpine nitrate in all 4 subjects, however was not evident with the lower concentrations of pilocarpine. Blood pressure and resting pulse rate were not affected by the concentrations of pilocarpine used,

nor did the subjects complain of excessive salivation, tear secretion or other cholinergic mediated side effects.

In all four subjects the transepidermal water loss measurement of control skin (contralateral arm) varied minimally over the 5 hour study:

	Mean TEWL control skin over 5 hours ± SEM
Male I	8.7 ± 0.1 g m^{-2} h^{-1}
Male II	11.5 ± 0.2 g m^{-2} h^{-1}
Female I	7.7 ± 0.1 g m^{-2} h^{-1}
Female II	7.0 ± 0.1 g m^{-2} h^{-1}

Table 9.1. The mean transepidermal water loss (basal sweating rate) in four subjects at rest over the 5 hour study duration. Measurements were taken from the volar surface of the right forearm.

In addition, the perfusion of plain Ringer's solution did not significantly increase transepidermal water loss as compared with control skin, therefore the effect of pilocarpine nitrate on sweating was calculated as the percentage increase in TEWL compared with plain Ringer's solution.

In both female subjects a concentration of $5x10^{-5}$ % pilocarpine nitrate was needed to significantly increase sweating rate above baseline (Ringer's solution) level ($p<0.001$, ANOVA) (figure 9.1). $5x10^{-6}$% pilocarpine nitrate did not increase sweat gland activity. There was a dose response of sweating with increasing pilocarpine concentration up to the highest concentration of 0.05%. The three highest concentrations of pilocarpine each caused maximal sweating at 80 minutes from the start of perfusion. From 180 minutes to the end of the study (80 minutes to the

end for $5x10^{-5}$% pilocarpine nitrate) a steady state of sweating was achieved with the 4 higher concentrations of pilocarpine. There was no significant difference in the increase in sweating caused by 0.05% pilocarpine as compared with 0.005% (p=0.13, ANOVA).

The average rate of transepidermal water loss (per 20 minute time point) measured for each of the concentrations of pilocarpine nitrate is shown in table 9.2.

Pilocarpine concentration	**Mean Transepidermal Water Loss (± SEM)**
0.05 %	47.8 ± 0.9 g $m^{-2} h^{-1}$
5 x 10^{-3} %	44.8 ± 0.9 g $m^{-2} h^{-1}$
5 x 10^{-4} %	29.9 ± 2.0 g $m^{-2} h^{-1}$
5 x 10^{-5} %	13.2 ± 0.7 g $m^{-2} h^{-1}$
5 x 10^{-6} %	8.6 ± 0.3 g $m^{-2} h^{-1}$
Ringer's solution	9.0 ± 0.4 g $m^{-2} h^{-1}$

Table 9.2 The mean rate of sweating over 5 hours for each concentration of pilocarpine in the 2 female subjects. There was no significant difference in TEWL between Ringer's solution and 5 $x10^{-6}$ % pilocarpine (ANOVA). The difference was significant between Ringer's solution and the four higher concentrations of pilocarpine.

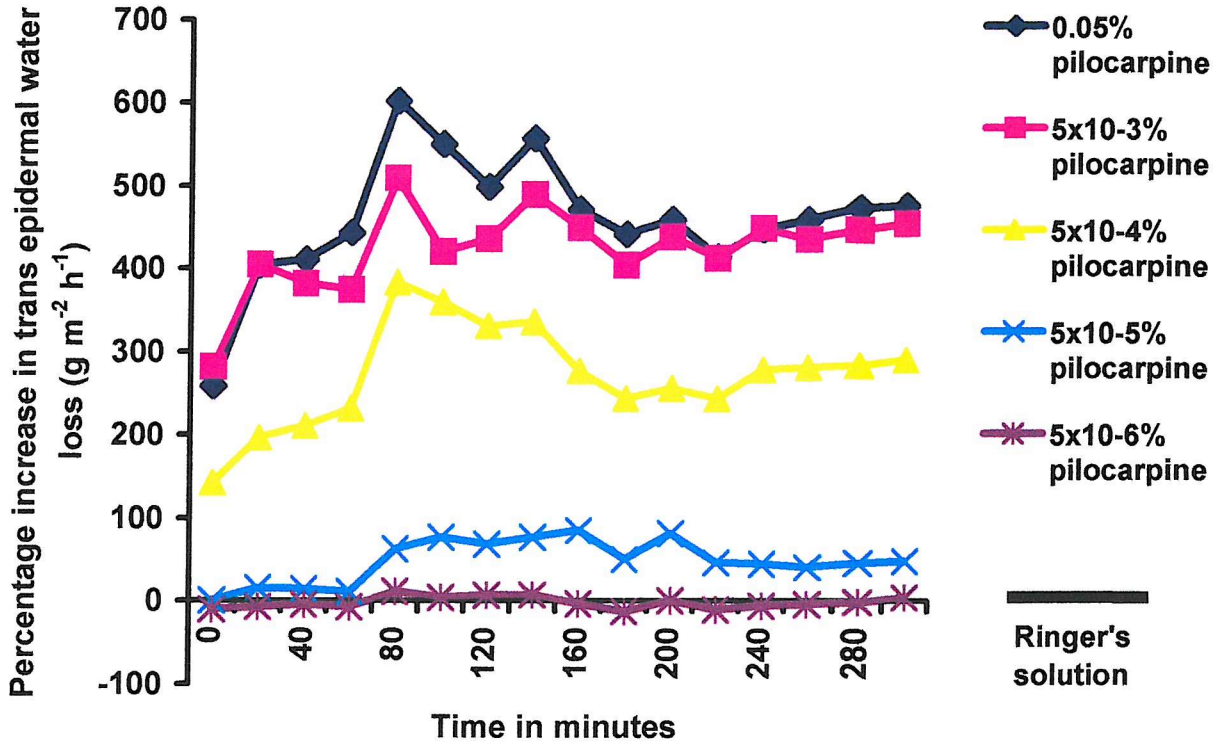


Figure 9.1 The mean effect of 5 different concentrations of pilocarpine nitrate (delivered continuously by microdialysis fibres) on TEWL in 2 female subjects. The TEWL is presented as the percentage change in TEWL compared with that caused by the perfusion of plain Ringer's solution.

Both male subjects had a higher basal rate of sweating as compared with the female subjects (table 9.1). The percentage increase in TEWL (over plain Ringer's solution perfusate) with time for each concentration of pilocarpine is shown in figures 9.2. The lowest concentration of pilocarpine ($5x10^{-6}$ %) significantly increased sweating rate above baseline (Ringer's solution) level ($p<0.001$, ANOVA). Increasing pilocarpine concentration caused a dose response increase in TEWL up to the highest concentration of pilocarpine of 0.05%. There

was a significant difference in TEWL induced by 0.05% pilocarpine compared with $5x10^{-3}$ % pilocarpine ($p<0.001$, ANOVA) with the more concentrated pilocarpine solution causing a higher rate of sweating. The greatest rate of sweating was at 100 to 120 minutes for the 3 highest concentrations of pilocarpine. A steady state of sweating was seen from 180 minutes to the end of the 5 hour study with these 3 solutions. The 2 lower concentrations of pilocarpine (5×10^{-5}% and 5×10^{-6}%) both caused a consistent degree of sweating from 100 minutes until the end of the study.

Table 9.3 demonstrates the mean TEWL (per 20 minute time point) for each of the concentrations of pilocarpine nitrate in the 2 male subjects.

Pilocarpine concentration	**Mean Transepidermal Water Loss (± SEM)**
0.05 %	77.2 ± 1.8 g $m^{-2} h^{-1}$
5×10^{-3} %	62.6 ± 1.4 g $m^{-2} h^{-1}$
5×10^{-4} %	57.0 ± 2.1 g $m^{-2} h^{-1}$
5×10^{-5} %	16.3 ± 1.3 g $m^{-2} h^{-1}$
5×10^{-6} %	12.5 ± 0.4 g $m^{-2} h^{-1}$
Ringer's solution	11.2 ± 0.3 g $m^{-2} h^{-1}$

Table 9.3 The mean rate of sweating over 5 hours for each concentration of pilocarpine in the 2 male subjects.

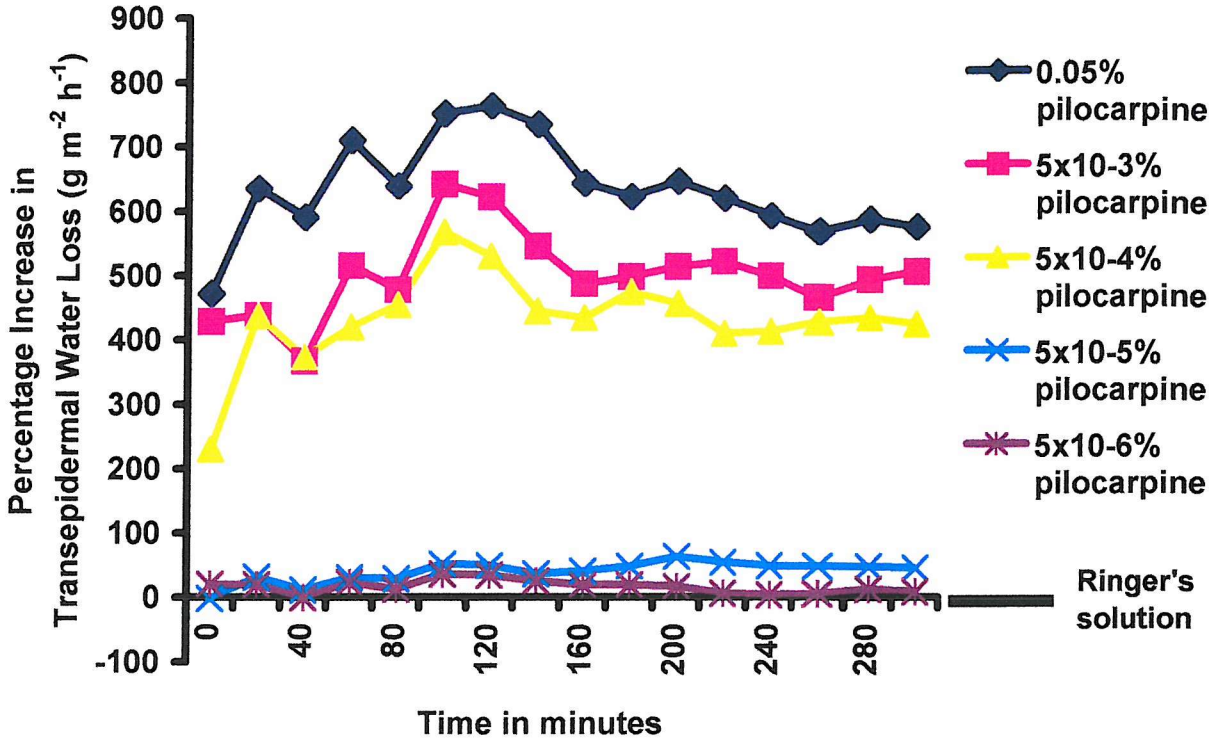


Figure 9.2 The mean effect of 5 different concentrations of pilocarpine nitrate (delivered continuously by microdialysis fibres) on TEWL in 2 male subjects.

The sex difference in pilocarpine mediated sweating is shown in figures 9.3a and 9.3b. At 60 minutes and 180 minutes into the study the mean percentage increase in sweating rate above that of Ringer's solution has been plotted for the 2 male and 2 female subjects. Pilocarpine nitrate caused a dose response increase in TEWL in both sexes. This was most evident for a concentration of 5×10^{-4} % pilocarpine and higher. The increase in TEWL over delivery of Ringer's solution was greater in the male subjects than the female subjects at all pilocarpine concentrations.

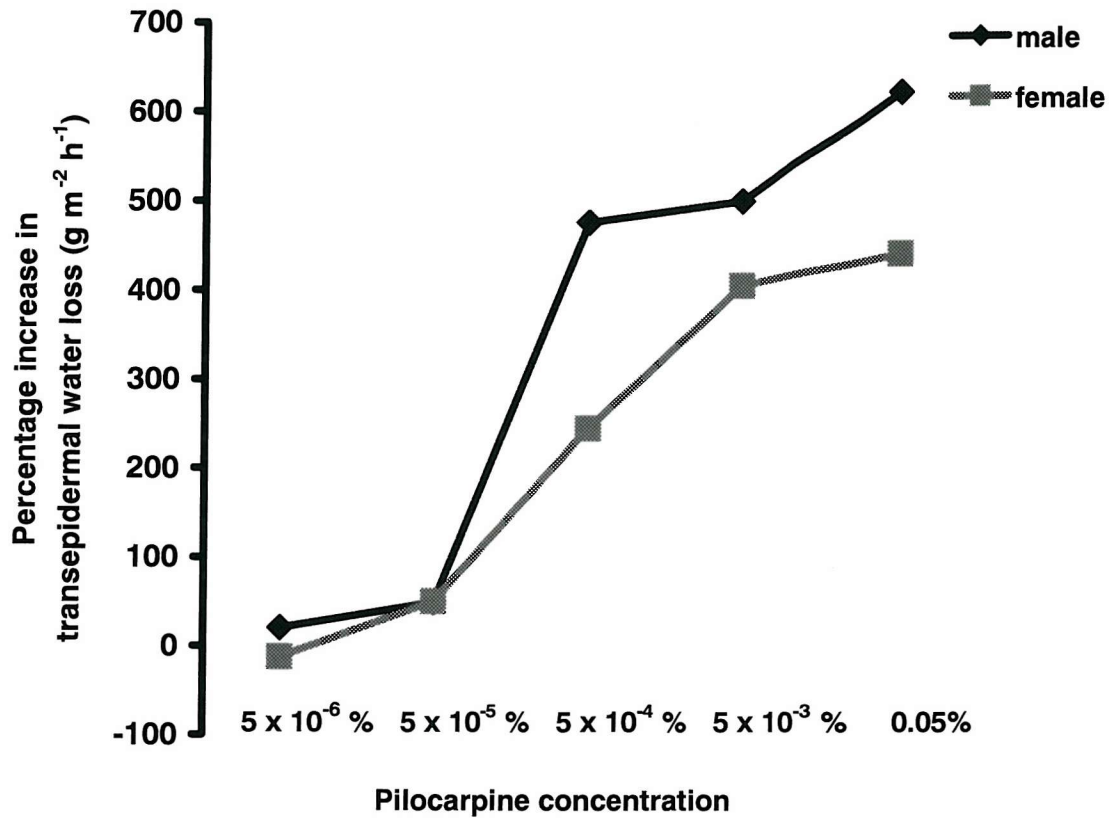


Figure 9.3a The increase in transepidermal water loss by continuous dermal delivery (via microdialysis fibres) of 5 concentrations of pilocarpine nitrate at 60 minutes in 2 male and 2 female subjects. Baseline level is the TEWL with delivery of plain Ringer's solution.

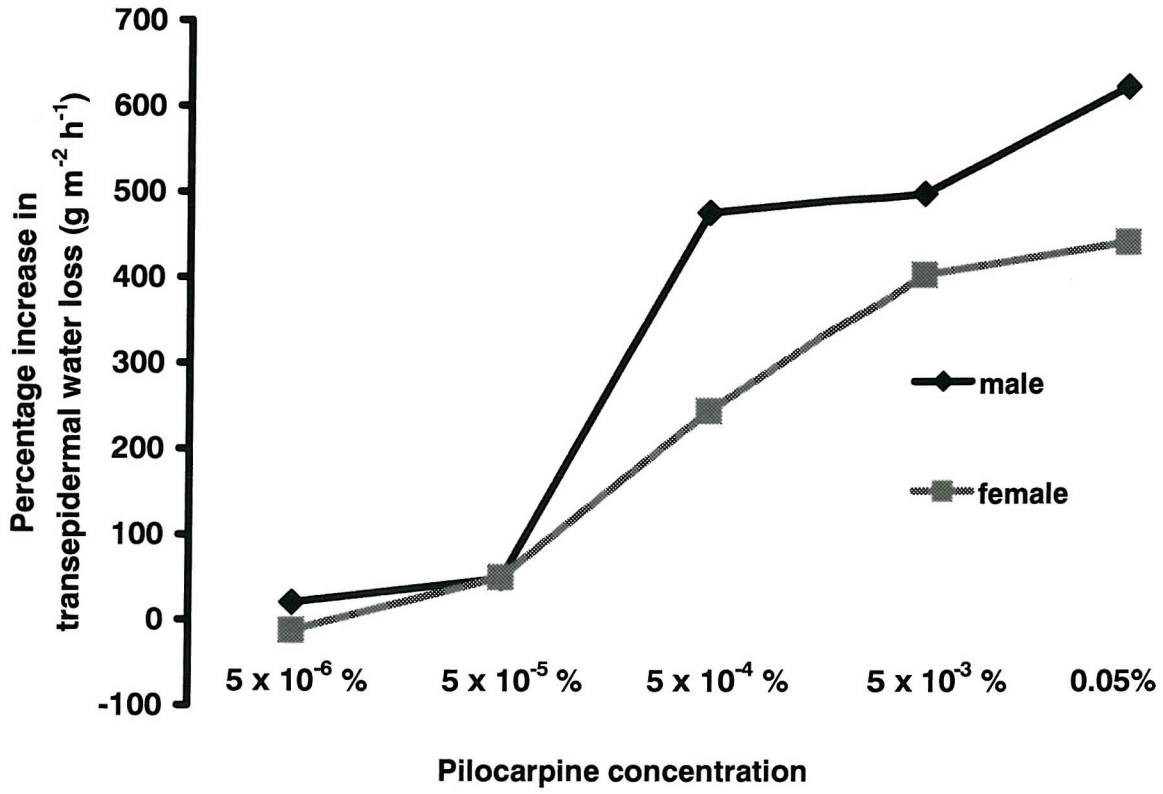


Figure 9.3b The increase in transepidermal water loss by continuous dermal delivery (via microdialysis fibres) of 5 concentrations of pilocarpine nitrate at 180 minutes in 2 male and 2 female subjects. Baseline level is the TEWL with delivery of plain Ringer's solution.

The close relationship between pilocarpine concentration and sweating is shown in figures 9.4a and 9.4b. These figures are similar to figures 9.3a and 9.3b and shown the percentage increase in sweating at 60 and 180 minutes for 5 x 10^{-5} % pilocarpine and above. At 60 minutes the correlation between sweating and pilocarpine concentration was: male $R^2 = 0.93$, female $R^2 = 0.95$; and at 180 minutes: male $R^2 = 0.81$, female $R^2 = 0.93$.

Mean fibre depth for the 4 subjects was 1.02 ± 0.1mm (SEM). Fibres were deliberately placed slightly deeper than is usual practice in order to place them in close vicinity to the secretory portion of the dermally sited eccrine sweat glands.

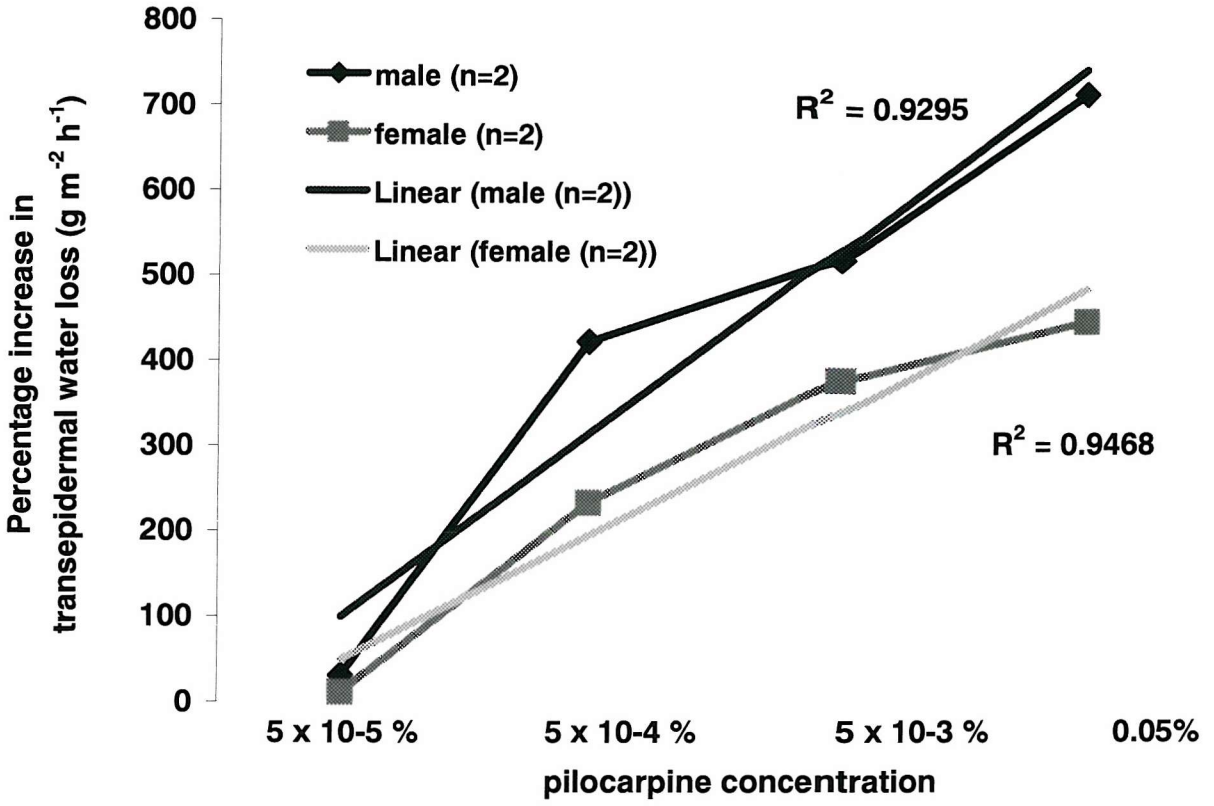


Figure 9.4a The dose response of transepidermal water loss by continuous dermal delivery (via microdialysis fibres) of 4 concentrations of pilocarpine nitrate at 60 minutes in 2 male and 2 female subjects. The baseline (zero) is the TEWL with delivery of plain Ringer's solution.

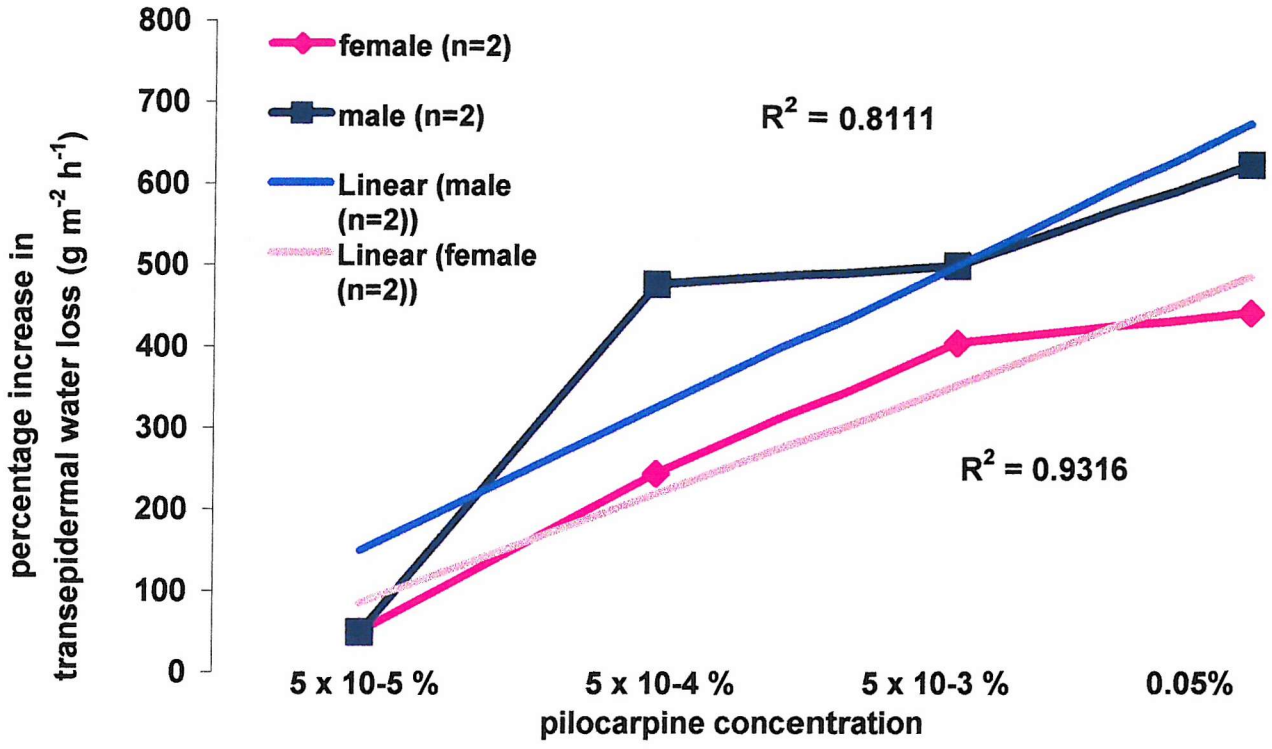


Figure 9.4b The dose response of transepidermal water loss by continuous dermal delivery (via microdialysis fibres) of 4 concentrations of pilocarpine nitrate at 180 minutes in 2 male and 2 female subjects. The baseline (zero) is the TEWL with delivery of plain Ringer's solution.

ii. Pilocarpine concentration greater than 0.05%

In a further subject (female aged 27) the effect of higher concentrations of pilocarpine nitrate on sweat gland function was measured. Six microdialysis fibres were inserted into the volar surface of the forearm. Fibres were randomized to be perfused with 0.5%, 0.05% or 0.005% pilocarpine nitrate (2 fibres per concentration). Flow rate was 5μl minute^{-1} and TEWL measurements were taken every 20 minutes for 320 minutes. Blood pressure and pulse were monitored throughout the study and fibre depth was measured using ultrasound at the end.

Results

The 0.5% and 0.05% concentrations of pilocarpine produced quite marked erythema extending for 1mm on either side of the fibre length. All three concentrations of pilocarpine produced similar rates of sweating with no significant difference in TEWL between any of the three concentrations. The mean rate of TEWL per time point at each of the three pilocarpine concentrations was: 0.5% 35.3 g m^{-2} h^{-1}; 0.05% 38.8 g m^{-2} h^{-1} ; 0.005% 36 g m^{-2} h^{-1} (figure 9.5). There was a drop in TEWL over the first two hours of the study from an average of approximately 50 g m^{-2} h^{-1} to a steady state for the remainder of the study of approximately 35 g m^{-2} h^{-1}. This was seen with all three concentrations of pilocarpine nitrate.

Fibre depth was a mean of 1.07 ± 0.04mm (SEM), and did not affect sweating rates.

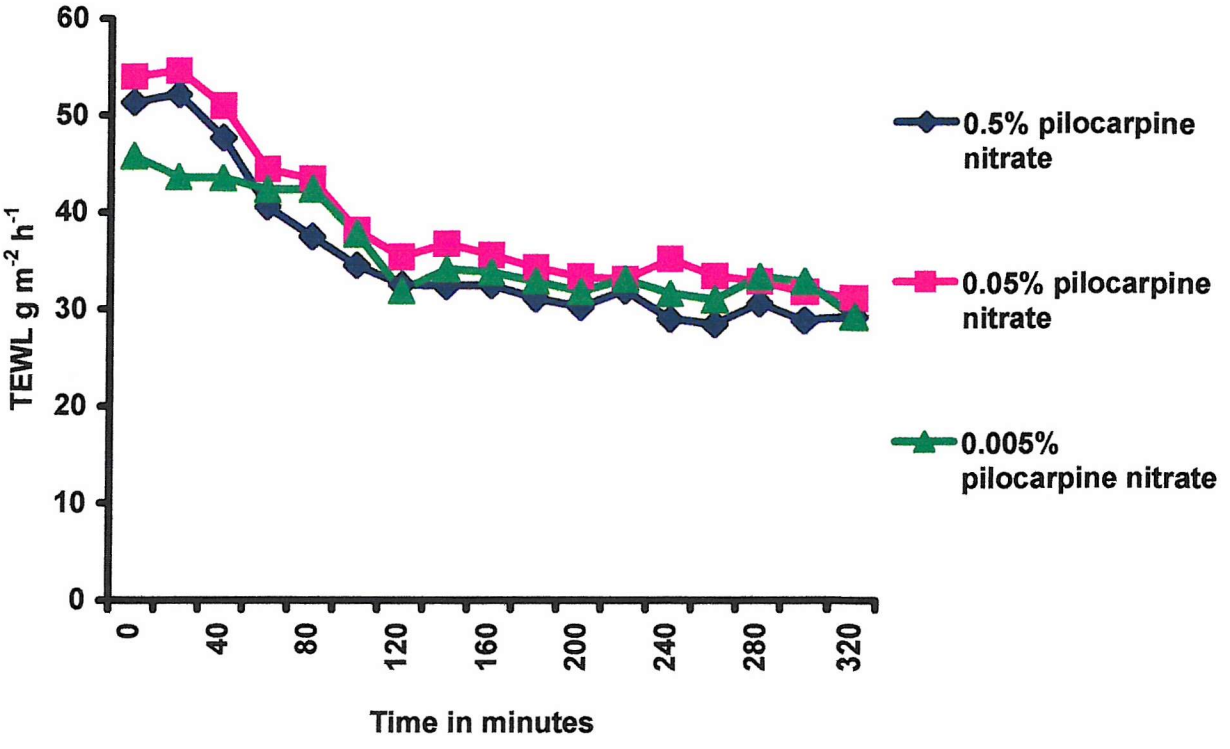


Figure 9.5 The effect of three different concentrations of pilocarpine nitrate (continuously delivered to the dermis by microdialysis fibres) on sweating as measured by transepidermal water loss in one subject.

2. Noradrenaline:

The effects of pilocarpine and noradrenaline on sweating

In 4 subjects (1 male, 3 female) the effect of continuous stimulation of sweat glands by pilocarpine, noradrenaline and a combination of pilocarpine with noradrenaline was investigated. Six fibres were inserted per subject with randomization of perfusate to fibre site. The concentrations of agonist used were:

0.166% pilocarpine nitrate in Ringer's solution.

5×10^{-4} % noradrenaline in Ringer's solution.

0.166% pilocarpine nitrate with 5×10^{-4} % noradrenaline in Ringer's solution.

As before, TEWL measurements were taken every 20 minutes for 5 hours, and readings taken from the contralateral arm as a control site.

Results

Data is presented as the increase in sweating above that of the control area of skin (i.e. TEWL of skin overlying fibre minus TEWL of control skin). It was not possible to have an additional Ringer's solution only fibre (6 perfusion pumps only available), and as has been found previously the perfusion of plain Ringer's solution does not significantly affect transepidermal water loss measurement as compared with normal skin. All subjects tolerated the perfusates well with no reported side effects.

There was a marked sex difference in the sweating response to the three different perfusates. Pilocarpine (0.166%) – induced sweating (TEWL) for the 4 subjects is shown in figure 9.6. There was no significant difference in TEWL between the two females aged 22 or either young female and the older female. However sweating was significantly different between

the male subject and both females aged 22 ($p < 0.005$ and $p < 0.0005$ (ANOVA)), and between the male and older female ($p < 0.0005$). There was a reasonably close correlation of sweating at each time point between the two females of the same age ($r = 0.7$).

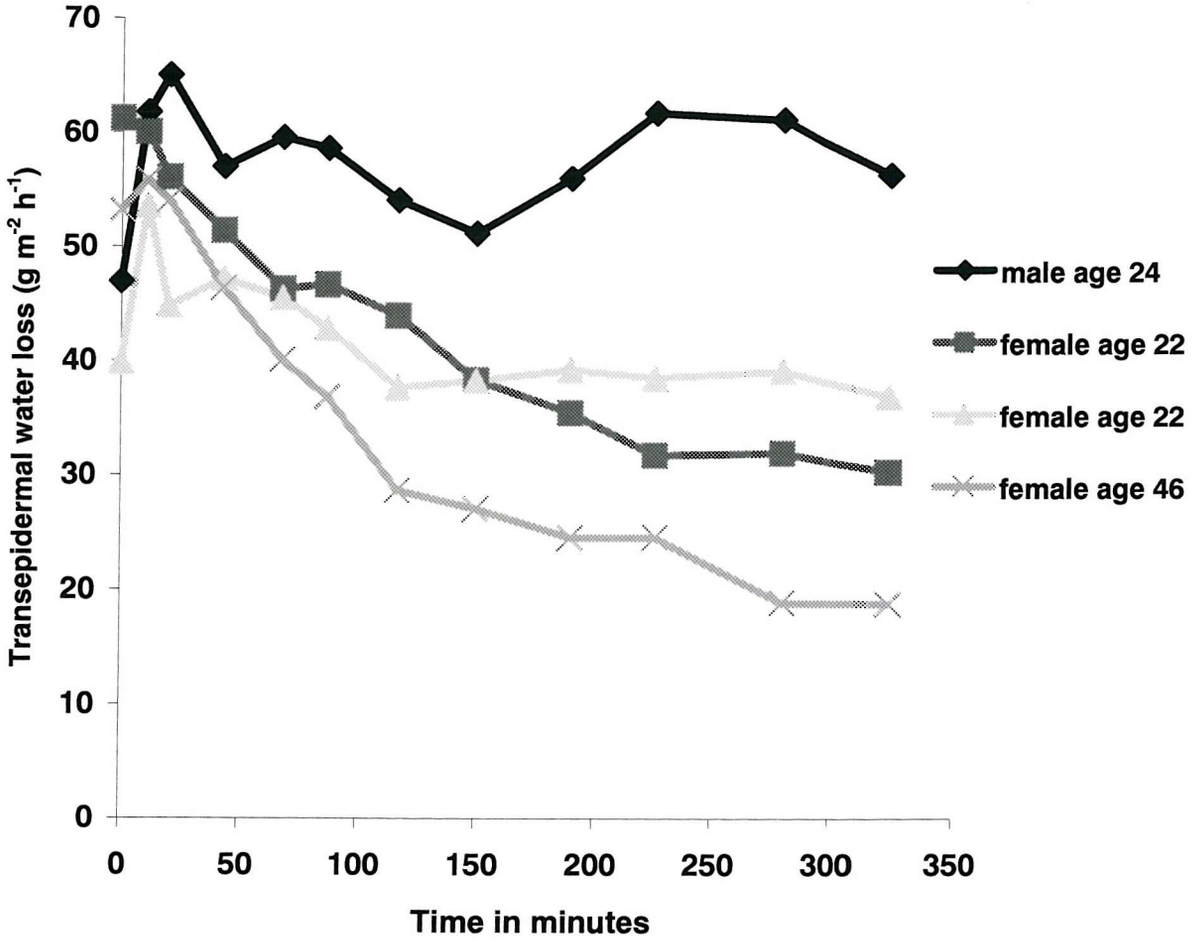


Figure 9.6 Increase in TEWL above normal skin with 0.166% pilocarpine nitrate as the perfusate in 4 subjects.

Noradrenaline–induced sweating for the 4 subjects is shown in figure 9.7; sweating was in general much lower than with pilocarpine. TEWL was significantly higher in the male subject compared with the three female subjects ($p < 0.0005$ for the older female and one of the younger female subjects, and $p < 0.001$ for the second younger female). In 2 subjects an increase in TEWL was seen for only the first hour of the study, after that noradrenaline had a minimal effect on sweat production.

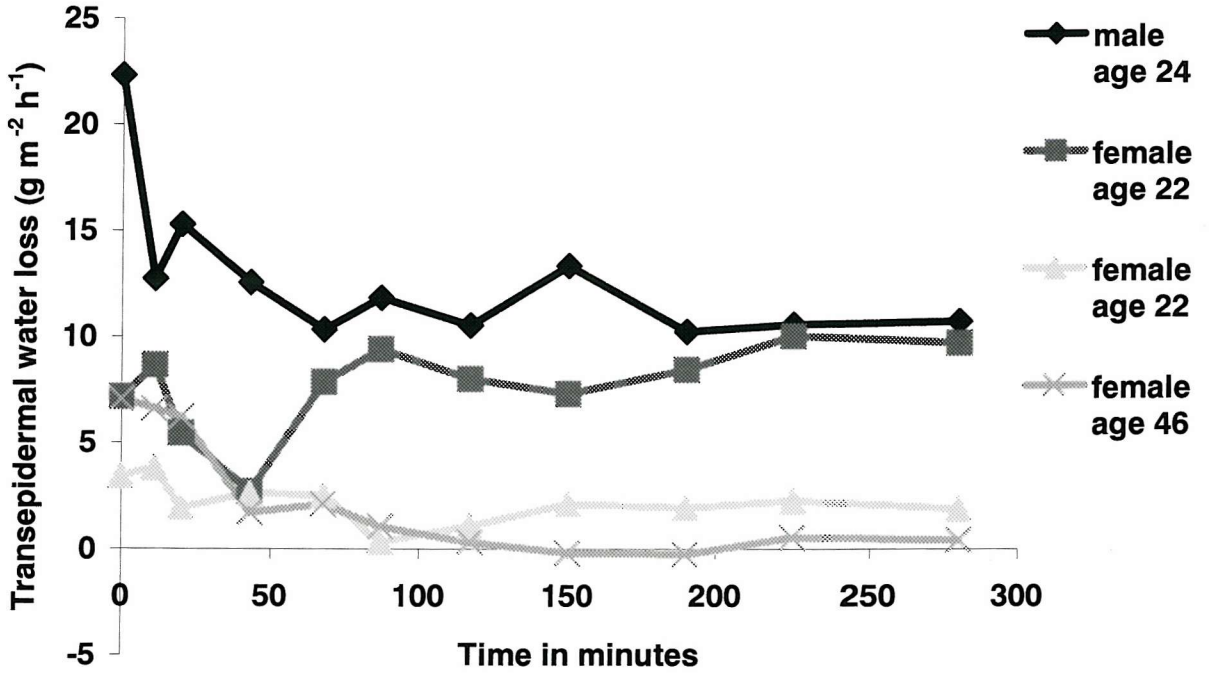


Figure 9.7 Increase in TEWL above normal skin with 5 x 10-4 % noradrenaline as the perfusate.

The mean TEWL with the three different perfusates is shown in figure 9.8. Sweating stimulated by pilocarpine alone was significantly higher than that induced by pilocarpine and noradrenaline combined ($p<0.0005$ (2 tailed paired student t test)), and sweating stimulated by either pilocarpine or pilocarpine with noradrenaline was significantly higher than that for noradrenaline alone ($p<0.0005$ (2 tailed paired student t test)). Sweating was maximal at 20 minutes with both pilocarpine-containing perfusates and peaked from the start of perfusion (0 minutes) with plain noradrenaline stimulation. Maximal sweating rates were: Pilocarpine 58.4g m^{-2} h^{-1}; pilocarpine and noradrenaline 53.8g m^{-2} h^{-1}; noradrenaline 10g m^{-2} h^{-1}. Steady state was obtained in all groups; at 120 minutes for plain pilocarpine (38.7g m^{-2} h^{-1}) and pilocarpine and noradrenaline (28.4g m^{-2} h^{-1}); and at 40 minutes for noradrenaline (5.4g m^{-2} h^{-1}).

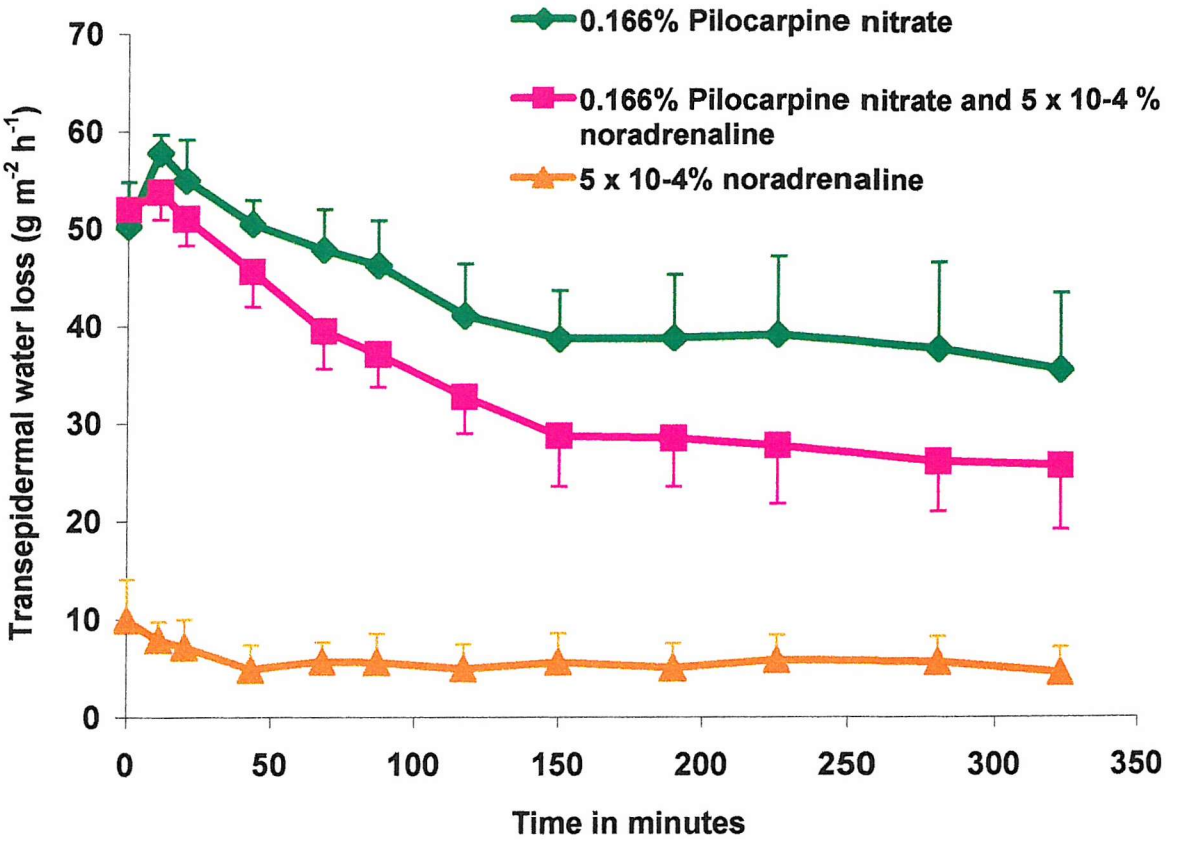


Figure 9.8. Transepidermal water loss above that of control skin with three different perfusates in 4 subjects. Error bars are SEM.

3. Atropine:

The effects of atropine on sweating induced by pilocarpine or noradrenaline

In three subjects (one male and two females), six microdialysis fibres were inserted and perfused with pilocarpine, noradrenaline or atropine (2 fibres per variable). As before flow rate was 5μl minute^{-1} and TEWL measurements were taken every 20 minutes. At two hours the perfusate in 1 fibre of each pair was changed as detailed in table 9.4 and TEWL measurements continued for a further 3 hours.

	Perfusate 0-2 hours	**Perfusate 2-5 hours**
Fibre 1	Noradrenaline	Noradrenaline
Fibre 2	Noradrenaline	Noradrenaline & Atropine
Fibre 3	Atropine	Atropine
Fibre 4	Atropine	Atropine and noradrenaline
Fibre 5	Pilocarpine	Pilocarpine
Fibre 6	Pilocarpine	Pilocarpine & Atropine

Table 9.4 Study protocol for the investigation of dual stimulation of eccrine sweat glands with a muscarinic agonist and antagonist, and an adrenergic agonist and muscarinic antagonist.

Results

Results are given as the change in TEWL as compared with control skin on the contralateral arm (figure 9.9). Perfusion with atropine alone reduced sweating to below basal levels from 60 minutes into the study until the end. Perfusion with noradrenaline caused a similar increase in sweat rate as that seen in study 2 (figure 9.7). The addition of atropine to the noradrenaline perfusate did not affect sweat rate as compared with noradrenaline alone. This effect was further seen when noradrenaline was added to an atropine perfusate, the TEWL increased from below basal levels to a 100% increase above that of control skin. This level was

similar to the noradrenaline only perfusate. The addition of atropine to the pilocarpine perfusate caused the sweating rate to fall rapidly (within one hour) from 350% that of control skin to below basal levels.

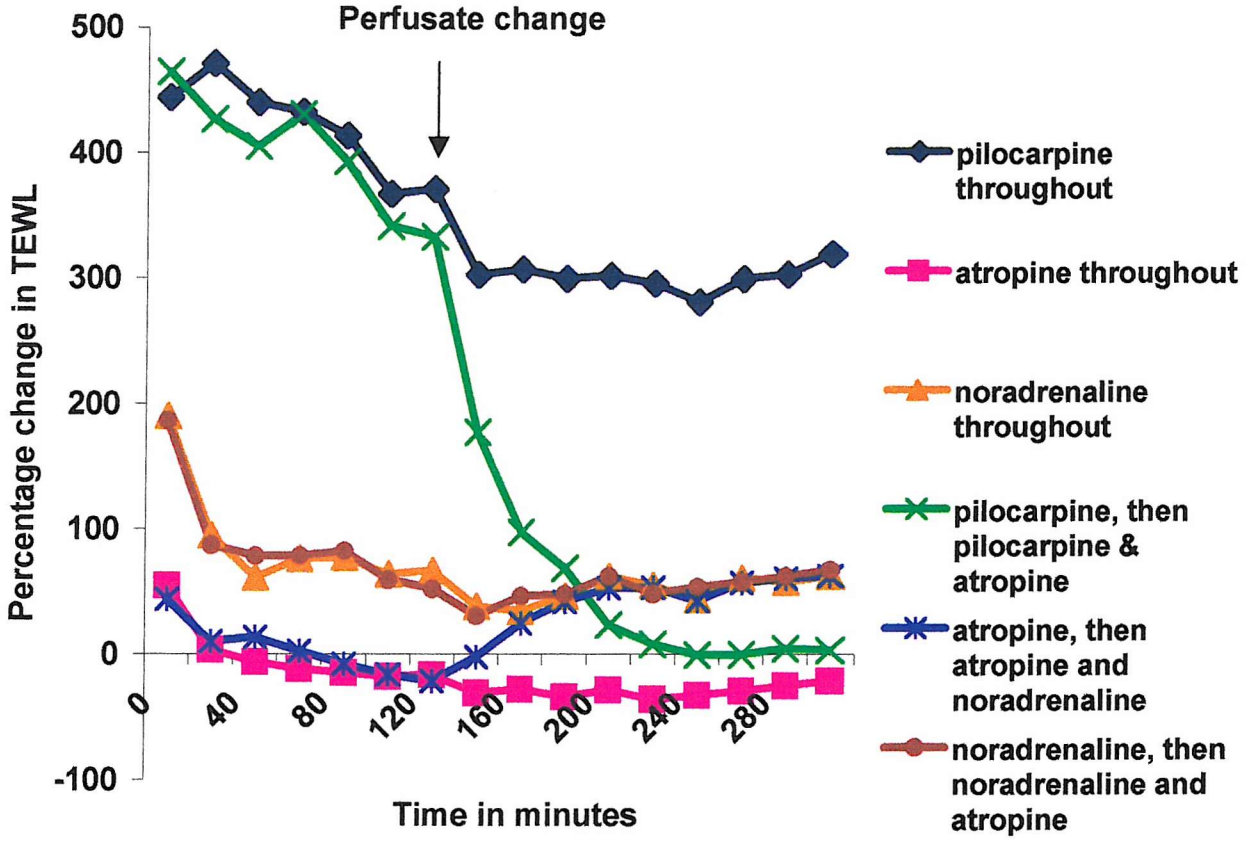


Figure 9.9 The effect of continuous sweat gland stimulation with noradrenaline, atropine or pilocarpine on sweat gland activity in 3 subjects.

Discussion

Most previous work on sweat gland physiology and pharmacology has involved *in vitro* work using isolated human sweat glands, animal studies or the intradermal injection of pharmacological agonists and antagonists *in vivo* at single time points. Microdialysis enables the continuous stimulation of sweat glands – in these studies up to 5 hours, with measurement of sweat gland activity. This unique method of investigating sweat gland function has highlighted some of the differences in sweating between the sexes and the effect of co-administration of both sweat gland agonists and antagonists.

With a resting individual and baseline measurements of control skin taken, transepidermal water loss is an accurate means of measuring sweat gland activity. Here we have used TEWL as a means of quantifying sweating with time, pharmacological agonist, sex and age.

In study a) i) sweating is depicted as the increase in TEWL above that caused by Ringer's solution. As subjects vary in their baseline sweating rates it is more accurate to compare a change in sweating rate per subject (here described as the percentage increase in sweating above that caused by a non active dialysate) rather than grouping together results. This would result in a loss of meaningful data. Baseline sweating was greater in the male subjects than the female. This is as expected; it is known that men have a higher basal rate of sweating than women at a range of skin temperatures (Fox *et al*, 1969).

A 5×10^{-6} % concentration of pilocarpine was sufficient to cause significant sweating in the male subjects. However in the female subjects a higher concentration of pilocarpine (5×10^{-5} %) was needed. It is known that female sweat glands demonstrate a higher threshold of sweating than male (Fox *et al*, 1969), that is, the stimulus for sweating (in this case an infusion of

pilocarpine) has to be of a greater concentration to induce sweating in women as compared with men.

The increase in sweating above basal level was greater in the male subjects than in the female at all pilocarpine concentrations. Therefore male sweat glands are both more sensitive to lower concentrations of pilocarpine and also demonstrate a higher rate of sweating following pilocarpine stimulation than female sweat glands. It is not known whether this sex difference is due a higher rate of sweating per gland in male subjects or the result of a greater recruitment of active sweat glands, with female sweat glands needing a higher concentration of agonist to stimulate individual sweat gland activity.

For each concentration of pilocarpine the highest rate of sweating was seen in the first two hours of the study with a slight fall in sweating rate after this time. This was seen in both male and female subjects. Such a time course of sweating with continuous stimulation by different pharmacological agonists has not been reported previously.

There was a very close correlation between the rate of sweating and concentration of pilocarpine from 5 x 10^{-5} % to 0.05%. This was seen in both sexes and continued throughout the 5 hour study duration although the correlation became slightly less close with time (table 9.4).

	male	**female**
60 minutes	0.93	0.95
180 minutes	0.81	0.93
300 minutes	0.83	0.89

Table 9.5 The correlation (R^2) between pilocarpine concentration and mean sweat rate for male and female subjects at 60, 180 and 300 minutes post start of pilocarpine perfusion.

All 4 subjects described themselves as being 'moderately fit', that is taking part in a form of reasonably strenuous exercise at least once a week. It is known that athletic individuals have sweat glands that respond to a lower concentration of agonist and produce a higher rate of

sweating per sweat gland. Physical training results in sweat gland hypertrophy although the number of sweat glands does not change. Therefore in a study comparing sweat rates between the sexes it would be more accurate to recruit a much higher number of subjects so that any differences in overall fitness and therefore 'induction' of sweat glands would become less important. Despite low numbers in this study (n=2 per sex), the difference in sweat rates between male and female subjects for the concentrations of pilocarpine from $5 \times 10^{-4}\%$ to 0.05% were highly significant.

The perfusion of plain Ringer's solution did increase transepidermal water loss as compared with normal (control) skin, however this difference was not significant. The increase may represent a slight increase in evaporation from the skin due to an increase in dermal hydrostatic pressure as fluid is pumped through the dermis. Alternatively it could be a function of measurement site on the forearm. Transepidermal water loss does vary according to forearm site – the wrist tends to have a slightly higher TEWL (Panisset *et al*, 1992). This has previously been attributed to emotional sweating. In all 4 subjects the position of each pilocarpine concentration and Ringer's solution was randomly allocated within the forearm. Hence close proximity to the wrist would not account for the higher (but not significant) TEWL seen with the perfusion of Ringer's solution. There have been no published studies examining the difference in TEWL between the dominant and non dominant arm in subjects. Here we have used the left arm as the experimental site with the right arm acting as control. Most of the subjects would have been right handed. If there is a difference in TEWL between the dominant and non dominant forearms it is likely that this difference is small and so would not affect the significant results seen here.

This study (i) did not establish what the maximum rate of sweating was with increasing pilocarpine concentration - the sweating response had not yet reached a plateau with increasing pilocarpine strength in the male subjects, there was a significant difference in the rate of sweating with the two greatest (0.05% and 5 x 10^{-3}%) concentrations of pilocarpine. With the female subjects the two highest concentrations of pilocarpine nitrate (0.05% and 5x10^{-3}%) resulted in similar sweating rates hence it is likely in females the maximal sweating rate had been reached. In order to investigate this, the concentration of pilocarpine nitrate used for cystic fibrosis sweat tests (0.5%) was perfused and sweating measured in a further subject (study ii).

At the higher concentrations of pilocarpine there was a general trend of sweating to lessen with time, this was not seen with the lower concentrations of pilocarpine. This was evident at all three concentrations of pilocarpine (0.5% to 0.005%) in study ii. For this subject a concentration of 0.005% pilocarpine maximally stimulated sweat glands with no further increase seen with the higher doses. In this female subject the very high TEWL rates seen in the male subjects of study i) were not attained.

The pattern of the graph of TEWL against time (figure 9.5) is seen throughout the study with high doses of pilocarpine; there is an initial fall in TEWL over the first two hours of the study and then sweating rate reaches a steady state at which it remains for the rest of the study. This fall in TEWL is not due to fluid being pumped through the dermis as it is not seen with the fibres perfused with plain Ringer's solution. It is also unlikely to be due to the vasodilatation caused by pilocarpine as TEWL is unaffected by vasoconstriction or vasodilation (Pinnagoda *et al*, 1990). Rather it most likely represents the sweat glands' inability to sustain such a high output of sweat, possibly due to a local deficiency of Ca^{2+}. This feature of sweat gland function has not been examined previously as research into the stimulation of sweating *in vivo* has

typically been by a single injection of agonist, short term administration of agonist by iontophoresis or continuous stimulation *in vitro* by placing isolated sweat glands in baths of various agonists or antagonists.

Both cholinergic and adrenergic stimulation cause sweating, however stimulation of the sweat glands with a mixture of pilocarpine and noradrenaline causes less sweating than pilocarpine alone (study 2). This may be due to noradrenaline-induced vasoconstriction resulting in a reduced supply of calcium, oxygen and other nutrients reaching the sweat gland or the reduced clearance of waste products. Alternatively the reason may be due to an imbalance between sweat production and reabsorption: Combined cholinergic and adrenergic stimulation of the sweat gland secretory cells may cause less sweat production by the secretory cells than cholinergic alone, or noradrenaline may be enhancing the reabsorption process in the sweat duct. It is known that beta-adrenergic stimulation enhances sweat reabsorption in the sweat duct, and noradrenaline does have some activity in stimulating beta-adrenergic receptors. Reabsorption in the sweat duct is due to the active process of uptake of sodium ions by the Na^+ pump situated in the basal ductal cell membrane. Chloride movement is also an active process, and there are thought to be sodium and chloride channels in the luminal membrane. What makes this explanation less likely is that although ductal reabsorption of salt results in a hypotonic sweat the same volume of sweat should be produced and so transepidermal water loss would be the same.

It is of interest that the shape of the pilocarpine and pilocarpine with noradrenaline TEWL-versus-time curves were very similar, with an initial fall in sweat rate over the first 150 minutes of stimulation and then an established steady state for the remaining 150 minutes. It may be expected that if vasoconstriction was severely affecting the delivery of essential products to the glands that the shape of the two curves would not be as similar – with a general decline of

sweat gland activity with time rather than steady state reached. Therefore, if vasoconstriction is affecting sweat gland nutrient supply, it is not an absolute rate limiting step in sweat production but rather is decreasing overall gland activity.

Here we have found that at steady state noradrenaline stimulation alone increases sweating by about 14% compared with pure cholinergic sweating (100%). This figure is similar to those previously quoted for adrenergic stimulation of sweat glands (Sato 1983; Szabadi *et al*, 1980; Foster *et al*, 1970). However previous studies that investigated sweat gland activity following adrenergic or cholinergic stimulation within the same subject and at the same time involved only a single injection of agonist. This study has provided information (for the first time) about sweat gland activity with prolonged stimulation both within the same individual and with both cholinergic and adrenergic agonists.

The continuous delivery of atropine sulphate to sweat glands reduced sweat gland activity to below basal level. This suggests, that under resting conditions, there is continuous muscarinic sweat gland stimulation. The combined delivery of pilocarpine with atropine following pilocarpine only stimulation rapidly reduced sweat production to below that of basal level. This confirms what was previously known - pilocarpine is a muscarinic agonist whose action is blocked by atropine.

The combined delivery of noradrenaline with atropine resulted in sweating that was not different in volume from the delivery of noradrenaline only. Blocking sweat production initially with atropine and then delivering atropine with noradrenaline also resulted in a rate of sweating similar to that produced by noradrenaline alone. This suggests that noradrenaline does not act via the sweat gland muscarinic receptor but probably via an alpha or beta-adrenoceptor. Previous studies (Wolf *et al*, 1974) have reported that adrenergic-induced sweating is not

affected by atropine blockade, as we have found here. However the continuous delivery of atropine with noradrenaline has not been investigated previously.

It is known that a large number of transmitters and peptides in addition to acetylcholine and catecholamines are found in the immediate vicinity of the sweat gland, these include vasoactive intestinal peptide (VIP), natriuretic peptide (ANP), calcitonin gene-related peptide, galanin and ATP (Sato 1995). The significance of these transmitters and their function is not fully understood. If they play a role in stimulating sweating at rest then it is probable that they act via the sweat gland muscarinic receptor as muscarinic blockade by atropine reduced sweat production to below basal level. If these transmitters were acting via an alternative mechanism then atropine would not be expected to reduce the resting sweat rate to below baseline. Alternatively these neurotransmitters and peptides may have no contributory role on basal sweat production. These conclusions can be drawn from the observation that atropine reduced TEWL to below basal resting level of normal skin.

Our method of continuous sweat gland stimulation is unique and opens up ideas for further work that will clarify sweat gland pharmacology and physiology. The use of pure α-adrenergic and β-adrenergic agonists and antagonists will dissect out the control and overall contribution to sweat production by the adrenergic system including ductal reabsorption. It would also be interesting to deliver some of the periductal neurotransmitters such as galanin, ANP, VIP and ATP via microdialysis fibres and measure transepidermal water loss in an attempt to elucidate if they have an active role in stimulating sweating.

Chapter 10

The absorption of drugs via sweat glands

Introduction

The eccrine sweat gland is a potential shunt for drug absorption. As sweat is 99% water it would be expected that if any molecules were to enter the skin via sweat glands it would be more likely to be water-soluble drugs, particularly as lipophilic substances penetrate the skin via the intercellular pathway relatively easily.

We have demonstrated that microdialysis is a means of continuously delivering agents to the sweat glands that can promote high levels of sweating as measured by transepidermal water loss. Penciclovir is a water soluble drug that is not absorbed to any appreciable degree through an intact skin. It is therefore a good molecule to test the hypothesis that enhanced sweating may open up a conduit for the passage of water soluble drugs.

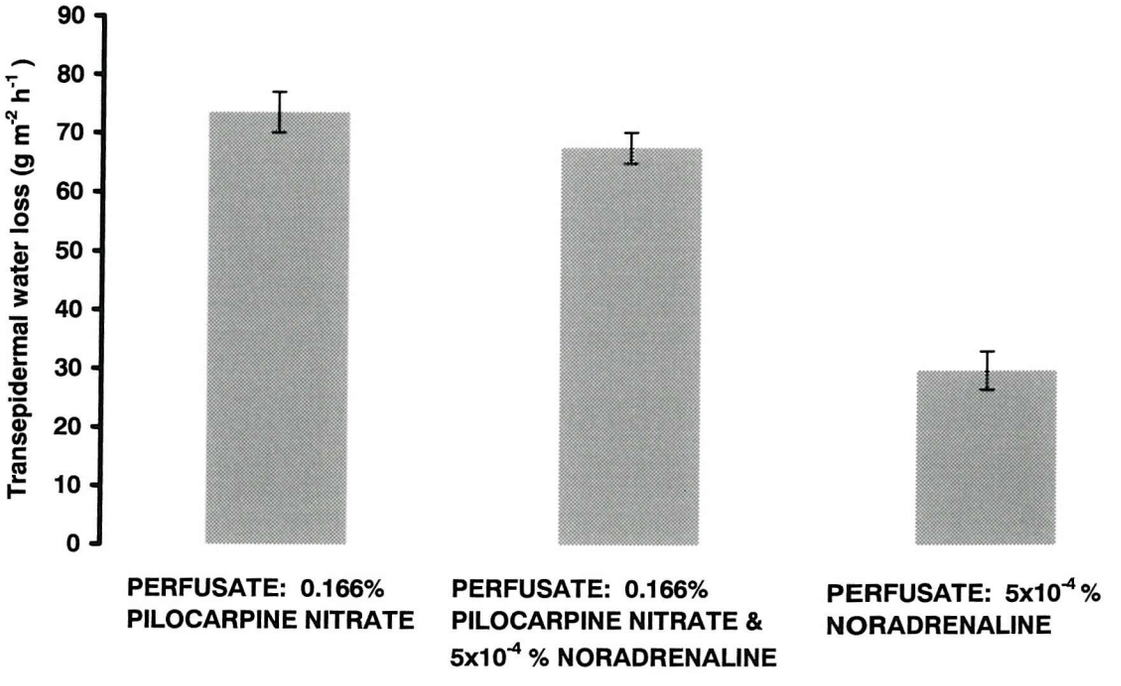
1. The absorption of topically applied penciclovir with a pilocarpine perfusate

The investigation of penciclovir absorption with a pilocarpine perfusate was performed in 9 subjects (6 male, 3 female). Each subject had 6 fibres inserted under EMLA anaesthesia. The fibres were perfused with Ringer's solution containing: 0.166% pilocarpine nitrate; 0.166% pilocarpine nitrate and $5x10^{-4}$% noradrenaline; and $5x10^{-4}$% noradrenaline. Perfusion flow rate was 5μl $minute^{-1}$. At 30 minutes after the start of perfusion, transepidermal water loss measurements were made on the skin overlying each fibre to ensure that the degree of sweating was within the expected range as outlined in previous experiments (chapter 9). 1% penciclovir cream (Vectavir) was applied in drug wells and dialysate collected every 30 minutes. At the end of the study, fibre depth was measured using ultrasound.

Results

Pilocarpine alone caused a significantly higher rate of sweating (detected as TEWL) at 30 minutes than pilocarpine with noradrenaline ($p<0.05$) or noradrenaline alone ($p<0.0001$(2 tailed t test)); TEWL after perfusion with pilocarpine with noradrenaline was significantly higher than induced by noradrenaline alone ($p<0.0001$(2 tailed t test)). The average TEWL readings (with standard error of the mean) were: pilocarpine 73.5 ± 3.5 g m^{-2} h^{-1}; pilocarpine with noradrenaline 67.6 ± 2.6g m^{-2} h^{-1} and noradrenaline 29.8 ± 3.3 g m^{-2} h^{-1} (figure 10.1).

Figure 10.1 Transepidermal water loss after fibre perfusion for 30 minutes in 9 subjects. Error bars are SEM.



The concentration of penciclovir recovered per 30 minutes over the three hour study duration is shown in figure 10.2a. In the presence of perfusion with plain pilocarpine, penciclovir absorption was over 100 fold higher than with the control perfusate of 5×10^{-4} % noradrenaline. The combination of pilocarpine and noradrenaline resulted in a 7 fold increase in PCV recovery compared with the control situation of noradrenaline perfusate only. The mean AUC with a pilocarpine perfusate was 1495 ± 476.2 ng ml^{-1} h (SEM) and with a pilocarpine and noradrenaline perfusate the mean AUC was 100.5 ± 46.9 ng ml^{-1} h (SEM). There was no correlation between fibre depth and penciclovir collected with either the pilocarpine ($r = - 0.04$) or pilocarpine and noradrenaline ($r = - 0.03$) perfusates.

Figure 10.2b shows the same data but with the y axis converted to a log scale. This enables easier appreciation of the shape of all 3 curves and the high degree of shape similarity between the pilocarpine, and pilocarpine with noradrenaline curves.

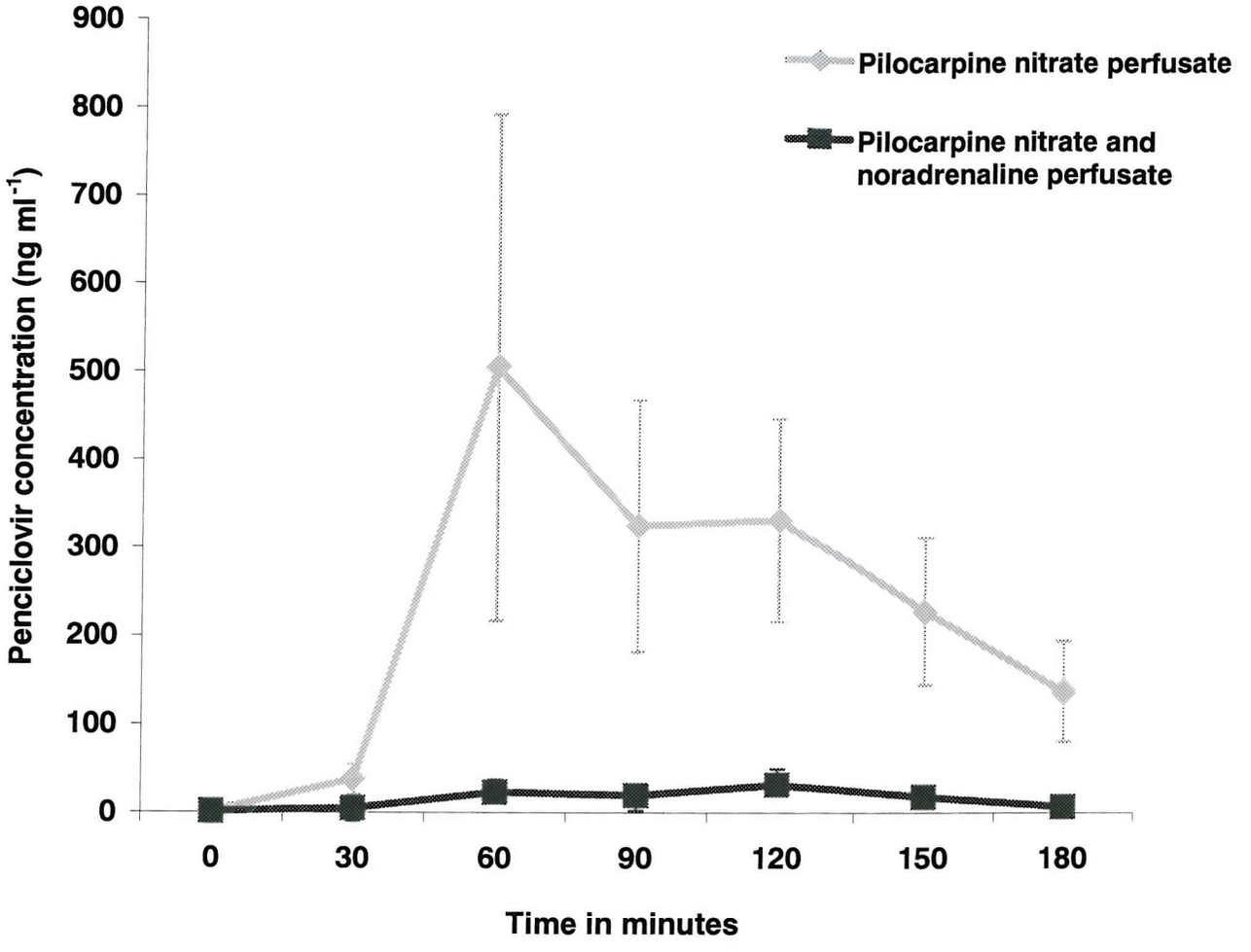


Figure 10.2a Penciclovir recovery with sweating induced by pilocarpine alone or pilocarpine with noradrenaline. n=9, error bars are SEM. The noradrenaline only line has not been included as it would lie very close to the baseline throughout.

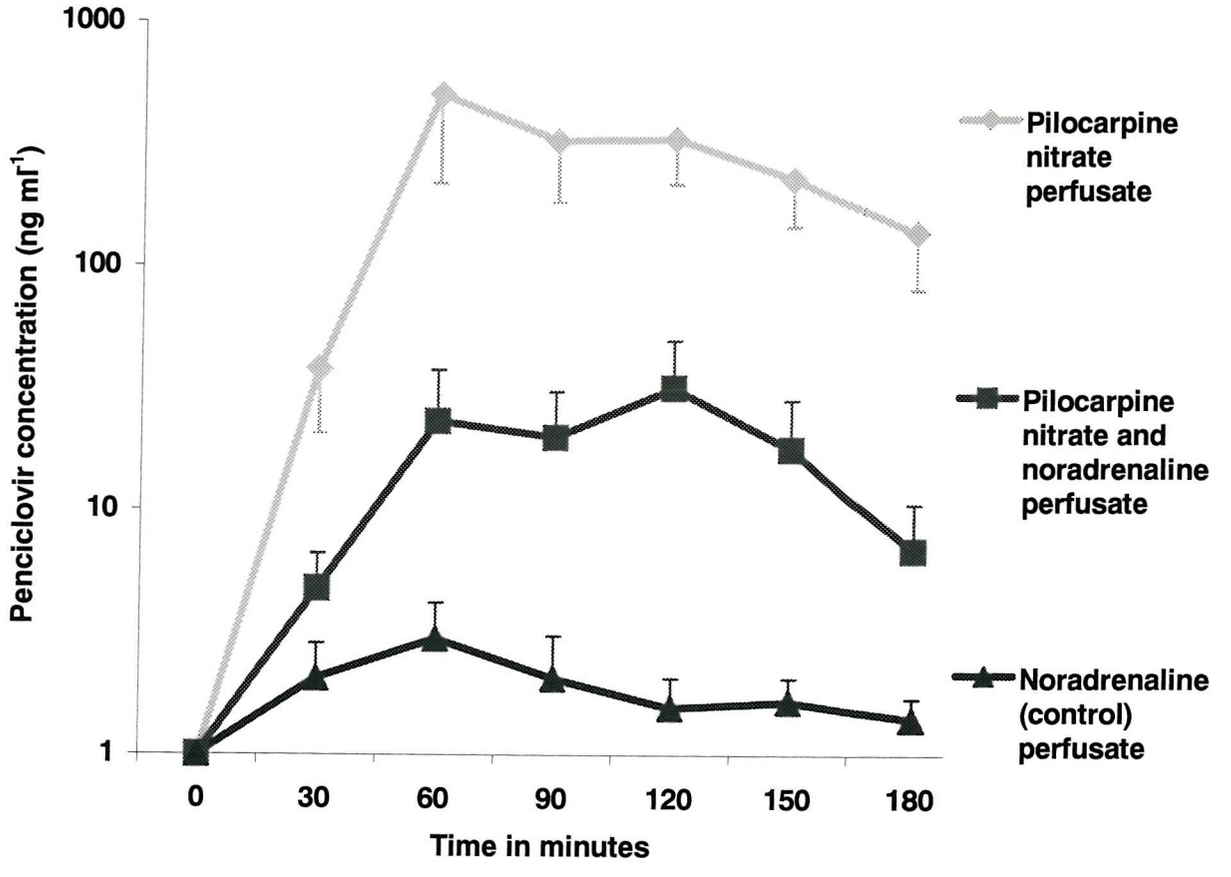


Figure 10.2b Log of penciclovir recovery with sweating induced by pilocarpine alone, pilocarpine with noradrenaline or noradrenaline alone. N=9, error bars are SEM.

2. The effect of topically applied pilocarpine on sweating

Having demonstrated that continuous delivery of pilocarpine nitrate to the dermis results in a higher level of penciclovir recovered by microdialysis we wanted to investigate the effect of topically applied pilocarpine. Initially it was essential to establish whether topically applied pilocarpine would induce a significant increase in sweating as measured by transepidermal water loss, and at what concentration.

Three concentrations of pilocarpine nitrate in aqua gel were made up; 7%, 12.5%, 25%. In order to achieve a concentration as high as 25% it was necessary to heat the suspension of pilocarpine nitrate in water in a water bath at 55° C for 30 minutes to ensure that the pilocarpine had fully dissolved. In 4 subjects (2 male, 2 female), each of the pilocarpine in aqua gel mixtures plus a plain aqua gel control were applied to the volar surface of the forearm in drug wells (internal diameter 1cm x 1cm). The drug wells were occluded and formulations left in place for 1 hour with the subjects supine.

After 1 hour, the drug wells were removed and the gel wiped off with cotton wool with care taken to ensure that all the gel was removed. Transepidermal water loss measurements were taken every 10 minutes to one hour with a final measurement taken at 100 minutes.

Results

All subjects tolerated the topically applied pilocarpine well with no side effects reported. At the end of the one hour application time the pilocarpine salt in the 25% gel had precipitated out of solution in all 4 subjects. The 12.5% pilocarpine gel and 7% did cause significant sweating as compared with aqua gel ($p < 0.01$ and $p < 0.005$ (2 tailed t test)). The 25% pilocarpine gel did not cause a significant increase in sweat rate (figure 10.3).

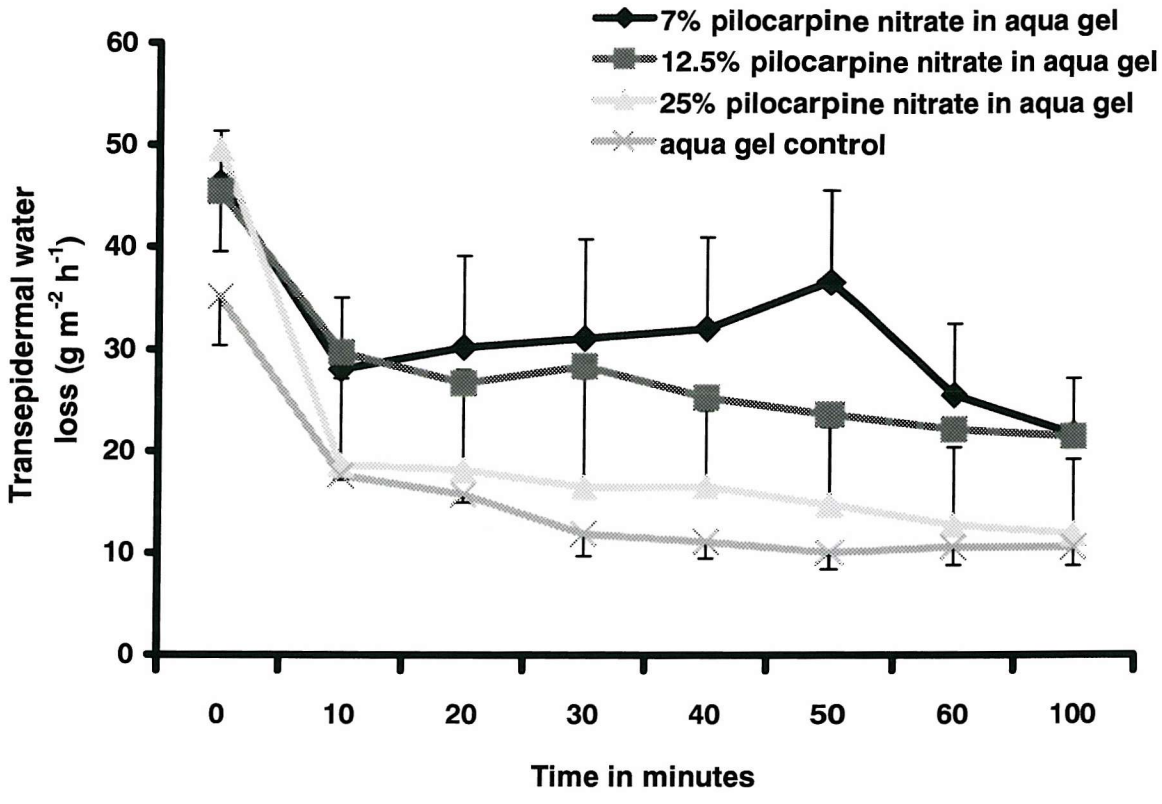


Figure 10.3. Transepidermal water loss with time following the topical application of 4 different concentrations of pilocarpine in an aqua gel vehicle. N=4, error bars are SEM.

3. The percutaneous absorption of penciclovir when applied topically with pilocarpine nitrate

The next step was to investigate if topically applied pilocarpine could improve the absorption of penciclovir. The 7% pilocarpine gel was used as it caused the highest rate of sweating. In 7 subjects (4 female, 3 male, age range 22 to 29), six microdialysis fibres were inserted. The 6 fibres were randomized to one of the following variables of perfusate and topically applied drug (2 fibres in each experiment per variable):

1. Perfusate: 5 $x10^{-4}$% noradrenaline in Ringer's solution
   Drug: 7% pilocarpine nitrate and 1% penciclovir in aqua gel
   (i.e. sweating induced and shutdown of dermal vascular perfusion)
2. Perfusate: Ringer's solution
   Drug: 7% pilocarpine nitrate and 1% penciclovir in aqua gel
   (i.e. sweating induced but dermal vasculature not shut down)
3. Perfusate: 5 $x10^{-4}$% noradrenaline in Ringer's solution
   Drug: 1% penciclovir in aqua gel. (control fibre)
   (i.e. no sweating, normal control with shut down of vasculature)

There was a ninety minute recovery period between fibre insertion and drug application. Perfusate flow rate was 5μl min^{-1}, dialysate was collected every 30 minutes for 150 minutes.

Results

The combination of 7% pilocarpine nitrate and penciclovir in aqua gel enhanced the absorption of penciclovir (figure 10.4). The mean area under the curve (± SEM) of penciclovir dialysed over the 150 minutes was:

Topical pilocarpine and penciclovir, noradrenaline perfusate	131.9 ± 56 ng $ml^{-1}h$
Topical pilocarpine and penciclovir, Ringer's perfusate	94.8 ± 36.8 ng $ml^{-1}h$
Topical penciclovir, noradrenaline perfusate	8.44 ± 1.4 ng $ml^{-1}h$

Although the addition of pilocarpine to penciclovir increased its recovery by up to 15 times when the mean area under the curve of penciclovir concentration was taken, examination of data for individual subjects demonstrated that there was a high degree of intersubject variation. Figure 10.5 shows the concentration of penciclovir collected per subject over the 150 minute study duration with topically applied pilocarpine and penciclovir and a noradrenaline perfusate, compared with the control situation of topical penciclovir and a noradrenaline perfusate. The PCV recovered under the two conditions was significantly different ($p < 0.05$ (ANOVA)). It was of note that the two subjects who did not absorb higher concentrations of penciclovir with topical pilocarpine were both female.

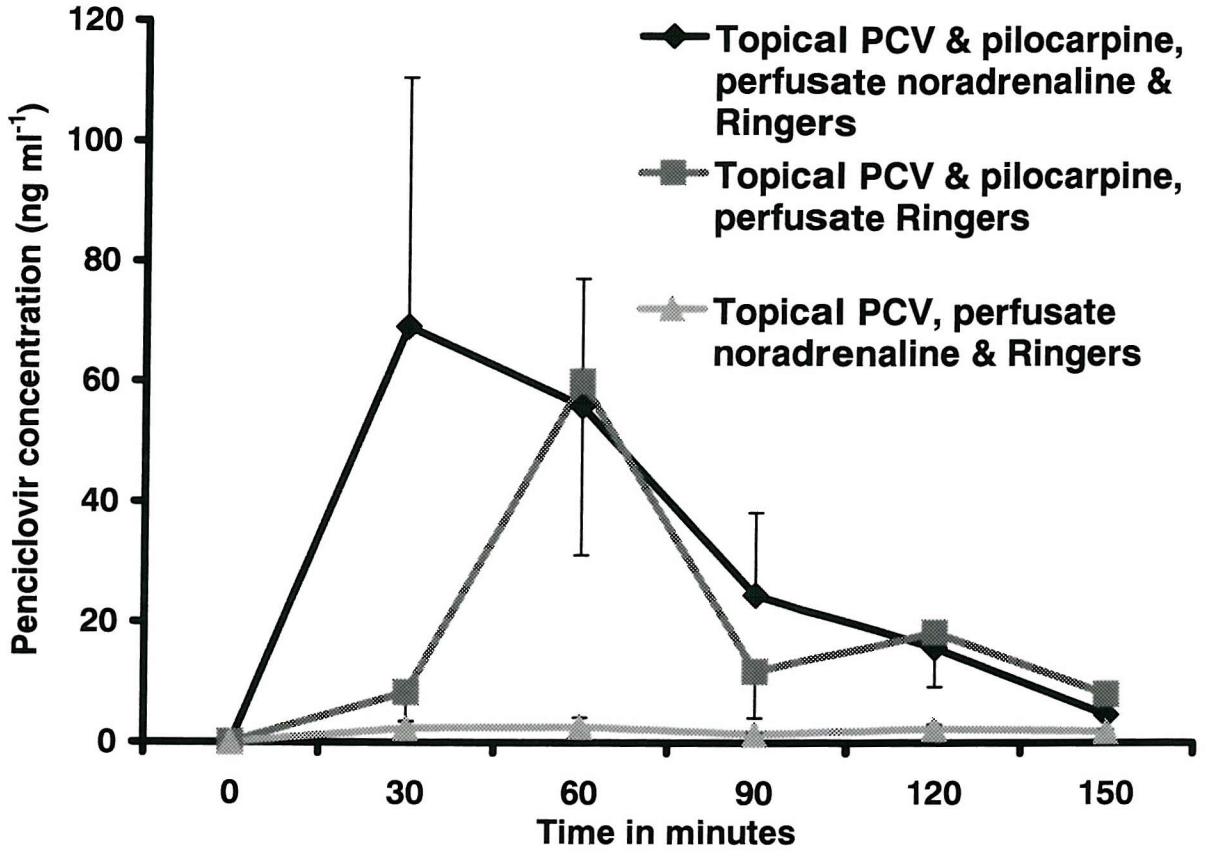


Figure 10.4 Recovery of penciclovir in 7 subjects when applied topically in a pilocarpine and aqua gel vehicle. Error bars are SEM.

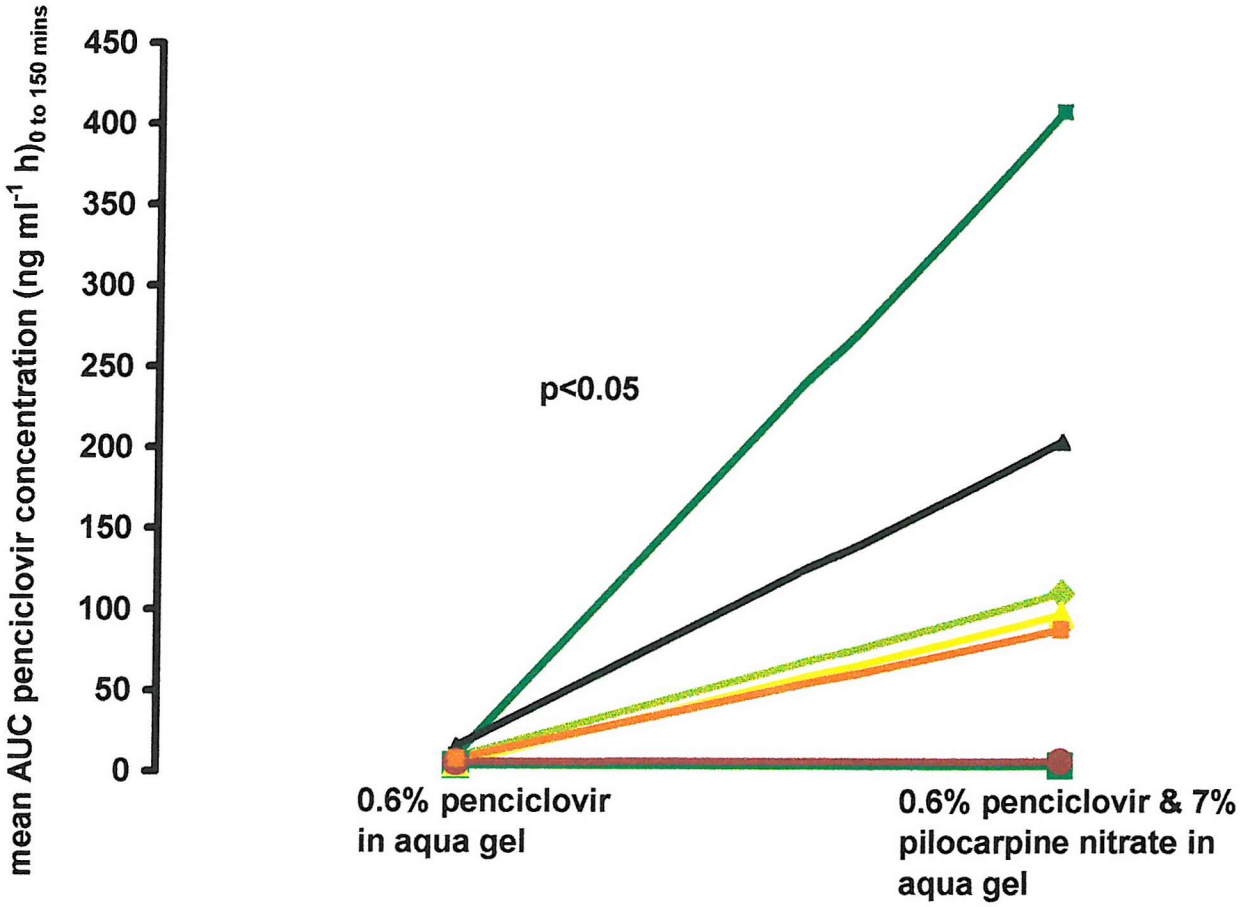


Figure 10.5 The effect of topically applied pilocarpine nitrate on penciclovir recovery over 150 minutes per subject (n=7) with a noradrenaline perfusate. The difference in penciclovir recovery was significant ($p<0.05$, ANOVA).

Discussion

Continuous delivery of pilocarpine to the dermis by microdialysis fibres has increased penciclovir recovery by over 100 fold as compared with a perfusate of noradrenaline in Ringer's solution, and an approximate increase of 1000 to 1500 fold as compared with plain Ringer's solution (see chapter 6). It is tempting to suggest that this increase is due to percutaneous absorption of the hydrophilic penciclovir molecules down the actively secreting sweat glands, however, other possibilities must be considered: Pilocarpine does cause vasodilatation which could be the mechanism for enhanced drug absorption but this is unlikely - in previous studies examining penciclovir absorption with a high degree of vasodilatation induced by glyceryl trinitrate (chapter 6, fig 6.2) we found only a minimal increase in penciclovir recovery. However, it is possible that both pilocarpine and GTN massively increase PCV absorption which when combined with very high clearance due to vasodilatation results in a net overall modest increase in PCV recovery. Alternatively, it is also possible that with pilocarpine induced sweating the increased PCV recovery is due to the PCV using the sweat gland as a conduit to the dermis.

It is of great interest that the addition of noradrenaline to the pilocarpine perfusate reduced the rate of sweating by only about 20% (chapter 9) but reduced penciclovir recovery by 15 fold (1500%) as compared with pilocarpine alone. However levels of penciclovir recovered are still much greater (7 fold) than with a perfusate of noradrenaline alone. Examining the time course of penciclovir recovery, there is maximal recovery in the first hour of dialysate collection – as there was a one hour interval between the start of perfusion and application of penciclovir topically the degree of sweating at this time is that seen in figure 9.8 from 1 to 2 hours.

Study 2 demonstrated that topically applied pilocarpine can stimulate sweating to a significant degree. Prior to this observation a pilot study was carried out using a 4% and 5% concentration of pilocarpine in aqua gel. These failed to stimulate sweating significantly, hence the decision to use the higher concentrations of pilocarpine. The cystic fibrosis sweat test uses a 0.5% concentration of pilocarpine that is introduced to the dermis by iontophoresis. Here we are having to use much higher concentrations of pilocarpine applied topically to stimulate sweating. It is not known how the pilocarpine enters the skin to have its effect on the secretory cells of the sweat gland – pilocarpine nitrate is a hydrophilic molecule with a low octanol-water coefficient (Dollery 1999b) and so will be poorly absorbed through the skin. This may explain why a significantly higher concentration of pilocarpine is needed to stimulate sweating when applied topically as compared with direct introduction to the dermis via microdialysis fibres or iontophoresis.

The application of aqua gel to the skin has a hydrating effect. This is seen as an increase in TEWL above resting skin conditions for the first 30 minutes of the study. Due to this, it is impossible to dissect out the real contribution to an increase in TEWL caused by pilocarpine during this initial 30 minutes, and a similar fall in TEWL was seen under all 4 experimental conditions following removal of the aqua gel. If the difference in TEWL is measured from 30 minutes to the end of the study, it is still significantly higher in the groups treated with 7% and 12.5% pilocarpine gels ($p<0.005$).

The sweating rate induced by topically applied pilocarpine is approximately 50% of that stimulated by pilocarpine delivered to the dermis by microdialysis fibres. This sweating rate could be improved by the use of higher concentrations of topically applied pilocarpine. However as demonstrated here, the solubility of pilocarpine does not permit its application as a 25% gel

as it precipitates out of solution. The use of solvents such as propylene glycol or dimethyl sulphoxide may permit higher concentrations of pilocarpine to be made in aqua gel, however this may be detrimental to the partitioning coefficient of pilocarpine from its vehicle to the skin and therefore affect its ability to stimulate sweating.

A mixture of pilocarpine and penciclovir in aqua gel promotes penciclovir recovery in the absence of any obvious side effects. It is of interest that there was no significant difference in penciclovir recovery with and without a noradrenaline perfusate. It would be expected that the vasoconstriction caused by noradrenaline would prevent drug clearance, however, as already seen, the addition of noradrenaline to the perfusate with pilocarpine (figure 10.2a) results in a much lower concentration of penciclovir collected. Therefore in this situation we may be seeing the effects of noradrenaline in both reducing the clearance of absorbed drug but also preventing sweat gland absorption of drug, hence the level of penciclovir under the two conditions being not dissimilar. It is not surprising that the 2 subjects who failed to respond to the topical pilocarpine were both female. It is likely that their eccrine sweat glands were not primed to be sensitive to the concentration of cholinergic stimulation used. This observation does lend weight to the hypothesis that penciclovir absorption is directly dependent on sweat gland activity.

There are three explanations for this apparent absorption of PCV down sweat glands seen here with a pilocarpine or pilocarpine with noradrenaline perfusate:

1. The pilocarpine-only perfusate is causing sweating that is well above the threshold for promoting penciclovir movement down the sweat glands in terms of sweat gland aperture and amount of aqueous sweat produced in which the penciclovir dissolves and is carried into the dermis. Although penciclovir transfer is against that of sweat movement it may

still be possible for the penciclovir to move in this way particularly as it is thought that there is no intrinsic pumping ability of sweat glands in terms of myoepithelial cell activity. As sweat rate falls with time, so penciclovir absorption is reduced in the pilocarpine only perfusate. With the pilocarpine and noradrenaline perfusate, it is only at the 60 minute point of TEWL with time (figure 9.8) that the rate of sweating is equivalent to that seen with pilocarpine only (at 180 minutes), so sweating doesn't quite reach high enough levels to allow penciclovir movement.

This is the 'threshold theory' of sweating and penciclovir absorption.

2. The second theory can be explained with reference to figure 10.2b which shows penciclovir absorption with time but with the log of the y axis taken. Both curves are similar in that there is an initial high concentration of penciclovir absorbed which falls with time. The downward part of both slopes are the same which suggests that the mechanism of penciclovir absorption is the same with the two perfusates but the addition of noradrenaline is affecting penciclovir levels to the same degree at all time points.

The leakiness of sweat glands is not fully understood. Frömter and Diamond (Fromter *et al*, 1972) tentatively classified the transporting epithelia of glands into two distinct groups, the leaky and tight epithelia. Leaky epithelia include the renal proximal tubule, the gall bladder and the choroid plexus. Work on monkey eccrine sweat glands have classified them as leaky epithelium – this means that the secretory epithelia transport Na^+ and water isotonically, have high values for osmotic water permeability and have a relatively high transport rate. The secretory epithelium of the sweat gland is considered to be as leaky as the renal proximal tubule (Sato 1983), however exact detail of transport by intercellular or

intracellular pathways and pharmacological stimulation of this is not yet understood. Therefore it is possible that on stimulation of sweat production by pilocarpine, there is the opening of a channel or initiation of a transport process for penciclovir movement from the lumen of the sweat duct into the dermal interstitial space. Noradrenaline partially inhibits this mechanism so that penciclovir is unable to enter the dermis to such a high degree.

3. The third theory is a variation on theory 2; penciclovir is moving from the sweat gland into the dermis through the sweat duct epithelium – details of the leakiness of this structure is not yet resolved. Pilocarpine alone promotes sodium chloride reabsorption in the sweat duct with production of a hypotonic sweat, the addition of noradrenaline – with relatively greater α-adrenergic effect than β-adrenergic, inhibits sweat reabsorption with the resultant inhibition of movement of molecules either intercellularly or intracellularly through the sweat duct epithelium. The effect of α-adrenergic stimulation on sweat ducts is not known. If noradrenaline does inhibit the resorption of sodium chloride in the sweat duct, this will not affect transepidermal water loss as the sweat produced will vary in tonicity but this will not affect the relative humidity of the air at the skin surface.

It is not possible to state categorically that penciclovir is definitely entering the dermis via the eccrine sweat glands. There are models in which this could be investigated; a fluorescent marker of penciclovir could be made and its absorption investigated in an animal model with skin biopsies. The usual laboratory animals could not be used as they only have sweat glands on their feet, rather a pig or monkey model would be necessary with the assumption that results obtained could be related to the human *in vivo* situation. The difficulty with studying absorption of water

soluble molecules down sweat glands in man, is that on taking the skin biopsy, the washing and fixing procedure of the tissue specimen is likely to result in loss of any substances of interest that were present in the sweat gland or dermis. This could be avoided by using PCV labeled with either a radioactive or fluorescent marker although such a technique may not be considered ethical for *in vivo* work.

Our knowledge of cholinergic drug action in the skin is limited and it is possible that although pilocarpine and noradrenaline significantly increase sweat production, it is actually by some other mechanism that penciclovir absorption has increased - such as pharmacological alteration of the stratum corneum barrier. At this stage we can only hypothesise as to what may be the mechanism of absorption. However, it is likely, that with the advent of techniques such as cutaneous microdialysis it will be possible to learn more about sweat gland physiology and pharmacology *in vivo* and the probability and so manipulation of drug transport down eccrine sweat glands.

DISCUSSION

This thesis has examined many of the factors surrounding percutaneous absorption through both the well described intercellular route and the poorly described transappendageal route. The antiherpes drugs penciclovir and aciclovir have been proven to be excellent probes to investigate percutaneous absorption. Their usefulness is that their absorption through normal skin with normal blood flow is undetectable using microdialysis as a sampling technique and high performance liquid chromatography as the assay. Therefore, increasing the absorption of penciclovir by any means, provides a clear indication of the contribution of that particular factor or variable to percutaneous absorption. The disadvantage of using such poorly absorbed drugs is that subtle differences in percutaneous absorption are not obvious. The addition of a range of penetration enhancers to a penciclovir-containing gel had no effect on overall absorption, whereas if a molecule whose absorption had been 100 fold greater under normal circumstances had been used, a difference may have been apparent.

A further slight disadvantage of using penciclovir is that it does not allow us to comment on its absolute concentration in the extracellular space on account of the impossibility of performing an *in vivo* calibration experiment: Many microdialysis studies use a calibration technique that enables accurate estimation of substances in the extracellular space. This diminishes the need for *in vitro* relative recovery and loss experiments (Lonnroth *et al*, 1990). The calibration procedure is based on the principle that different concentrations of the substance to be measured in the extracellular space are added to the perfusate *in vivo*. The concentration that does not cause any change in the dialysate is therefore the concentration present in the extracellular fluid, that is by regression analysis:

$$\textbf{concentration}_{in} = \textbf{concentration}_{out} = \textbf{intercellular concentration.}$$

It has been impossible to do this experiment with penciclovir as in the absence of vasoconstriction there is no detectable drug in the dermis (following topical application). Theoretically it could have been attempted following the oral ingestion of famciclovir, however the extracellular concentration of penciclovir did not reach a steady state in the absence of vasoconstriction and so such an experiment would not have given a consistent value for dermal drug concentration. Therefore we have had to rely on other indirect means of determining fibre efficiency for penciclovir. It has been reassuring that the *in vitro* relative recovery and loss measurement, and *in vivo* loss measurement gave consistently similar values for fibre efficiency of 15 to 20%. However without a true *in vivo* calibration experiment it is not possible to comment directly on dermal concentrations of penciclovir but rather on the concentration recovered and how this is altered by manipulation of that particular factor.

It was possible to determine a class effect for the absorption of the acyclic nucleosides by comparing the absorption of penciclovir with aciclovir. Having found that they were absorbed to an appreciably similar degree, only penciclovir was used in the subsequent experiments as duplication of studies with aciclovir would have reduced the number of different experiments that could be performed in the time available.

The extent of the extremely high barrier to hydrophilic drug absorption has been explored. It is not surprising that removal of the stratum corneum allowed such high absorption of penciclovir and aciclovir and serves to reiterate the importance of the stratum corneum barrier to hydrophilic drug absorption which should perhaps be re-endorsed to the manufacturers of such topically applied drugs. The derivation of a figure for the contribution of blood flow to drug clearance is extremely important for topical drug formulation and is the first time that an attempt has been made to quantify the role of cutaneous vasculature on dermal drug levels *in vivo*.

A few studies have used cutaneous microdialysis to measure dermal levels of orally or systemically administered drugs (Borg *et al*, 1999a; Borg *et al*, 1999b; Petersen *et al*, 1999a; Petersen *et al*, 1999b). As shown here microdialysis can provide essential pharmacokinetic information about a drug whose site of action is within the skin. It would be interesting to use microdialysis to measure dermal levels of systemic drugs used in dermatology such as cyclosporin, methotrexate or azathioprine in order to establish effective concentrations in the skin. This may make it easier to arrive at an appropriate dose for a patient rather than stepwise increase in dose until clinical improvement is seen.

There are many other experiments that can be performed using microdialysis to investigate the skin barrier and percutaneous absorption: Using a probe that is absorbed better than penciclovir (although penciclovir may be suitable if large changes are seen) it would be interesting to assess the effect of exposure to UV light of different wavelengths and doses on the barrier and blood flow. The effect of temperature could also be explored.

The use of microdialysis to continuously stimulate or inhibit sweat glands *in vivo* is unique and has demonstrated the capacity of sweat glands for continued sweat production over a long period of time. It has been possible to explore the relative effects of cholinergic and adrenergic stimulation and the effect of dual stimulation. In common with other successful investigative studies, having shed light on some aspects of the problem, this leads to a whole series of new questions. The mechanism of penciclovir absorption through sweat glands requires a lot of further investigation to clarify its importance. However it is interesting that out of all the techniques described in this thesis in attempting to enhance percutaneous absorption, apart from physical removal of the stratum corneum, pharmacological manipulation of the sweat glands has enhanced penciclovir absorption the most.

In conclusion, percutaneous absorption remains an elusive pathway into the body that can only be traversed by drugs with certain physicochemical properties. Here we have succeeded in examining some of the key factors affecting drug absorption and clearance in a large number of subjects. Knowledge of these variables should aid in the design and formulation of topically applied drugs for the benefit of all.

Appendix

Vectavir cold sore cream – list of exipients:

1% w/w penciclovir

white soft paraffin

liquid paraffin

propylene glycol

cetostearyl alcohol

cetomacragol 1000

purified water

Zovirax cold sore cream – list of exipients:

5% w/w aciclovir

poloxamer 407

cetostearyl alcohol

sodium lauryl sulphate

white soft paraffin

liquid paraffin

propylene glycol

purified water

Famvir tablets - list of exipients:

famciclovir 250mg

hydroxypropyl cellulose PhEur

lactose anhydrous NF

sodium starch glycollate BP

magnesium stearate PhEur

hydroxypropyl methyl cellulose PhEur

titanium dioxide PhEur

polyethylene glycol NF

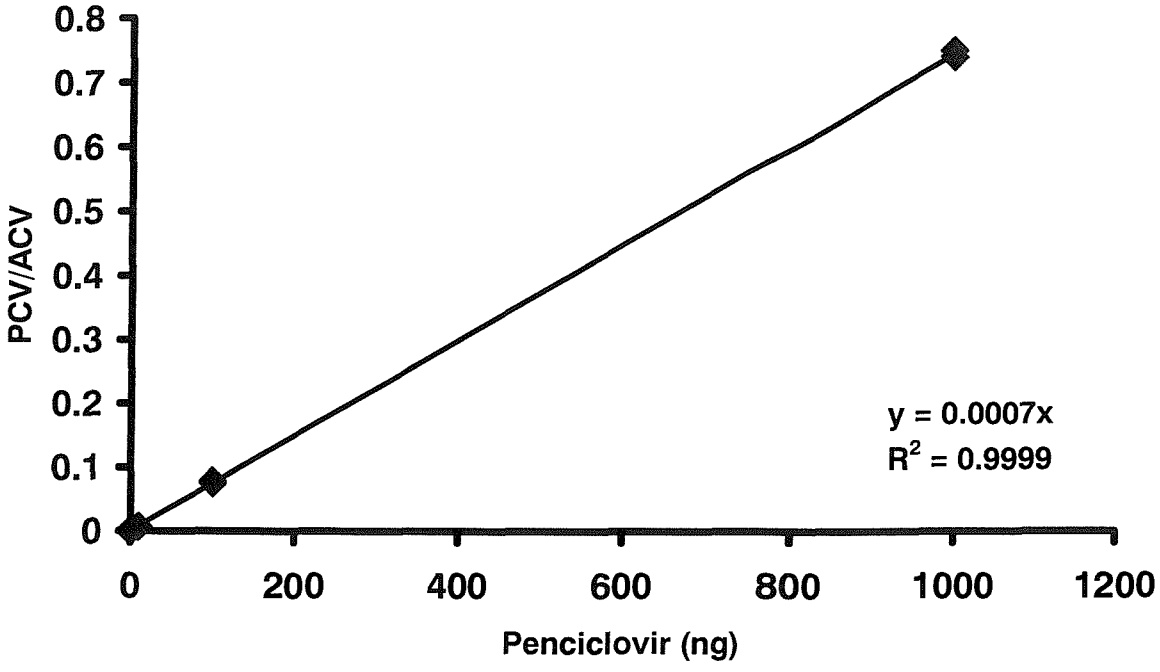


Figure A1 : Typical standard curve for penciclovir from HPLC analysis. Standards range from 0.5ng to 1000ng

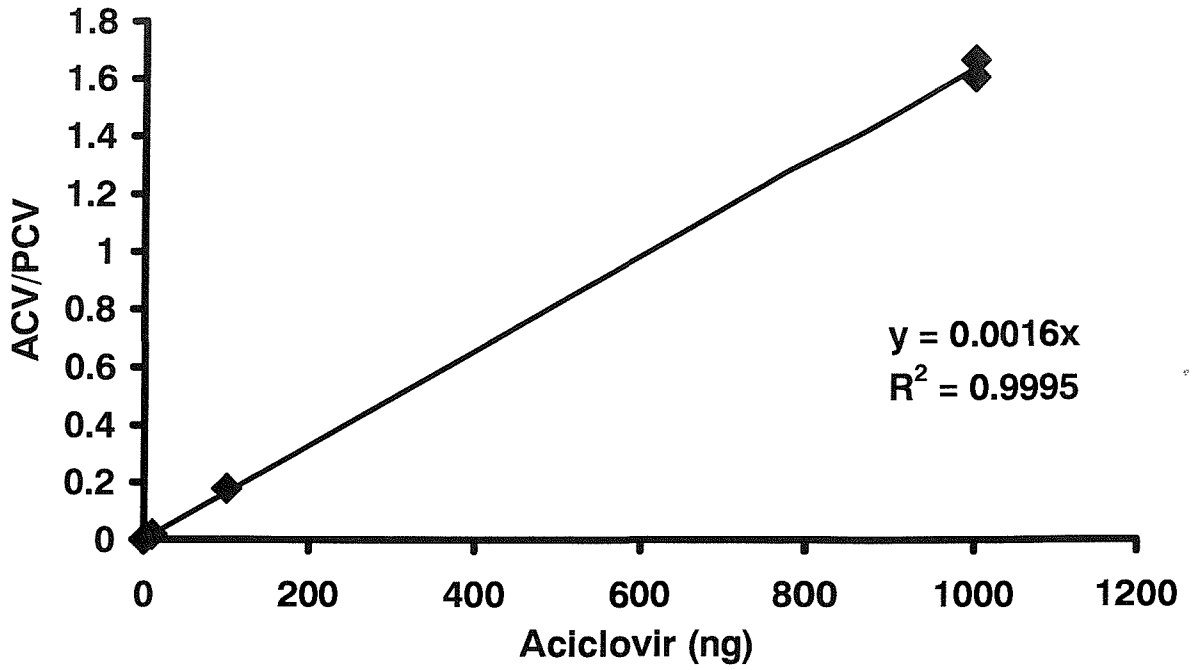


Figure A2 : Typical standard curve for aciclovir from HPLC analysis. Standards range from 0.5ng to 1000ng.

Abbreviations

ACV	Aciclovir
ANOVA	Analysis of variance
AUC	Area under the curve
Ca^{2+}	Calcium
Cl^-	Chloride ion
DMSO	Dimethyl sulphoxide
ECF	Extracellular fluid
GTN	Glyceryl trinitrate
K^+	Potassium ion
LDI	Laser Doppler imaging
Na^+	Sodium ion
NaCl	Sodium Chloride
NA	Noradrenaline
NO	Nitric oxide
PCV	Penciclovir
PG	Propylene glycol
Ringer's	Ringer's intravenous solution
SEM	Standard error of the mean
SLS	Sodium lauryl sulphate
TEWL	Transepidermal water loss

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