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Percutaneous Penetration of Penciclovir.

Assessment of Dermal Drug Concentrations Using Cutaneous Microdialysis.

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Doctor of Philosophy

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ABSTRACT

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCES

CLINICAL PHARMACOLOGY

Doctor of Philosophy

PERCUTANEOUS PENETRATION OF PENCICLOVIR. ASSESSMENT OF DERMAL DRUG CONCENTRATIONS USING CUTANEOUS MICRODIALYSIS.

By Catherine S. Kay

The aims of this thesis were to evaluate and develop the microdialysis technique, to assess microdialysis recovery in vivo following transdermal absorption of penciclovir; to identify a topical formulation to promote penciclovir absorption with the use of penetration enhancers; to investigate the effect of blood flow on dermal levels of penciclovir following delivery with microdialysis; and to demonstrate diffusion of the drug through the dermal tissue.

Cutaneous microdialysis is a technique used for the measurement of in vivo drug concentrations. Penciclovir is an antiviral drug used for the treatment of herpes labialis.

An in vitro microdialysis technique, together with in vivo studies using healthy adult volunteers were used. Skin for the in vitro absorption studies was obtained from adult male Wistar rats, and was mounted in Ussing diffusion chambers. Samples from microdialysis and diffusion chambers were analysed for penciclovir using HPLC. Data were analysed using unpaired t-tests.

In vitro microdialysis studies showed that recovery of penciclovir could be enhanced by increased ambient temperature and decreased perfusion flow rate. Protein-binding did not affect recovery.

In vivo transdermal studies demonstrated that the recovery of penciclovir in the dermis was very low.

In vitro skin studies showed that the penetration enhancers, oleic acid and salicylic acid, appeared to act synergistically when applied together with penciclovir, resulting in highly significant increases in the percutaneous penetration of penciclovir ($p < 0.001$). An in vivo study of transdermal absorption of penciclovir with oleic acid and salicylic acid showed that microdialysis recovery of penciclovir in the dermis was very low.

In vivo microdialysis delivery of penciclovir showed that in conditions of restricted dermal blood flow, the drug accumulated in the tissue surrounding the probe and delivery was reduced ($p < 0.001$). With normal skin blood flow, penciclovir was cleared from the tissue, and microdialysis delivery was increased. In the same study, diffusion of the drug was assessed by a second probe. Penciclovir concentrations showed an exponential decrease with distance from the delivery probe. The concentrations recovered were higher and showed diffusion to greater distances when the dermal blood supply was restricted.

Microdialysis is an effective technique for the assessment of transdermal drug absorption. The low microdialysis recovery of penciclovir in vivo following topical application may be due to inadequate skin penetration, or efficient clearance of penciclovir by the dermal microvasculature. Penetration enhancers may be useful in the promotion of percutaneous penetration of penciclovir.

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There are a number of people to whom I owe a great debt of thanks:

To Ben, for knowing I could do it and for telling me so frequently, and to our unborn baby, who gave me a reason to get on with it.

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And to Smithkline Beecham for funding this work.

List of Abbreviations.

μg	Microgram
ACV	Aciclovir
ACV-TP	Aciclovir triphosphate
AUC	Area under curve
cm	Centimetre
C_{max}	Maximum concentration
Da	Dalton
dGTP	Deoxyguanosine triphosphate
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EMLA	Eutectic mixture of local anaesthetics
GTN	Glyceryl trinitrate
HPLC	High pressure liquid chromatography
HSV	Herpes simplex virus
ID	Inner diameter
KDa	Kilodalton
mg	Milligram
min	Minute
ml	Millilitre
mm	Millimetre
NA	Noradrenaline
ng	Nanogram
nl	Nanolitre
OD	Outer diameter
PCV	Penciclovir
PCV-DP	Penciclovir diphosphate
PCV-MP	Penciclovir monophosphate
PCV-TP	Penciclovir triphosphate
PEG	Polyethylene glycol
TEWL	Transepidermal water loss
VZV	Varicella zoster virus

Aims of the Thesis.

The experiments fall into four studies.

An *in vitro* study of microdialysis to define and evaluate different aspects of this technique.

The assessment of *in vivo* recovery of penciclovir using dermal microdialysis, following topical application of the drug.

The identification of a modified vehicle formulation in which to topically apply penciclovir, with the use of penetration enhancers.

Finally, to demonstrate, using dermal microdialysis, the delivery of penciclovir *in vivo* into the skin and assessment of the influence of the dermal microcirculation on the extent of drug diffusion through the tissue.

Aims I. *In Vitro* Microdialysis Studies.

- to determine the effects of the following factors on recovery of penciclovir into the perfusate:

  sterilisation of the probes, presence of protein in the perfusate or the bath solution, presence of wire in the probes, external penciclovir concentration, perfusate flow rate, length of probes and temperature.
- to investigate if recovery and delivery of penciclovir using microdialysis are equal.

Aims II. *In Vivo* Transdermal Absorption Studies.

- to determine penciclovir absorption using dermal microdialysis, and to assess the effect of the anaesthetic cream, vasoconstriction, skin occlusion and probe depth on penciclovir recovery.

Aims III. Effect of Penetration Enhancers of Penciclovir Absorption.

- to investigate the value of salicylic acid as a penetration enhancer to promote *in vivo* transdermal absorption of penciclovir, assessed by dermal microdialysis.
- to determine the effects of salicylic acid and oleic acid on the *in vitro* absorption of penciclovir across excised rat skin, using diffusion cells.

- to study the *in vivo* absorption of penciclovir when applied topically with oleic acid and salicylic acid, using cutaneous microdialysis.

Aims IV. Dual Probe Study *In Vivo*.

- to determine *in vivo* delivery of penciclovir using dermal microdialysis under conditions of vasoconstriction and normal skin blood flow.
- to assess *in vivo* penciclovir delivery by microdialysis at different perfusate flow rates.
- to estimate the diffusion of penciclovir through the dermal tissue following microdialysis delivery, by recovery of the drug at various distances from the site of delivery, and the effect of dermal blood flow on drug concentrations.

This thesis is the result of work performed wholly while a registered postgraduate student at the University of Southampton.

Chapter 1

Introduction

1. Introduction.

The aim of percutaneous penetration of a drug from a topically applied pharmaceutical formulation is to deliver a compound across the skin to its target site at a therapeutic level.

Transdermal drug delivery is becoming increasingly popular as a route for the administration of compounds, both for systemic and localised applications. However, the limitations to percutaneous penetration, posed by the barrier function of the skin, mean that the development of pharmaceutical formulations is of considerable interest. The aim of most of these formulations is to deliver the maximum amount of drug into the skin without causing irritancy, sensitisation or inducing irreversible changes to the skin barrier.

There are several advantages of transdermal delivery of drugs over to oral administration. It avoids enzymatic degradation of the compound in the gastrointestinal tract and metabolism via the first-pass effect in the liver. Orally administered compounds require frequent dosing in order to maintain therapeutic levels, whereas topically applied drugs need less frequent application as absorption is slow, which also improves patient compliance. Transdermal application delivers the compound in a more constant manner, and does not result in the peaks and troughs in plasma concentrations seen in repeated oral dosing. However, there are also some limitations of topical drug absorption. One of the main functions of the skin is to act as a barrier and prevent the entry of exogenous substances. The body absorbs a drug across the skin slowly and incompletely, and generates the need for potent compounds to be used. Often a large proportion of the formulation is lost from the skin surface due to washing, adherence to clothing and by shedding of the upper layers of the skin. Other problems include large variability in the permeability of the skin between individuals, and at different sites of the same individual.

Historically, methods for determining the amount of drug absorbed across the skin relied on the measurement of the levels of the compound detected in the blood or excreta, or by very invasive techniques such as

punch biopsies of the skin. Drug measured in the blood and excreta undergoes extensive dilution after transdermal absorption, and does not account for drug that may not enter the systemic circulation, such as compounds with a very lipophilic nature, which remain in the skin, forming a reservoir for extended periods of time. Invasive techniques may be undesirable as they allow only one measurement of drug concentrations for that particular site, and also may cause long-term effects, such as scarring of the skin.

Dermal microdialysis is a new technique for the measurement of drug levels at the site of absorption i.e. the dermis. This technique takes advantage of the fact that it samples the drug concentration at the site of administration, to yield more accurate measurements of the drug absorbed. It is minimally invasive and can be used for continuous sampling of the same tissue space for hours and even days after dosage.

1.1 The Skin.

1.1.1 Functions of the skin.

The main functions of the skin are to protect the internal organs and fluids of the body from the external environment, to prevent water loss from the body, to maintain the body temperature, and to prevent the entry of micro-organisms and other exogenous substances. The skin also has a sensory role with regard to touch, temperature and pain.

1.1.2 Structure of the skin.

Mammalian skin is composed of two distinct layers, the epidermis and the dermis. Beneath the dermis lies the subcutaneous fatty layer, which comprises mainly of adipocytes arranged in lobules.

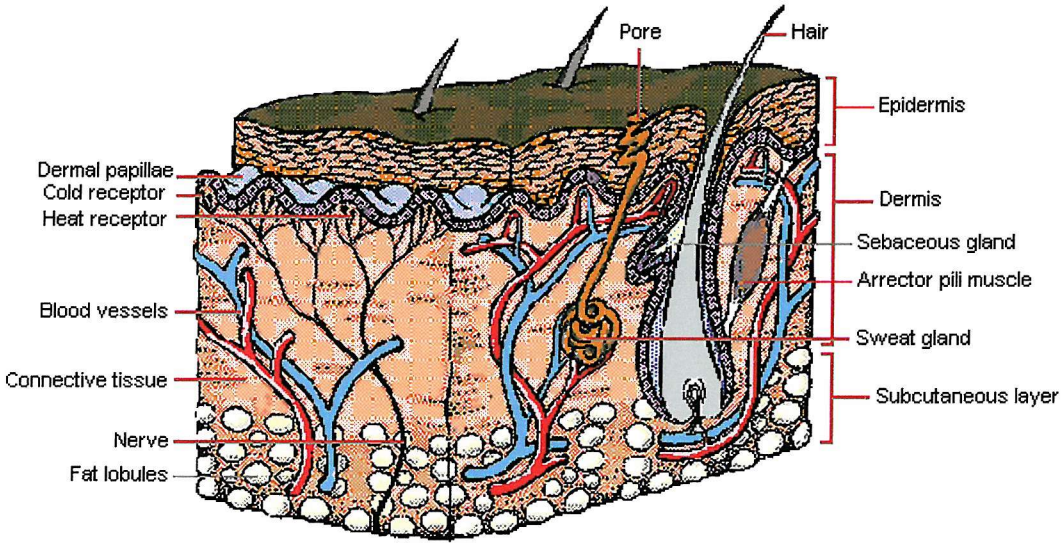


Figure 1 Schematic representation of mammalian skin

(Encarta, Microsoft, printed with permission).

1.1.3 Epidermis.

The epidermis has an average thickness of 0.12mm (Wiechers J.W. 1989 and Chien Y. 1982). The thickness varies due the cell size and number of cell layers depending on anatomical site, ranging from as thick as 0.8 mm on the soles of the feet, to its thinnest on the eyelids of 0.06 mm (Chien Y. 1982 and Bucks D.A.W. 1984). The viable epidermis does not contain any vascular supply, so nutrition for the keratinocytes is obtained from the underlying dermis by passive diffusion through the interstitial fluid.

The most abundant cell of the epidermis is the keratinocyte, but other cells present in this skin layer include the pigmented melanocytes, the Merkel cells, associated with sensory reception and the Langerhans cells which have an immunological role, being important in antigen presentation. Nerve fibres permeate the viable epidermis and are in contact with the keratinocyte cell bodies

The epidermis is further divided into separate layers, which are characterised by the state of differentiation of the cells. Differentiation is the process by which dividing stem cells migrate upwards through the epidermal layers and are lost from the skin surface. During the process of differentiation, undifferentiated, proliferative keratinocytes are converted into highly differentiated, non-dividing cells, the corneocytes. The epidermis replaces itself every 12-24 days (Eckert R.L. 1989, Elias P.M. 1983 and Wiechers J.W. 1989).

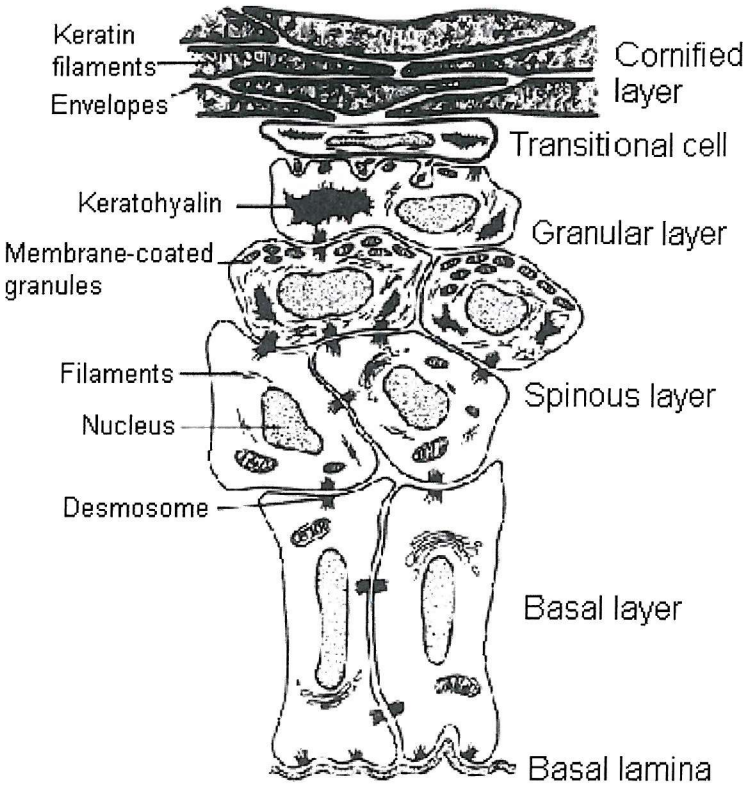


Figure 2 Schematic representation of the epidermis layers (Eckert R.L. 1989).

1.1.3.1 The Basal Layer.

The cells in this layer continuously undergo mitosis to renew the epidermis. This proliferation compensates for the stratum corneum cells lost by desquamation from the surface of the skin. The basal cells are attached to the basal lamina, which forms the division between the epidermis and the dermis. Basal cells are columnar and have a prominent nucleus surrounded by keratin filaments. The cells have junctions linking them to the basal lamina (hemi-desmosomes), and to adjacent basal cells (desmosomes) (Eckert R.L. 1989, Elias P.M. 1983 and Wiechers J.W. 1989).

1.1.3.2 The Spinous Layer.

This layer is immediately above the basal layer. The cells have a spine-like appearance due to the desmosomes between adjacent cells. The cells become more rounded than the basal cells. The synthesis of large molecules of keratin and lamellar bodies begins in this layer. Lamellar bodies are ovoid lipid-filled organelles (Eckert R.L. 1989, Elias P.M. 1983 and Wiechers J.W. 1989).

1.1.3.3 The Granular Layer.

This lies above the spinous layer. The cells are still living, as they exhibit organelles and cellular activities associated with intact cellular metabolic function. Granular cells produce keratohyalin granules, which are thought to aid with keratin aggregation. The lamellar bodies migrate towards the cell periphery and will eventually fuse with the plasma membrane (Eckert R.L. 1989, Elias P.M. 1983 and Wiechers J.W. 1989).

1.1.3.4 The Transition Zone.

This layer lies between the living granular layer and the dead cornified layer. The action of proteases, DNA-ases, RNA-ases and acid hydrolases destroys the cellular organelles. In this layer, the lamellar bodies fuse with the plasma membrane and their lipid contents are extruded into the extracellular space. These lipids then become modeled into the multi-lamellar bilayers of the inter-cellular space of the stratum corneum. The expelled contents of the lamellar bodies prevent the outward movement of water and so prevent water loss from the skin (Elias P. 1981b). The plasma membrane becomes a cornified envelope, a sheath of covalently cross-linked protein, and the keratin filaments start to become restructured (Eckert R.L. 1989, Elias P.M. 1983 and Wiechers J.W. 1989).

1.1.3.5 The Cornified Layer (Stratum Corneum).

The stratum corneum consists of several (8-16) layers of flattened cornified dead cells, and is approximately 10 μm thick (Barry B. 1991b, Ohman H. and Vahlquist A. 1994, Chien Y. 1982 and Wiechers J.W. 1989). This epidermal layer is a dual-compartment system comprised of dead, flattened corneocytes surrounded by a matrix of non-polar lipid lamellar bilayers. This has been described as the brick and mortar representation of the stratum corneum. The corneocytes represent the bricks and the extracellular lipid, the mortar. Within the corneocytes, the intracellular spaces are protein-rich and are hydrophilic. Conversely, the cell membranes and the extracellular lipid are hydrophobic.

The cells are highly water-retardant due to the extracellular lipid, but can also be water absorbant due to the hydrophilic intracellular compartment. Thus the stratum corneum can be considered to be a hydrophilic-lipophilic multilayered structure. This composition suggests that substances with both of these properties will penetrate the stratum corneum.

Keratin accounts for approximately 80% of the intracellular space and is formed into macrofibrillar bundles. These bundles provide a rigid structure for the corneocyte, which are ultimately shed from the skin surface (Eckert R.L. 1989, Elias P.M. 1983 and Wiechers J.W. 1989, Barry B. 1991 and 1991b).

It is generally agreed that the main location of the skin barrier function lies in the stratum corneum, and is mediated by the lipid enriched lamellar bilayers of the intercellular spaces (Barry B. 1991b, Bucks D.A. 1984, Denda M. *et al* 1998 and Wiechers J.W. 1989). However, even if the stratum corneum were removed there is still a substantial barrier to penetration. The secretion and coagulation of the interstitial fluid following barrier perturbation are rapid, so it is difficult to measure barrier function in the absence of this response. Also, the lipid from lamellar bodies in the stratum granulosum may reduce trans-epidermal water loss (Elias P. 1981b and Shaefer H. and Redelmeier T. 1996).

Evidence for the structure of the stratum corneum can be derived from the use of differential scanning calorimetry. This technique can show phase transitions in the tissue which relate to individual components of the stratum corneum, and are expressed as a temperature at which a particular phase transition occurs. In normal human stratum corneum there a four main phase transitions:

Endotherm T1 relates to lipid melting, mainly sebaceous lipids, at around 40^0C.

Endotherm T2 is due to melting of the lipid chains which are present within the bilayer structure, this occurs at approximately 70^0C.

Endotherm T3 accounts for the loss of associations between the polar head groups of the lipids and is observed at approximately 85^0C.

Endotherm T4 relates to the protein denaturation of the intracellular keratin. This occurs at around 100^0C (Barry B. 1987, 1991 and 1991b).

1.1.4 Dermis.

The dermis is composed of cells which are interconnected by collagen and elastin fibres, all embedded in an amorphous ground matrix of glycosoaminoglycans. The dermis forms the bulk of the skin and is approximately 3-5 mm thick (Wiechers J.W. 1989 and Barry B. 1991b). The network of fibrous, filamentous connective tissue provides the tensile strength and elasticity of the skin, and also forms a physical support for the nerve fibres and blood vessels. The glycosoaminoglycans are able to bind up to 1000 times their weight in water and are responsible for the water retention capacity of the dermis. The dermis also contains fibroblasts, endothelial cells and mast cells. The fibroblasts produce the components of connective tissue, including collagen. Mast cells play a role in defense against infective organisms through the release of histamine and proteases.

The dermis requires an efficient blood supply to transport nutrients, remove waste and to regulate skin temperature (Barry B. 1991b).

The dermis can be further distinguished as the upper papillary dermis and the underlying reticular dermis. The papillary dermis is folded to form

ridges or papillae, which matches the basal layer of the epidermis. This results in an increase in surface area contact between the dermis and epidermis, thus facilitating diffusion of substances, such as nutrients and growth factors, between these two layers. The papillary dermis has an average thickness of 0.1-0.2 mm (Chien Y. 1982). The reticular dermis is much thicker and has a large role in structural support, having an extensive collagen and elastin network.

1.1.5 Skin appendages.

Sweat glands are the most numerous of the skin appendages, and have a role in thermal regulation. They respond to increases in temperature by secreting an almost isotonic solution which is similar to the low molecular weight portion of the plasma (Bucks D.A. 1984).

Hair follicles are distributed unevenly through the skin, with increased numbers in certain locations in humans, the scalp, for example, has a high concentration of hair follicles. Each follicle is associated with an arrector pili muscle and a sebaceous gland, which joins the follicle via a duct. The entrance to the hair follicle may form a route of entry into the skin for formulations applied topically. Compounds may diffuse along the hair shaft and be delivered directly to the hair bulb, the sebaceous glands and the dermis (Bucks D.A. 1984).

Sebaceous glands consist of a lobe connected to the hair follicle by a duct. They secrete sebum which is mainly composed of triglycerides, free fatty acids and waxes. It is thought to be unlikely that sebum either enhances or hinders the transdermal absorption of topically applied compounds (Kligman A. 1983).

1.1.6 The Skin Vasculature.

The vascular supply of the skin is located in the dermis. It is comprised of arterioles and venules, which form two distinct plexuses, which

lie parallel to the skin surface. The upper horizontal plexus is found in the papillary dermis. From here, capillary loops arise which protrude into the dermal papillae, which reach to within 0.2 mm of the skin surface (Bronaugh R. *et al* 1986). This ensures that the blood supply rapidly absorbs and systemically dilutes any compound that penetrates through the epidermis. This constant removal maintains the concentration of penetrants low in the dermis (Barry B. 1991b).

The lower horizontal plexus is positioned 3-4 mm away at the dermal-subcutaneous interface. This lower plexus is formed from vessels from the underlying muscles and subcutaneous fat. The lower plexus is directly connected to the upper plexus by arterioles and venules, and also forms the blood supply for the hair follicles and sweat glands.

The walls of the arterioles and venules walls in the dermis are much thicker than those in the subcutaneous fat, 2-3μm and 0.1-0.3μm thick, respectively. This suggests a protective role against the external shearing forces to which the skin, and in consequence these vessels, are subjected (Braverman I. and Keh Y. A. 1981).

1.2 Cutaneous Drug Penetration.

For a compound to be absorbed through the skin, it must first penetrate the stratum corneum. From there it must enter the viable epidermis and diffuse into the dermal layer. Here, a compound can either undergo further diffusion into the underlying subcutaneous fat and beyond into muscle, or the drug may enter the systemic circulation via the dermal vasculature. The compound may form a reservoir in the skin, for example in the stratum corneum, where it may persist for several days, from which it may subsequently be slowly released. The applied drug can be lost from the skin surface by abrasion, evaporation and desquamation, thus reducing the amount of drug available for absorption.

For the majority of topically applied drugs, the stratum corneum is the rate limiting barrier to penetration. Percutaneous absorption of a compound is generally related to the length of exposure. Following topical application of a compound, a concentration gradient across the skin arises, which decreases exponentially with depth (Schaefer H. and Redelmeier T. 1996).

1.2.1 Routes of Drug Absorption.

It has been proposed that there are three possible routes of drug absorption across intact skin, the intercellular route, the intracellular route and the trans-appendageal route.

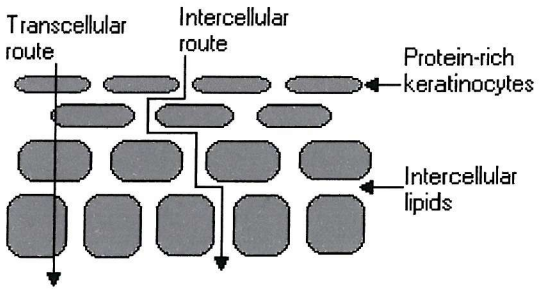


Figure 3 Routes of absorption through the skin.

1.2.1.1 Intercellular Route.

The intercellular pathway is via the lipid component between the cells of the stratum corneum. It is thought that low-molecular-weight, non-ionised, lipophilic molecules follow this route. The compounds dissolve in and diffuse through the lipid matrix.

The intercellular space is comprised of two alternating layers; firstly there are the lipids, which are organised into multi-laminated sheets (bilayers). These bilayers consist of straight, closely packed hydrocarbon chains, the majority of which are saturated. This provides the intercellular space with a highly ordered, rigid structure which accounts for the relative impermeability of the skin to many compounds including water (Wiechers J.W. 1989). The second of the two layers comprises watery channels between the polar head groups of the lipids, which may provide a polar route of absorption through the bilayers (Wiechers J.W. 1989).

The highly organised, multi-lamellar packing of the lipid region of the stratum corneum means that a penetrating molecule must diffuse across a large number of structural bilayers, and must move sequentially between hydrophilic and lipophilic domains (Bodde H.E. *et al* 1991 and Hadgraft J. and Pugh W. 1998). Small hydrophilic molecules may be able to diffuse along the polar head groups of the lipids. This suggests that the intercellular pathway may comprise of lipophilic and a hydrophilic route of absorption, between the corneocytes (Bodde H.E. *et al* 1991).

A penetrating molecule must diffuse around the numerous overlapping corneocytes of the stratum corneum, resulting in a pathway of high tortuosity. Although the stratum corneum has a thickness of approximately 10 μm, the diffusional pathlength may be as much as 500 μm (Kalia Y. *et al* 1998 and Hadgraft J. 1999).

There have been sites identified within the intercellular space which have a depletion of lipid lamellae. These sites generally occur at lateral sites of the corneocytes and between three-cell junctions. These cavities or lacunae may provide sites of access for water and other compounds to move through the stratum corneum (Barry B. 1991b).

The partition coefficient of the penetrating compound (a measure of affinity of the compound for different tissue layers) is correlated to the rate of penetration via this route. If the compound is extremely lipid soluble, it may enter the stratum corneum, but then remain there forming a reservoir, unable to diffuse into the more aqueous layers of the viable epidermis and dermis.

1.2.1.2 Intracellular Route

The intracellular pathway is via the corneocytes of the stratum corneum. It is thought that this pathway is followed by more hydrophilic molecules. These cells are filled with keratin and are bounded by an envelope which has a hydrophilic interior but a lipoidal exterior, which anchors the cells to the intercellular lipid lamellae (Barry B. 1991b and Wiechers J.W. 1989). The intracellular route is thought to be provided by a restricted, open portion of the hydrated protein (keratin) within the corneocytes (Barry B. 1991b).

It was considered that the only pathway through the stratum corneum was via the intercellular lipid route, but it was then observed that polar penetrants had higher than expected permeation rates through the skin. It would be expected that the polar compounds would have reduced partitioning if the lipoidal route was the only pathway available (Barry B. 1991b). Hence, it is likely that there is a non-lipoidal pathway through the membrane (Peck K.D. *et al* 1995). It is thought that hydrophilic molecules may diffuse through the corneocytes until an intercellular space is reached. Further diffusion through the stratum corneum may occur by movement of the compound out of the cells via desmosomes and passage to the next corneocyte may be facilitated by the lipid-depleted lacunae, that were described above (Barry B. 1991b).

A demonstration that compounds may enter the corneocytes was provided by Bodde H.E. *et al* (1991). They showed that mercuric chloride, when applied to excised human skin, entered the intercellular spaces, indicating that this route was predominantly used by this compound. However, following extended periods of time (up to 48 hours), the mercuric chloride was also seen inside the apical corneocytes. It was proposed that

this uptake into the cells was through desmosomes. Although it was proposed that the intracellular route of absorption was not likely to contribute significantly to the total absorption of mercuric chloride, this route may be significant for compounds that favour more hydrophilic conditions.

1.2.1.3 Trans-appendageal Route.

The appendages of the skin, the hair follicles with associated sebaceous glands and the sweat glands, may be considered as channels through the stratum corneum. There is abundant evidence that compounds can penetrate the skin appendages and so bypass the rate-limiting barrier of the stratum corneum (Tregear R. 1961, Kao J. *et al* 1988 and Schaefer H. and Redelmeier T. 1996).

This route of absorption was originally considered to be of little importance, as the number of appendages accounts for approximately 0.1-1% of the total surface of human skin (Schaefer H. and Redelmeier T. 1996 and Tur E. *et al* 1991). However, more recently it has become apparent that this route of absorption is important for some compounds. Using the Wahlberg guinea pig, which has an area of skin behind the ear which is devoid of any skin appendages, Illel B. *et al* (1991) showed that the *in vitro* diffusion of hydrocortisone, niflumic acid, caffeine and p-aminobenzoic acid was 2-4 times greater in normal haired skin compared to the diffusion across the hairless skin. Hydrocortisone absorption was also investigated across haired and hairless rat skin. The trans-appendageal route was shown to be important for this compound, particularly in the first 24 hours after topical application (Illel B. and Shaefer H. 1988).

Kao J. *et al* (1988) demonstrated that benzo[a]pyrene permeation across mouse skin *in vitro* was greater across haired skin than across hairless skin. Testosterone absorption was also studied. This compound is extensively absorbed, and showed no differences in absorption between hairless and normal mouse skin. However, the initial rates of absorption were higher in the haired mouse skin, indicating the appendageal diffusion may play a significant role.

A study using low intensity ultrasound on rat skin was used to investigate the penetration routes of several compounds. The ultrasound did not cause any change to the integrity of the skin, but did cause a discharge of sebum from the sebaceous glands which filled the hair follicle shafts. This caused the trans-follicular route to become blocked. Absorption of sucrose and mannitol was non-existent following ultrasound treatment of the skin. This indicates that these compounds penetrate the skin almost entirely by the trans-follicular route. Approximately half of hydrocortisone absorption could be accounted for by this route, but there was no difference in absorption of 5-fluorouracil and aminopyrine, indicating that the latter two compounds penetrate the skin across the stratum corneum and not via the appendages (Meidan V.M. *et al* 1998).

The sweat glands may represent an aqueous pore through the epidermis. This may provide an absorption route for polar compounds, which could diffuse through the sweat fluid produced by the glands. A possible hindering effect may be the outflow of sweat, which could oppose the inward diffusion of a penetrating molecule.

It is still not known to what extent the trans-appendageal route contributes to the total percutaneous absorption. The contribution appears to vary in degree between compounds. It is thought that this route may be more important for very slow diffusing compounds, and play a significant role immediately following topical application (Kao J. *et al* 1988).

1.2.2 Factors Affecting the Trans-dermal Penetration of Drugs.

1.2.2.1 Drug Characteristics.

The physicochemical characteristics of a compound govern its percutaneous penetration, but the most reliable parameter for prediction of penetration is the lipophilicity of the drug (Goosen C. *et al* 1998, Hadgraft J. 1999 and Roberts M.S. and Walters K.A. 1998). The molecular mass,

solubility and percentage of ionised compound do not correlate well with absorption (Goosen C. *et al* 1998, Wiechers J.W. 1989 and Hadgraft J. 1999).

The octanol-water partition coefficient (P_{oct}) of a compound is a measure of its lipophilicity, and is usually expressed as its $\log_{10}$ (log P). The value represents the equilibrium concentrations of a dissolved substance in a two phase system consisting of two immiscible solvents (octanol and water) (Idson B. 1983). Low P_{oct} and small molecular size indicate good penetration. Highly lipophilic drugs (with a high log P) may partition easily from the vehicle into the stratum corneum, but may not be able to partition from the stratum corneum into the more hydrophilic viable epidermis (Barry B. 1991b). Penetrating drugs ideally have both lipophilic and hydrophilic characteristics with a log $P \leq 2$ (Goosen C. *et al* 1998, Hadgraft J. 1999 and Roberts M.S. and Walters K.A. 1998). A lipid penetrant (log $P > 2$) is likely to be constrained to the hydrocarbon part of the lipid bilayer structure of the stratum corneum. A compound which is hydrophilic (log $P < 2$, often negative) will concentrate in the aqueous regions of the tissue (Barry B. 1991).

Movement of the molecule through the stratum corneum is via passive diffusion. This can be described by Ficks first law which states that the molecular flux per unit area (J) is proportional to the negative concentration gradient of the drug across the skin.

$$J = - D (dC/dx) \qquad \textbf{Equation 1}$$

D is the diffusion coefficient (reflects the ease with which a molecule moves through the skin), dC/dx is the concentration gradient (rate of change of concentration with distance within the skin). The negative sign indicates that the flux is in the direction of the lower concentration (Flynn G. 1989).

The flux and the penetration rate constant are directly related to the drug permeability of the stratum corneum. There are a number of ways in which to increase flux; by increasing the drug diffusion coefficient in the stratum corneum, and enhancing the drug solubility in the barrier, or by

increasing the drug concentration in the vehicle or reducing the drug solubility in the vehicle (Bach M. and Lippold B. 1998).

The electrical charge of the drug can affect its penetration into the stratum corneum. If the compound is ionisable, then it can exist in both its charged and uncharged forms, depending on the pH of the environment. The non-ionised form of a compound is more lipophilic and so may dissolve rapidly in the lipid component of the skin. The ionised form is less lipid soluble and so permeation is limited (reviewed in Goosen C. *et al* 1998).

The presence of polar groups on a penetrating compound causes a reduction in the permeability of the compound in the skin, due to an increase in the degree of chemical interaction between the polar groups and the stratum corneum (Idson B. 1983). Hydrogen-bonding between the permeating molecule and the stratum corneum is a major negative determinant in the diffusion of the molecule (Pugh W. *et al* 1996 and Roberts M.S. *et al* 1996). The permeability of the molecule is inversely related to the number of hydrogen-bonding groups present (Roberts M.S. *et al* 1996, Roberts M.S. and Walters K.A 1998, Pugh W. *et al* 1996 and Hadgraft J. 1999). There is a decrease in diffusion with the increase in hydrogen-bonding groups up until there are three groups present on the molecule. Thereafter, additional hydrogen-bonding groups cause negligible effects (Hadgraft J. 1999).

1.2.2.2 Effects of Vehicle Formulation.

The vehicle in which it is applied can influence the cutaneous penetration of a penetrating compound. The most common vehicles used are emulsions, either in the form of creams or lotions. The general requirement of a vehicle is that it doesn't induce irritation and sensitisation of the skin. The vehicle must also be capable of dissolving the drug, but must also allow the drug to diffuse out of the vehicle and into the stratum corneum.

The thermodynamic activity of the drug in the vehicle is the driving force for drug diffusion into the skin. Generally, the rate of release of the drug increases with increasing concentration in the vehicle, reaching a maximum at

the saturation concentration (Idson B. 1983 and Schaefer H. and Redelmeier T. 1996). The rate of release may by further increased from a vehicle that is supersaturated, but these formulations tend to be unstable and the compound may precipitate, thus rendering it unable to penetrate the stratum corneum (only the soluble fraction of the drug in the vehicle is able to diffuse into the skin) (Idson B. 1983 and Schaefer H. and Redelmeier T. 1996).

The partition coefficient is a measure of the affinity between the drug and the vehicle. If the partition coefficient is high, this indicates that the vehicle has a poor affinity for the drug, and the compound is able to leave the vehicle more easily. A low partition coefficient suggests that the drug will be retained by the vehicle, and so will have poor penetration of the skin (Idson B. 1983).

When the drug formulation is applied to the skin, it may undergo compositional changes. The formulation is usually applied in a thin layer with a large surface area, which can result in a high degree of surface evaporation. If the drug itself is highly volatile evaporation can result in a decrease in the concentration of the drug in the vehicle and so a reduction in drug available for skin penetration. The components of the vehicle may evaporate leading to an increase in the viscosity of the formulation and the possible formation of a supersaturated solution. This may then lead to precipitation of the drug, but may possibly also transiently enhance the therapeutic efficacy of the drug (Barry B. 1991b). The thinness of the applied layer also restricts the amount of drug that is applied to the skin (Schaefer H. and Redelmeier T. 1996).

1.2.2.3 Penetration Enhancers.

A penetration enhancer has the function of reversibly reducing the barrier resistance of the stratum corneum (Barry B. 1987). The result is that drug penetration is greater and may be more rapid. A large proportion of enhancers interact with the lipid structure of the stratum corneum, so as to disrupt the organisation of the lipids and increase their fluidity, for example, the non-polar enhancers oleic acid and azone. Many enhancers affect the

intracellular protein and so reduce the diffusional resistance of this route to penetration. The intracellular diffusional resistance can be reduced substantially by water. When dry, the intracellular contents are more solid, but become more fluid when hydrated. Some enhancers, particularly small polar molecules such as DMSO (dimethylsulfoxide) and propylene glycol, can accumulate in both the intercellular lipid and the intracellular protein regions, increasing drug partitioning into the skin and so promoting drug absorption via both routes (Barry B. 1987).

1.2.2.4 Effects of Occlusion.

Skin hydration plays a major role in the rate and extent of cutaneous penetration. In normal, unoccluded skin, there is a continuous outward diffusion of water from within the body, through the stratum corneum and out into the environment, this is called the transepidermal water loss (TEWL) (Wiechers J.W. 1989, Barry B. 1987, Wester R.C. and Maibach H.I. 1995 and Idson B. 1983). This means that the stratum corneum is a partially hydrated tissue, with continuous passive movement of water through it, dependent on a concentration gradient (Wiechers J.W. 1989). Transepidermal water loss (TEWL) measurements demonstrate evaporation of endogenous water from the skin surface, and can be rapidly performed using a TEWL meter (Nangia A. *et al* 1998). TEWL increases with increasing perturbation of the stratum corneum, by tape-stripping for example. It has therefore been proposed that TEWL can be considered to be an index of the integrity and efficiency of the stratum corneum (Van der Valk P. and Maibach H. 1990 and Nangia A. *et al* 1998). It has been demonstrated that there are substantial regional variations in TEWL. For example, the TEWL is greater on the volar, compared to the dorsal forearm, and it increases from proximal to distal forearm (Oestmann E. *et al* 1993). Lotte C. *et al* (1987) showed that the TEWL measurements were linearly correlated to penetration of several compounds, regardless of number of cell layers of the stratum corneum.

The primary method of increasing skin hydration is occlusion of the skin. This can be achieved by various methods such as bandaging, the use

of polythene dressings and the wearing of clothes. Partial skin occlusion can be the result of applying some topical formulations, such as petrolatum, ointments or creams (Schaefer H. and Redelmeier T. 1996). The occlusive layer prevents the normal evaporation of water from the skin surface, and also results in a build up of the water diffusing from the deeper layers of the epidermis. Normally, the water content of the stratum corneum is 5-15%, but this can be increased to up to 50% by occlusion (Treffel P. *et al* 1992). Measurements of TEWL are also increased, as would be expected with the increased hydration of the tissue.

It is the general opinion that an increase in stratum corneum hydration produced by occlusion can lead to the promotion of percutaneous penetration (Ryatt K.S. *et al* 1988). The main effect of occlusion is the increased hydration of the stratum corneum. One of the effects of increased tissue hydration is the swelling of the corneocytes, which reduces the density of their structure and their resistance to diffusion. The swollen corneocytes may serve to provide a pathway for penetrating substances, by facilitating entry into the corneocytes and so increasing the diffusivity of the penetrating compound through these cells (Idson B. 1983 and Schaefer H. and Redelmeier T. 1996).

The increased water content also affects the lipid region of the stratum corneum, and causes alterations to the stratum corneum phase transition temperatures. T1 is not altered by the increased concentration of water, but T2 and T3 are reduced slightly (T2 accounts for the melting of the lipid chains within the bilayers, and T3 relates to the breakdown of associations with the lipid polar head region). T4 falls considerably (irreversible protein denaturation) (Barry B. 1987). It is of interest to note that the two major endotherms relating to the stratum corneum lipids become reduced. This suggests that the lipid bilayer region has an increased in fluidity and that the barrier function afforded by this area is reduced by the presence of water. The water molecules become associated with the polar head groups of the lipids via hydrogen-bonding. The insertion of these water molecules around the head groups forces a loosening of the lipid packing, and decreases the inter-molecular forces between the lipids. This is the cause of the reduction in

the transition temperatures T2 and T3 (Barry B. 1987). The reason for the fall in T4 is that the presence of the water molecules results in swelling of the cells in the tissue. This allows more freedom of movement for protein rearrangement within the corneocytes, and also that the water molecules compete for hydrogen-bonding sites that would normally interact with other proteins. These two factors result in a greater ease to denature the protein structures (Barry B. 1987). The more fluid structure of the stratum corneum lipids in the presence of increased hydration, together with the increased volume of water and the competitive binding of water molecules to the tissue binding sites, results in a general increase in drug motility and so permeation in the stratum corneum.

The absorption of several compounds has been assessed in relation to occlusion of the skin. The absorption across human skin of ethyl nicotinate and nitrobenzene (reviewed in Wester R.C. and Maibach H.I. 1995), hexyl nicotinate (Ryatt K.S. *et al* 1988) and citropten (Treffel P. *et al* 1992) was increased due to effect of occlusion, but substances such as caffeine, methanol, ethanol and hydrocortisone did not have increased absorption (Treffel P. *et al* 1992). It is a general opinion that occlusion of the skin has a more pronounced effect on the absorption of lipophilic compounds, with limited or no increases in skin absorption for hydrophilic substances. This may be related to the route of absorption that these compounds favour. It has already been discussed that the lipid region of the stratum corneum is affected by the presence of water, and that the barrier function becomes impaired. This is the predominant route of absorption of lipophilic compounds, and drug motility is increased. However, for hydrophilic substances, it is thought that they favour the intracellular route across the stratum corneum. The corneocytes, although they become swollen and may allow a greater diffusivity of compounds into and through the cells, are generally already hydrophilic in nature (Schaefer H. and Redelmeier T. 1996 and Treffel P. *et al* 1992). There may not be such a dramatic difference in absorption seen via this route due to an increase in water content.

Occlusion results in an increase in skin temperature from 32^0C to 37^0C (Kligman A. 1983). However, this is unlikely to affect the permeation of a

penetrating species, as it has been shown *in vitro* that the changes in absorption were small as the temperature increased up to 70^0C, and that it was only above this temperature that large increases in skin permeability were observed (reviewed in Wiechers J.W. 1989). This temperature relates to the T2 phase transition of the stratum corneum, accounting for lipid melting in the bilayers. Above this the other phase transitions occur, including breakdown of the associations with the polar head groups and denaturation of the proteins of the corneocytes (Barry B. 1987). It is expected then, that as these high temperatures are approached, the integrity of the stratum corneum is compromised, thus permeability increases. Below 70^0C the stratum corneum structure is likely to be maintained, accounting for the minor differences in permeant absorption due to small changes in the temperature of the skin.

The effects of occlusion on the stratum corneum are transitory. Upon removal of the occlusive dressing, the level of tissue hydration, measurements of TEWL and degree of drug absorption all return to baseline levels (unhydrated skin) within one hour (Schaefer H. and Redelmeier T. 1996 and Ryatt K.S. *et al* 1988).

1.2.2.5 Effects of Heat Exposure.

Exposure to high ambient temperatures may have an effect on the transdermal penetration of some compounds. Increased plasma levels of nicotine (Vanakoski J. *et al* 1996), methyl salicylate (Danon A. *et al* 1986), glyceryl trinitrate (GTN) (Barkve T. *et al* 1986) and dihydrotestosterone (Clarys P. *et al* 1998) have been demonstrated in situations of increased temperature following topical administration. Clonidine was not shown to have an increased dermal penetration following external heating, but the absorption was significantly increased in summer as compared to winter (Fujimura A. *et al* 1996), although this could be due to the suspected decrease in the barrier function of the stratum corneum in the winter months (see Section 1.2.2.6.5).

The increases in drug penetration of the skin seem to be mainly related to the heat-induced vasodilation of the skin (Vanakoski J. and Seppala T. 1998). Skin perfusion and total dermal blood flow are linearly related between 30-38^{0}C (Banic A. *et al* 1990), and the cutaneous blood flow can increase up to 10-12 fold in high ambient temperatures, such as in a sauna. The high plasma levels of these drugs are thought to be due to an increase in dermal washout of the compounds which results from the enhanced perfusion of the skin. The increased temperature may also produce greater diffusion of the drug across the skin.

Exercise resulted in similarly increased plasma levels of GTN and methyl salicylate. The increase in dermal blood flow in this situation is also thought to be a major determinant of drug penetration (Barkve T. *et al* 1986 and Danon A. *et al* 1986).

1.2.2.6 Biological Factors Affecting Absorption.

The skin functions as a barrier when the stratum corneum is intact, but changes in the barrier function of this layer can greatly influence percutaneous penetration. Mechanical perturbation of the stratum corneum, such as tape-stripping and delipidisation, enhance the absorption of many compounds. Delipidisation generally increases penetration of hydrophilic compounds, as the barrier due to the intercellular lipid is lost.

1.2.2.6.1 Ageing of the Skin.

Ageing of the skin may have an effect on drug permeability. When barrier function was compared between young (20-30 years) and aged (> 80 years) human skin, the perturbation of the barrier with acetone and by tape-stripping was much more marked, and tissue recovery was slower in aged skin. (Ghadially R. et al 1995) Ageing of the skin causes increased dryness of the stratum corneum and reduces sebaceous gland activity, which results in a decrease in the

amount of skin surface lipids. The lipid content in aged skin is substantially decreased, although the distribution of the lipids e.g. sterols, free fatty acids and triglycerides, and the sphingolipids is not altered. This indicates that the ageing of the epidermis is accompanied by a global reduction in stratum corneum lipid content, but that there is no selective decrease in any specific species of lipid (Ghadially R. *et al* 1995 and Rogers J. *et al* 1996). There is a decrease in the thickness of the stratum corneum in aged skin, but there is no alteration in the number of cell layers (Schaefer H. and Redelmeier T. 1996). There is a flattening of the dermal-epidermal junction, and some atrophy of the capillary network of the skin. The reduced blood supply in the skin of the elderly results in a decreased ability of the microcirculation to clear transdermally absorbed compounds (Roskos K. *et al* 1989).

1.2.2.6.2 Regional Variations in Skin.

Regional differences in skin permeability have been demonstrated. Using the topical application of various compounds (benzoic acid and its sodium salt, caffeine and salicylic acid), Rougier A. *et al* (1987) demonstrated that the skin permeability increases in the rank order of arm $\leq$ abdomen $<$ post-auricular (behind the ear) $<$ forehead. In all cases, the forehead was approximately twice as permeable as the arm or abdomen. It was proposed that the increased number of sebaceous glands present on the forehead may contribute to the increased permeability, by facilitating absorption through the follicles rather than across the epidermis. However, the number of glands is greater by a factor of 50 to 100, whereas the permeability only increased by a factor of two.

It has been shown that the lipid content of the stratum corneum correlates inversely with penetration of water and salicylic acid (Elias P. *et al* 1981). The mean percentage lipid weight of the stratum corneum in the leg is 3%, and is 6.8% in the abdomen. Penetration through the stratum corneum of the leg was around two fold greater than through the abdominal stratum corneum. The absorption was not affected by the stratum corneum thickness, or the number of cell layers. This suggests that the thickness of

the stratum corneum may not be a determining factor in predicting the permeability of the skin as was thought by Holbrook K. and Odland G. (1974), but that the content of the stratum corneum lipid may be a much more important predictor (Elias P. *et al* 1981).

1.2.2.6.3 Skin Type.

Skin types show differences in structure, composition and percutaneous penetration. There are no observed differences between Asian and white skin, but black (African American) skin exhibits a greater number of cell layers, but has the same stratum corneum thickness as white skin (Berardesca E. and Maibach H. 1996 and Reed J. *et al* 1995). Black skin has a greater amount of stratum corneum lipids, but no alteration in the lipid distribution (Berardesca E. and Maibach H. 1996). The penetration of fluocinolone acetonide and the effectiveness of EMLA cream were both reduced in black skin compared to white skin (Berardesca E. and Maibach H. 1996).

There were no gender differences in stratum corneum thickness, number of cell layers and lipid composition (Reed J. *et al* 1995).

1.2.2.6.4 Effect of Dermal Microcirculation.

It has been assumed that the dermal microcirculation efficiently removes a penetrating compound from the skin tissue into the systemic circulation. However, some lipophilic compounds such as lignocaine, testosterone and progesterone have been shown to penetrate into the subcutaneous layer, beyond the dermal microvasculature. These compounds are unionised at the dermal pH of 7.4, and so may not enter the blood supply effectively, where-as salicylic acid, for example, is ionised at this physiological pH, and will be readily taken up by the blood supply. This demonstrates that the microcirculation is not a perfect sink for all topically applied compounds (Singh P. and Roberts M.S. 1994). It is interesting to note that some

salicylates have been seen to penetrate into the underlying tissues, and that diclofenac has been found in the synovial fluid of joints where the drug was topically applied. These compounds are unlikely to reach these regions purely by diffusion, as the dermal blood supply should have largely cleared them from the tissue. It is possible that these drugs were redistributed to these regions by the systemic circulation (Singh P. and Roberts M.S. 1994).

In the absence of a blood supply, i.e. excised skin, removal of transdermally absorbed compounds is by simple diffusion into the deeper tissues. When the blood supply is present, the compound is cleared by a combination of deeper diffusion and by removal into the systemic circulation (Singh P. and Roberts M.S. 1994). *In vitro* absorption studies have shown that the tissue concentrations are greatest in skin with no blood supply, decreasing in vasoconstricted skin, and further in normal skin, with the lowest concentrations seen in vasodilated skin (Roberts M.S. and Walters K.A. 1998).

1.2.2.6.5 Environmental Factors.

Recently, it has been shown that percutaneous penetration of compounds is likely to be affected by circadian rhythm. Yosipovitch G. *et al* (1998) showed that in intact skin, the TEWL is increased in the evening and night, being at its minimum in the morning. This suggests that the barrier function is reduced in the evening and night, and so skin permeability is likely to be increased. Pershing L. *et al* (1994) demonstrated that betamethasone absorption, following topical application, was maximal at midnight.

Seasonal variations in TEWL have also been noted. Topically applied sodium lauryl sulphate (SLS) induces irritant dermatitis, with increased dermal blood flow and TEWL. Agner T. and Serup J. (1989) reported an increased dermal reactivity to SLS and raised TEWL measurements in the winter as compared to the summer. This indicates that in the winter, the barrier function of the stratum corneum is reduced. Rogers J. *et al* (1996) noted that the lipid content of the stratum corneum is reduced in the winter, and suggests that this may explain the diminished barrier function of the skin in

the winter months. It was also noted that the hydration of the stratum corneum was reduced in the winter compared to summer months (Agner T. and Serup J. 1989). Again, this indicates that it is the lipid content of the skin which is the primary determinant of the barrier function of the stratum corneum and so determines the skin permeability.

1.3 Methods of *In Vivo* Measurement of Percutaneous Absorption.

There are several generally accepted methods for the determination of percutaneous drug absorption. Some of these methods rely on the interpretation of plasma concentrations, time profiles and urinary excretion. However, these may be of limited use if the compound is poorly absorbed, resulting in dilution of already small concentrations. Other techniques such as skin stripping and suction blister fluid analysis give more direct measurements of concentration, but are also of limited use as they are destructive to the skin tissue, and they are unable to provide multiple samples from the same site. These techniques may also lead to permanent scarring.

1.3.1 The Radiochemical Method.

This indirect method involves the measurement of radioactivity in urine and faeces, following topical application of the radiolabelled compound. To correct for radioactivity that may be retained in the body, the same radiolabelled compound is administered intravenously, and the amount of radioactivity excreted is compared to that following topical administration. This method does not distinguish between parent drug and metabolites, is very time consuming and expensive, and is not always applicable to human use (Wester R.C. and Maibach H.I. 1989).

1.3.2 Skin Stripping Method.

This method determines the concentration of the compound in the stratum corneum following a period of topical application. The compound is applied to the skin for a short length of time, approximately 30 minutes. The stratum corneum is then removed by successive tape-strippings, which are then assayed to determine the drug concentration (Wester R.C. and Maibach H.I. 1989, Surber C. 1996 and Schalla W. *et al* 1989). However, the number

of stratum corneum cells being removed may not be linearly proportional to the number of strippings. This is influenced by several factors including the hydration of the skin, anatomical site, interindividual variability in stratum corneum thickness and number of cell layers, and tape-stripping technique (Marttin E. *et al* 1996).

1.3.3 Suction Blister Method.

Blisters can be raised in the skin, sub-epidermally, by mild suction. The blister fluid is generally accepted to represent interstitial fluid, containing proteins and lipids. Drugs can be applied topically before, during or after the blisters are raised, and the fluid is sampled by puncture of the blister roof with a fine needle. Suction blisters can also be used to determine skin drug concentrations following systemic administration.

This method is suitable for use in humans, but it takes around 5-10 days for the skin to heal. The technique is often not suitable for use in diseased skin (Surber C. 1996 and Schalla W. *et al* 1989).

1.3.4 Skin Biopsy Method.

This method is the most invasive, as it involves the excision of a full-thickness portion of skin. The compound of interest is applied topically, followed by a punch biopsy of that region of the skin, under local anaesthesia. The biopsy should produce full thickness skin, and can be sectioned parallel to the skin surface, to obtain distinct layers of epidermis, dermis and subcutaneous tissue. The sections are analysed for their drug concentration, and a concentration gradient from the surface through the layers of the skin is obtained (Surber C. 1996).

1.4 Principles of Microdialysis.

Microdialysis is a technique for the *in vivo* sampling of substances, both endogenous and exogenous, in the extracellular fluid. The technique was first used to measure the chemical changes in extracellular fluid via a semi-permeable membrane (Bito L. *et al* 1966). It then became established as a technique for the determination of neurotransmitter levels in rat brain studies (Understedt U. 1984). Since the 1980's, microdialysis has been performed in many other tissues, including muscle, systemic blood, liver, heart and adipose tissue (reviewed in Elmquist W. and Sawchuck R. 1997). The first use of microdialysis in a human subject was reported by Lonnroth P. *et al* in 1987, who performed an experiment to measure the glucose concentration in the subcutaneous adipose tissue of the abdomen.

Human dermal microdialysis was first reported by Anderson C. *et al* in 1991, measuring absorption of ethanol across normal skin. This was closely followed by a report of dermal glucose concentration measurement (Petersen L. *et al* 1992). Dermal microdialysis is being increasingly used to assess the pharmacokinetic and pharmacodynamic properties of drugs, both when administered systemically and topically.

The basic principle of the microdialysis membrane is to mimic the passive function of a blood capillary. The microdialysis probe is a very thin tubular semi-permeable membrane, which can be inserted into the organ of interest, in this case, the skin. The membrane is continuously perfused with an isotonic solution (the perfusate) at a very low flow rate. The membrane allows the diffusion of small molecules either into or out of the perfusate, without any net transfer of liquid in either direction at low flow rates. This is termed 'dialysis'. Diffusion takes place down a concentration gradient, i.e. if a substance is in a higher concentration in the tissue than in the perfusate, the compound will diffuse through the microdialysis membrane and enter the perfusate. If a substance is in a higher concentration in the perfusate, then the opposite will occur. This means that substances in the extracellular fluid

can be sampled, or a compound can be delivered into the tissue using microdialysis.

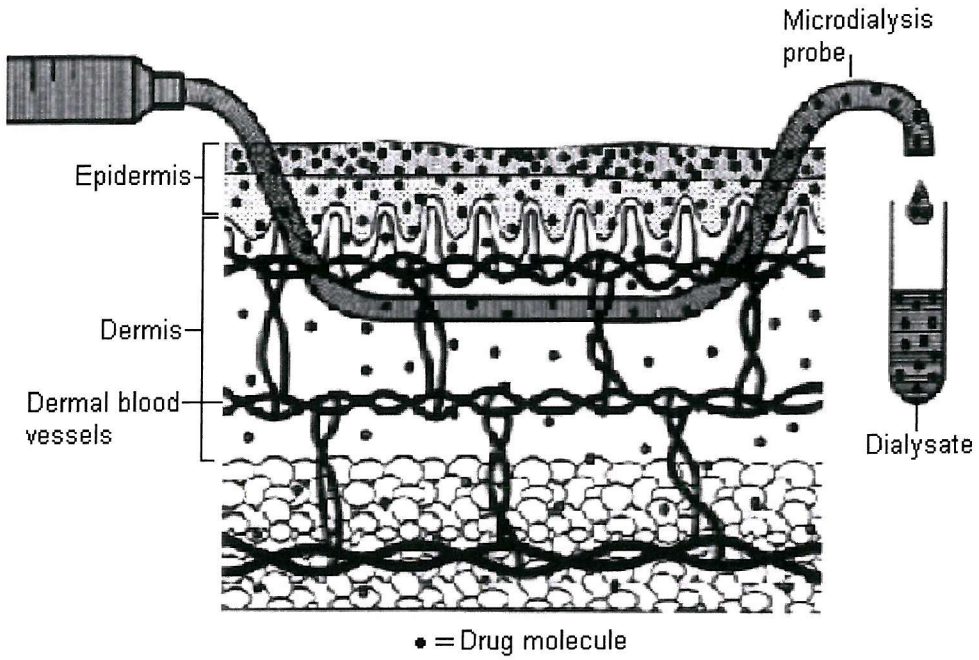


Figure 4 Schematic representation of the microdialysis in the dermis following topical drug application (Benfeldt E. 1999).

The size of the pores in the microdialysis membrane determine the size of the molecule that can pass through it. In this project, the probes had a molecular cut-off of 2000 Daltons. This resulted in restricted diffusion of large molecules. Substances with high molecular weight, such as proteins, were not able to pass across the membrane and enter the perfusate. This has the advantage of rendering the samples protein-free, so there is minimal sample preparation prior to analysis.

The concentration of a given substance in the dialysate reflects the actual concentration of that substance in the tissue. The dialysate concentration will not equal the actual tissue concentration, as the perfusion fluid does not reach equilibrium, due to the constant flow. The relationship between the two concentrations can be calculated, whereby the dialysate concentration is expressed as a percentage of the tissue concentration, and is termed relative recovery. As the perfusate flow rate decreases, the relative

recovery increases. The slower flow allows more time for diffusion of the molecules across the microdialysis membrane. For a wide range of compounds, recovery into the probe and delivery from the perfusate are equal. This is true when using a variety of probe types with molecular cut-off ranging from 5-29 KDa (Zhao Y. *et al* 1995).

It is generally accepted that the recovery *in vitro* cannot be used to estimate *in vivo* recovery. *In vitro*, the rate-limiting step of diffusion is due to the resistance of the dialysis membrane. The diffusion characteristics for an aqueous solution, as used in the *in vitro* situation, and for skin tissue are different (Fettweis G. and Borlak J. 1996). The extracellular matrix of a tissue has a gelatinous character due to glycoproteins, collagen and elastin fibres. This produces an increased viscosity when compared to a simple aqueous solution. *In vitro* recovery is generally higher than recovery *in vivo*, indicating that the diffusion of the compound in the tissue is the rate-limiting factor for *in vivo* recovery studies (Song Y. and Lunte C. 1999). The ground substance, together with the tissue tortuosity, which arises due to the large numbers of cells, can impede diffusion.

During *in vivo* microdialysis, the tissue immediately surrounding the probe is sampled. The compound of interest diffuses from this area down a concentration gradient, and enters the perfusate. In the freely diffusible aqueous situation of an *in vitro* study, the area around the probe is continuously and rapidly replenished with drug from the bath solution. However, *in vivo,* movement of the drug in the skin is likely to be hindered. If the drug cannot diffuse through the tissue sufficiently rapidly to replace the sampled compound, then the area of tissue around the probe may become depleted. The result of this could be that, due to the initial depletion, a concentration gradient arises in the tissue. The amount of drug being sampled by the microdialysis probe may be a reflection on how fast the compound can be delivered into the sampling area, rather than the actual concentration of the compound in the tissue.

The structure of the substance being dialysed can affect recovery. Recovery is inversely proportional to the molecular weight of the compound of interest. The smaller the molecule, the more easily it will diffuse across the

membrane. The hydrophobicity of the compound can also affect the recovery. In this project, the type of microdialysis probe used was composed of cellulose, and was hydrophilic. This is more permeable to hydrophilic substances (Groth L. and Jorgensen A. 1997). It has been found that as the lipophilicity of the compound increases, the recovery decreases (Groth L. and Jorgensen A. 1997). It has also been demonstrated that for more lipophilic compounds, the recovery into the perfusate and delivery of the compound from the perfusate may not be equal, with delivery being greater than recovery. Amberg G. and Lindefors N. (1989) offer the explanation that these differences may be due to interactions between the compound and the membrane. Groth L. and Jorgensen A. (1997) also noted that compound-membrane interaction (adherence) is more pronounced with more lipophilic compounds.

The extent of protein-binding of the compound will affect recovery. If the substance is highly protein-bound in the extracellular fluid, then less would be available for diffusion into the perfusate. It is possible to enhance the recovery of such compounds by modification of the perfusion fluid, for example by the addition of human serum albumin. This has the effect of binding the compound and retaining it within the perfusate (Carneheim C. and Stahle L. 1991). The same effect can also be obtained by using a microdialysis probe with a much larger pore size and greater molecular cut-off. This would allow the diffusion of large molecules, such as proteins, across the membrane so enabling the recovery of the bound fraction, as well as the free fraction of the compound. The recovery of lipophilic compounds can also be enhanced by perfusion fluid modification. The perfusion of a lipophilic fluid, such as Intralipid (an emulsion of soy bean oil and egg yolk phospholipid in water, used clinically for parenteral feeding), through the probe improves the diffusion of lipophilic compounds across the microdialysis membrane, and so improves recovery (Carneheim C. and Stahle L. 1991).

Another factor affecting diffusion of a molecule across the membrane is the thickness of the probe walls. The thinner the membrane, the lower the resistance of the membrane to diffusion. This results in greater mobility of molecules within the membrane, and so increased diffusion (Flynn G. 1989).

Zhao Y. *et al* (1995) demonstrated that recovery decreased as the membrane wall thickness increased.

1.5 Advantages and Limitations of Microdialysis.

Some of the previous methods of assessing the extent of transdermal absorption of a compound rely on the measurement of the substance in the blood or excreta. Generally, the amount of drug absorbed via the skin is very small, and the uptake of the compound into the systemic circulation results in a huge dilution effect, which may reduce the concentration of the substance to a level below the limit of detection. The microdialysis technique samples drug concentrations immediately below the site of application and has several advantages over traditional methods of measurement of dermal drug concentrations, but has few disadvantages or limitations.

1.5.1 Advantages.

- Microdialysis allows the continuous monitoring of a defined compartment of a tissue for prolonged periods of time, from hours, to days, to weeks.
- The studies can be performed in freely moving, conscious animals or humans.
- Microdialysis probes can be inserted into any organ or tissue.
- It is possible to monitor several sites on the same animal or human. This has the advantages of reducing variability, as intra-animal/human variability is generally less than inter-animal/human variation. This reduces the number of animals or humans involved in the study, and also means that the animal does not need to be sacrificed at the end of the experiment.
- Microdialysis can be used not only to monitor drug concentrations in the tissue, but can be used to deliver compounds to defined areas.
- The membrane excludes the diffusion of protein into the perfusate, so the samples need minimal or no preparation prior to analysis.

- The exclusion of protein is useful in pharmacokinetic studies where it is the concentration of the unbound fraction of the drug that is required.
- In the absence of proteins, there is no possibility of enzymatic degradation once the compound has entered the microdialysis probe.
- Microdialysis can also be useful in metabolic studies, having the ability to sample the parent compound and its metabolites simultaneously.
- Microdialysis is a very easy technique to master and probes can be inserted reproducibly after only a few practices.

1.5.2 Disadvantages.

- The concentration of the drug measured in the dialysate is not the true extracellular concentration.
- Probe calibration is required to determine the percentage recovery for each compound, probe type and perfusion flow rate.
- The insertion of the microdialysis probe produces mild trauma to the skin, although only transient, it does alter the normal extracellular environment but to a lesser extent than a suction blister.
- To improve recovery, the flow rate can be decreased to very slow rates, but this produces very small volumes of sample which may then be difficult to analyse, leading to the need for very sensitive analysis methods.
- Microdialysis of lipophilic or highly protein-bound compounds may not be feasible. Although modification of the perfusion fluid can enhance their recovery.

1.5.3 Needle Insertion Trauma

One of the main disadvantages of the microdialysis technique is the resultant skin trauma following probe insertion. The insertion produces tissue damage, and temporarily alters the extracellular environment around the probe. The introduction of the needle into the skin produces an increase in skin blood flow, skin thickness, erythema and skin histamine levels. The

primary cause of increased skin thickness is the formation of oedema. Using ultrasound imaging, Groth L. *et al* (1998) demonstrated that the oedema in rat skin remains prominent even after 120 minutes.

The oedema may alter the diffusion characteristics of the skin by increasing the volume of the extracellular space around the probe. The oedematous tissue is more aqueous in nature than normal tissue, so diffusion may occur more rapidly. This may affect the recovery of the drug into the microdialysis probe. The drug is sampled from the extracellular fluid directly around the probe, and may deplete this area of the compound. The rate of delivery of the drug into this small area from the tissue surrounding it, can then determine the rate of recovery into the probe. In normal tissue the rate-limiting factor of this delivery can be hindered by the tortuous and complex nature of the tissue. In oedematous skin, the tortuosity is reduced and diffusion through this more aqueous tissue may be more rapid. The result is an increase in delivery of the drug into the immediate vicinity of the probe, and so may increase recovery.

The skin blood flow is also increased. Groth L. and Serup J. (1998) suggested that the dermal blood flow requires 90-120 minutes to stabilise in human skin. They reported that the erythema subsided to baseline after 90 minutes, but the increase in skin thickness remained. The probe itself was likely to have contributed to the thickness of the skin, as it was 216 μm in diameter, but the oedema also appeared to persist throughout the study (Groth L. 1998).

With microdialysis probes inserted in rat brain, Clapp-Lilly K.L. *et al* (1999) observed that there was tissue damage up to 1.4 mm away from the probe site. This damage was seen in both dialysed and non-dialysed brain tissue.

1.5.4 Determination of Extracellular Concentrations.

Values of recovery derived from *in vitro* studies are generally not reliable indicators of *in vivo* recovery. Therefore, it is necessary to ascertain recovery *in vivo*. There are various methods proposed to perform *in vivo*

calibration of the microdialysis probe. These include extrapolation to zero flow method (Jacobson I. *et al* 1985 and Lonnroth P. and Strindberg L. 1995), the slow-flow (stop-flow) method (Menacherry S. *et al* 1992) and the point of no-net-flux method (Lonnroth P. *et al* 1987 and Lonnroth P. and Strindberg L. 1995). These three methods are useful for measuring the concentrations of endogenous compounds, as they all require a steady-state concentration of the substance in the tissue. Methods for probe calibration that are useful for measuring exogenous compounds include the internal reference technique (Larsson C. 1991) and the retrodialysis method (Muller M. *et al* 1995). The latter relies on the assumption that recovery and delivery of the compound through the microdialysis probe are equal. It is important to assess the ratio between recovery and delivery *in vitro* prior to probe calibration *in vivo*.

1.5.4.1 Extrapolation to Zero Flow Method.

This method involves the perfusion of the microdialysis probe at different flow rates. The concentrations (recovery) of the compound of interest are measured in the dialysate for each flow rate. The concentration of the compound of interest is plotted against the flow rate, and it is possible to extrapolate to zero flow and so estimate the external concentration. At zero flow the compound of interest should be at equilibrium between the external tissue and the internal dialysate (Jacobson I. *et al* 1985 and Lonnroth P. and Strindberg L. 1995).

1.5.4.2 The Slow (Stop) Flow Method.

This method relies on the assumption that at very low flow rates, the concentration of the compound of interest in the dialysate is very close to an equilibrium with the external concentration. In theory, it would require zero flow to establish an equilibrium, but practically, flow is needed to collect the dialysate and analyse for the concentration of the compound. Above 90% efficiency is seen at flow rates of around 50nl/min (Menacherry S. *et al* 1992).

1.5.4.3 Point of No-Net-Flux Method.

Here it is assumed that, with a constant flow rate, the recovery of the compound of interest is also constant over a range of concentrations. The compound of interest is added to the perfusate in varying concentrations. If the concentration is lower in the perfusate than in the tissue, the compound will diffuse into the microdialysis probe. If the concentration is higher in the perfusate, the substance will diffuse out into the tissue. Net transport of the compound is calculated by subtracting the concentration in the dialysate from the concentration that was added to the perfusate. The point where there is no net transfer is when the external and internal concentrations are equal (Lonnroth P. *et al* 1987 and Lonnroth P. and Strindberg L. 1995).

1.5.4.4 Internal Reference Technique.

This method requires the use of a compound that is chemically very similar to the compound of interest. This ensures that the diffusion rates in the tissue are likely to be similar. The chemically-similar compound (internal reference) is added to the perfusate. The principle is, that the internal reference compound diffuses out of the perfusate at the same rate as the compound of interest diffuses in. The concentration of the internal reference compound may increase in the tissue around the microdialysis probe with time, and so the concentration gradient across the membrane may decrease. However, if the diffusion rate through the tissue is similar for both compounds, the concentration of the compound of interest should decrease to the same extent as the concentration of the internal reference compound increases. The concentration gradient across the microdialysis membrane for the compound of interest will decrease in a similar way as for the internal reference compound. The decrease in delivery of the internal reference compound will reflect the decrease in recovery of the compound of interest (Larsson C. 1991).

1.5.4.5 Retrodialysis Method.

The retrodialysis method relies on the assumption that the diffusion of the compound of interest is quantitatively equal in both directions across the microdialysis membrane. The compound of interest is added to the perfusion fluid. The diffusion of the compound out of the probe (delivery) can be taken as the recovery for a particular flow rate. It should be noted that in this method the probe is used for delivery only and recovery would be assessed using a separate microdialysis probe, at a site remote from the calibration probe.

This method is often preferred, as the others are slow and laborious. However, there are some limitations. The method relies on the fact that there is a sink in the vicinity of the probe, into which the compound diffuses. Decreases in delivery of the compound over prolonged periods of time indicate that the drug has accumulated around the probe, reducing the concentration gradient, and so lowering diffusion out of the perfusate. The sink effect becomes compromised. Over shorter periods of time, it should be possible to obtain a reliable value for *in vivo* recovery of the compound (Muller M. *et al* 1995).

1.6 Penciclovir.

Penciclovir is currently marketed as Vectavir cream for the topical treatment of herpes labialis (cold sores), and is a prescription only drug. Interest in the determination of the transdermal absorption of penciclovir arose from the pharmaceutical company that manufactures Vectavir cream. The use of this drug as model substrate for the evaluation and development of the microdialysis technique seemed ideal in the light of preliminary studies which indicated that the compound has good, but variable skin penetration.

1.6.1 Structure of Penciclovir.

9-(4-hydroxy-3-hydroxymethylbut-1-yl)guanine.

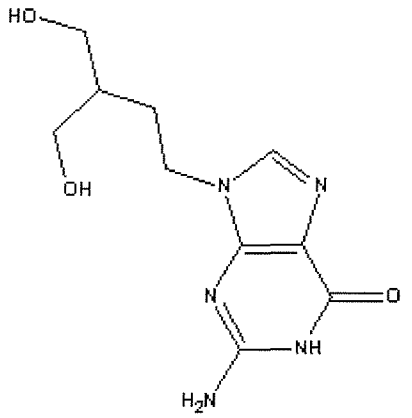


Figure 5 Structure of Penciclovir. Molecular weight : 253 Da.

Penciclovir is slightly soluble in water (1.7 mg/ml at 20^{0}C). It has a log P of -1.8, and has the dissociation constants pKa1 of 3.1 and pKa2 of 9.76. The molecule has three possible sites that could bind with the tissue forming hydrogen bonds. This indicates that the absorption of penciclovir could be hindered due to these drug-tissue interactions (Pugh W. *et al* 1996, Roberts M.S. *et al* 1996, Roberts M.S. and Walters K.A. 1998 and Hadgraft J. 1999)
(Properties of penciclovir from Material Safety Data Sheet, SKB)

Penciclovir is a potent and highly selective antiviral compound. It is active against herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), varicella zoster virus (VZV) and Epstein-Barr virus (EBV).

Penciclovir itself is poorly absorbed following oral administration, but is used in a topical formulation for the treatment of herpes labialis (HSV-1 infection or cold sores).

1.6.2 Absorption, Distribution, Metabolism and Elimination.

Famciclovir is the diacetyl 6-deoxy derivative of penciclovir, and is well absorbed following oral administration. Famciclovir is converted to penciclovir via deacetylation in the intestinal wall. This is followed by further deacetylation and oxidation in the liver by the enzyme aldehyde oxidase (Crumpacker C. 1996 and Clarke S. *et al* 1995). Famciclovir is used as an antiherpetic agent for the treatment of acute herpes zoster and recurrent genital herpes infections.

The absolute bioavailability of penciclovir from oral famciclovir is 77% (Pue M. and Benet L. 1993). The volume of distribution of penciclovir has been reported as 112 litres (Fowles S. *et al* 1992), which indicates that the drug is distributed into the tissue compartment. Levels in whole blood are similar to levels in plasma, suggesting that penciclovir can penetrate the erythrocytes, but does not accumulate within them (Rolan P. 1995).

The plasma protein binding of penciclovir has been reported as being less than 20% (Pue M. and Benet L. 1993 and Boyd M. *et al* 1988).

Penciclovir is excreted rapidly and almost exclusively by the kidneys, by active tubular secretion and glomerular filtration. There is negligible metabolism of penciclovir prior to elimination (Filer C. *et al* 1994).

1.6.3 Mechanism of Action.

Penciclovir can freely move in and out of cells in the body, and has been demonstrated to cross the blood-brain barrier (Borg N. and Stahle L.

1997). For penciclovir to become active against a virally-infected cell, it must first be converted to its triphosphate. The first step in its activation is the conversion of penciclovir to its monophosphate (PCV-MP) by thymidine kinase. In uninfected cells only a very small amount of penciclovir is phosphorylated due to the low affinity of the cellular thymidine kinase. However, viral thymidine kinase has a high affinity for penciclovir, so in an infected cell this compound is rapidly phosphorylated. Further phosphorylation of PCV-MP by cellular enzymes produces the diphosphate and then the antivirally-active triphosphate (PCV-TP). The intracellular triphosphate of penciclovir is retained inside the cells. The molecule PCV-TP is structurally similar to deoxyguanosine triphosphate (dGTP), a naturally occurring nucleotide, which is the substrate for the viral DNA polymerase. This enzyme mediates viral DNA elongation.

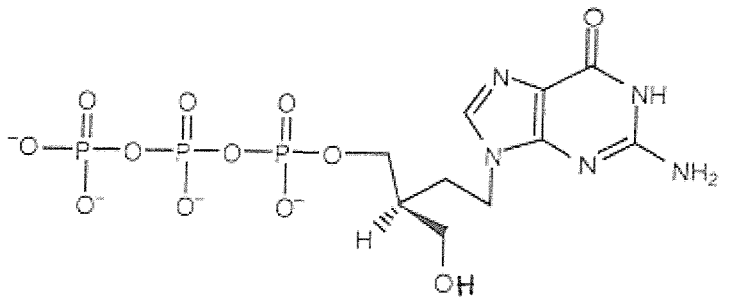


Figure 6 Structure of (*S*)-PCV-TP (Earnshaw D.L. *et al* 1992)

The proposed mechanism of action of PCV-TP is to inhibit the viral DNA polymerase through competition with dGTP binding, and so halts DNA chain elongation.

Interest in penciclovir arose from the limitations of aciclovir, another antiviral agent. Aciclovir has been used for almost two decades for the treatment of various herpes-virus infections. Although it is a much safer drug than some of the older antiviral drugs, such as vidarabine and idoxuridine, which are toxic to mammalian cells (idoxuridine also exhibits the adverse

reactions of stinging, nausea and an aftertaste (Hamuy R. and Berman B. 1998)), aciclovir is not without its limitations. The oral bioavailability of aciclovir is only 10-20% and the intracellular half-life of its triphosphate is only 0.7 hour in HSV-1 and one hour in HSV-2 infected cells, compared to 10 and 20 hours for PCV-TP (Vere Hodge R. and Cheng Y. 1993).

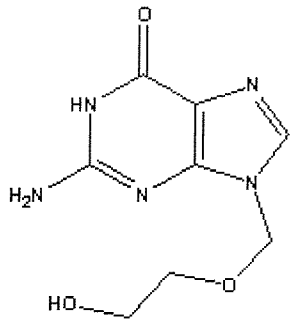


Figure 7 Structure of aciclovir. Molecular weight: 225 Da.

Topical aciclovir has been used in ointment and cream bases for the treatment of herpes labialis with limited success. It was first demonstrated that 5% aciclovir in an aqueous cream, applied 5 times per day, had clinical benefit for the treatment of cold sores by Fiddian A.P. *et al* (1983). The time to lesion healing was reduced from 6 days to 4 days when topical aciclovir was used compared to the vehicle alone. Raborn G. *et al* (1989) also reported decreased healing times (by one day) with the same formulation and study design, and also observed a reduction in the size of the lesions. Although in this study the differences between the drug and placebo groups were not significant, there was a trend of accelerated healing with aciclovir treatment. Both studies indicated that early treatment, in the prodromal and erythematous stages (see Section 1.7) was necessary for the accelerated healing due to aciclovir. Topical aciclovir (1%) in aqueous cream was shown to have some therapeutic benefit in HSV-1 infected guinea pigs. When applied twice a day to the lesions the healing time was reduced from 16 to 11 days (Boyd M. *et al* 1988). Using excised human skin Parry G.E. *et al* (1992)

demonstrated a concentration gradient of aciclovir when applied topically (5%) in a cream base. The highest levels were seen in the stratum corneum, decreasing in the epidermis and dermis. The epidermal concentrations were two-fold greater than the dermal concentrations. The drug concentrations in each layer increased with time.

However, studies by Shaw M. *et al* (1985) and Horwitz E. *et al* (1999) showed that treatment with 5% aciclovir cream did not have any clinical benefit for the treatment of herpes labialis. In the former study, the healing times for the drug and placebo groups were equally reduced in comparison to untreated lesions (10 days as compared to 13 days respectively). This indicated that the cream formulation may have beneficial effect for the lesion treatment.

Aciclovir applied to the lesions in a polyethylene glycol (PEG) ointment, 5 times per day, was found to be ineffective. The first study to show this indicated no difference in healing times, size of lesions and time to cessation of viral shedding between the drug and placebo groups (Spruance S.L. and Crumpacker C.S. 1982). This was initially thought to be due to a delay in treatment of the lesions as this was not a patient-initiated trial. A second, larger study, which was patient-initiated, demonstrated no decrease in healing time and only a slight decrease in lesion size. Early treatment was again thought to improve the effects of the drug formulation (Spruance S.L. *et al* 1984). A study using HSV-1 infected guinea pigs reinforced these findings and also showed no difference in the time to cessation of viral shedding (Spruance S.L. *et al* 1984b). Freeman D. *et al* (1986) showed that aciclovir absorption across excised human skin was very low from the PEG formulation, and was increased by 8 times from the aqueous cream base. It was concluded that topical aciclovir is minimally effective and has little clinical benefit for the treatment of herpes labialis (Hamuy R. and Berman B. 1998).

Penciclovir is structurally similar to aciclovir, but has the distinct advantage of an extended half-life in virally-infected cells. The half-lives *in vitro* of PCV-TP in HSV-1 infected cells is 10 hours, and 20 hours in HSV-2 infected cells. Also, the phosphorylation by the viral thymidine kinase seems

to be more marked for penciclovir than aciclovir in herpes virus-infected cells, indicating a higher affinity of the enzyme for penciclovir (Vere Hodge R. and Cheng Y. 1993).

Although penciclovir is phosphorylated to produce more of its triphosphate than aciclovir, they have similar antiviral activities *in vitro*. It was suggested that PCV-TP may be a less powerful inhibitor of viral DNA polymerase than ACV-TP (Vere Hodge R. and Cheng Y. 1993). The free hydroxyl group of the PCV-TP molecule (see Figure 6) allows further DNA chain extension, but this is inefficient. Thus, it is not an immediate chain terminator, as is ACV-TP. However, the synthesis of viral DNA is effectively blocked by the high concentrations of PCV-TP, which is rapidly formed, and accumulates, in the virally-infected cells (Earnshaw D.L. *et al* 1992).

Topically applied penciclovir (1% in aqueous cream) appears to have had some clinical success. Two large studies, which were patient-initiated, showed that when penciclovir cream was applied to the lesions every 2 hours for 4 days, the healing times were significantly decreased by 0.7 days and the time to loss of pain was reduced by 0.6 days. The clinical benefits were observed when the treatment was commenced in both early (prodromal and erythematous) and late (papular and vesicular) stages of lesion progression (see Section 1.7) (reviewed in Sacks S.L. and Wilson B. 1999 and Spruance S.L. *et al* 1997). Although the differences in healing times and loss of pain between the drug and placebo groups were small, this was accounted for by the inclusion of all patients, including non-compliant individuals, in the data (Spruance S.L. *et al* 1997).

It has been reported by Boyd M. *et al* (1987) that the *in vitro* concentration of penciclovir required to inhibit viral replication by 50% (IC_{50}) is 0.4μg/ml for HSV-1 and 1.5μg/ml for HSV-2 in cell culture. This indicates that for the drug to be pharmacologically active *in vivo*, these concentrations are needed at the site of viral infection. The basal epidermis is generally considered to be the site of HSV infection. It is necessary then, that when penciclovir is applied topically to the surface of the skin, that it is absorbed

and travels through the stratum corneum and the layers of the viable epidermis, to reach the basal layer.

1.7 Herpes Labialis Pathology.

Cold sores are most commonly seen on the skin around the mucosal orifices of the face, the lips and nose. The cold sores progress through eight stages: prodrome, erythema, papule (elevated solid area), vesicle (or blister; fluid-filled raised area), ulcer (discontinuity of the skin exhibiting complete loss of epidermis), crusting, dry flaking and residual swelling (Spruance S.L. 1995 and Robbins S. 1994). The lesions commence with prodromal symptoms of pain, itching and tingling, with subsequent development of grouped vesicles on an erythematous base (Higgins C.R. *et al* 1993). The mean size of the lesions is 75mm^2 (Spruance S.L. 1995).

Drug delivery through intact skin is necessary for the treatment of herpes labialis. In the early stages of virally induced epidermal pathology, i.e. the prodromal, erythematous and papular stages, the overlying stratum corneum is intact, and only becomes disrupted as the disease progresses to the vesicular and ulcerative stages. It is thought that once the epidermis is disrupted, chemotherapy, in the form of topical antiviral agents, becomes irrelevant as the immune system begins to clear the disease (Freeman D. *et al* 1986). Studies on guinea pig skin, infected with HSV showed no differences in aciclovir absorption, in the early stages of infection (prodrome, erythema and papule) compared to absorption through uninfected skin (Freeman D. *et al* 1986).

1.8 Previous Unpublished Data on Transdermal Penciclovir Absorption.

Studies performed in this department on the transdermal absorption of penciclovir using dermal microdialysis, demonstrated that penciclovir was absorbed through the skin in significant but variable amounts.

The studies were performed with a similar protocol to that used in this project. The probes were inserted into the skin of the forearm to result in a total length of microdialysis membrane available for dialysis of 3cm. The perfusion fluid consisted of 0.25% human serum albumin in Ringer's solution and was perfused at a rate of 6.6μl/min through the probes. The dialysate samples were collected hourly and analysed for penciclovir concentrations using high pressure liquid chromatography (HPLC).

A sample of the results of this study are shown below. Figure 8 shows penciclovir recovery via the microdialysis probes following topical application of Vectavir cream to the skin overlying the probes. The results demonstrate the recovery of penciclovir from 4 probes in one subject. The penciclovir recovery was substantial with peak concentrations seen at 3 to 4 hours after Vectavir cream application.

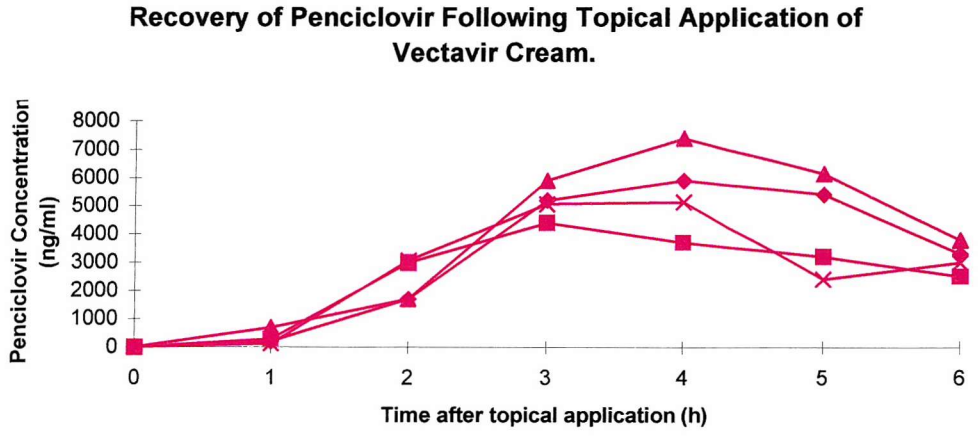


Figure 8 Recovery of penciclovir using dermal microdialysis following topical application of Vectavir cream. 1 subject, 4 probes (Walter E. 1998)

The next study involved the addition of noradrenaline to the perfusion fluid. The rationale behind this was that the noradrenaline would diffuse out of the perfusate and have the effect of restricting the dermal blood supply in the immediate vicinity of the microdialysis probe. It was thought that the penciclovir absorbed through the skin would be taken up by the blood supply and removed from the sampling site into the systemic circulation. By reducing the blood flow in the sampling area, then penciclovir would be expected to accumulate in the tissue due to diminished removal. This would lead to a greater amount of the drug being available for diffusion across the microdialysis membrane and into the dialysate.

Figure 9 shows the recovery of penciclovir when noradrenaline was added to the perfusate at a concentration of 0.001mg/ml. The graph represents data derived from one subject and 4 microdialysis probes.

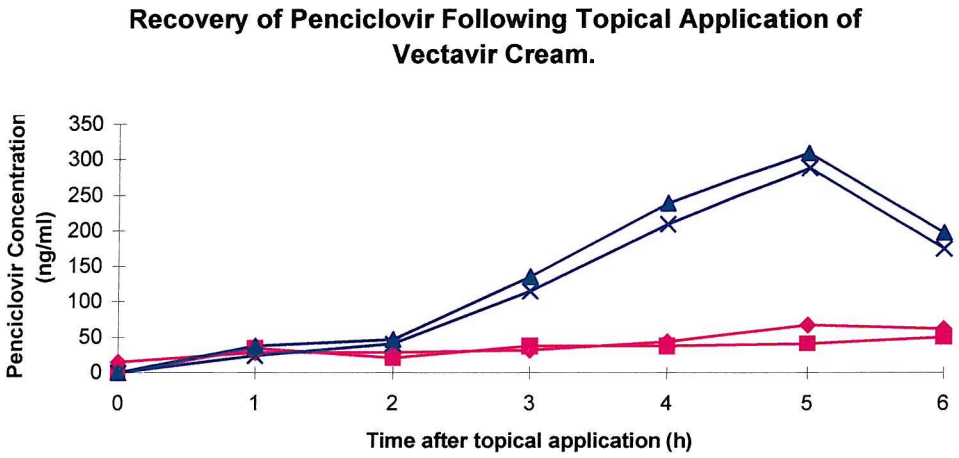


Figure 9 Recovery of penciclovir following topical application, when 0.001mg/ml noradrenaline was added to the perfusate (—■—), and with no noradrenaline present (—■—). One subject, 4 probes (Walter E. 1998)

The graph shows that in the presence of noradrenaline, the recovery of penciclovir was increased, with the peak concentration seen at 5 hours after application of the Vectavir cream.

It is interesting to note here that the penciclovir recovery without noradrenaline (the pink data), was at much lower levels than that seen in Figure 8. The maximum concentration in Figure 8 was 7400ng/ml compared

to just 67ng/ml seen in Figure 9. The two studies were performed using the same protocol but using different subjects.

Figures 8 and 9 illustrate a profile of penciclovir absorption. There was an increase in penciclovir recovery after topical application, becoming maximal at around 3 to 5 hours. This was followed by a decrease in recovery.

Table 1 outlines the concentrations of penciclovir recovered in a number of subjects, both with and without noradrenaline (NA). For each subject the presence or absence of noradrenaline in the perfusate is specified, as are the number of probes for these parameters. The values represent mean total area under the curve (AUC) ± one standard error of the mean. Also shown are the mean peak penciclovir concentrations (C_{max}) ± one standard error of the mean (SEM).

Subject		No. of probes	AUC ± SEM ng.h/ml	C_{max} ± SEM ng/ml
D	+ NA	2	4268 ± 492	2850 ± 200
	- NA	2	649 ± 104	450 ± 100
F	+ NA	1	1719	960
	- NA	2	131 ± 18	38 ± 3
G	+ NA	2	935 ± 30	590 ± 40
	- NA	2	98 ± 18	35 ± 5
H	+ NA	2	878 ± 57	330 ± 10
	- NA	2	228 ± 20	63 ± 8
S	+ NA	3	96 ± 4	61.7 ± 4.7
	- NA	2	15 ± 2	9.2 ± 1.3

Table 1 Summary of total AUC of penciclovir absorption, with and without noradrenaline in the perfusate to reduce dermal blood flow (Walter E. 1998).

These data demonstrate that there were large variations in penciclovir recovery following topical application. The variability was slight between

different probes used in the same subject, but was substantial between subjects.

Chapter 2

Experimental Methods

2. Experimental Methods.

2.1 List of Reagents.

Acetic Acid (glacial) AnalaR	BDH, UK.
Aciclovir	Donated by Smithkline Beecham Pharmaceuticals, UK.
Aquagel	Adams Healthcare, UK
Aqueous cream	The Boots Company PLC, UK.
Carboxymethylcellulose (sodium salt) medium viscosity.	Sigma Chemicals Co. UK
EMLA anaesthetic cream 5%, 25mg lidocaine, 25mg prilocaine per gram.	Astra Pharmaceuticals Ltd. UK.
Ethanol	100%, BDH, UK.
Methanol	Hipersolv, BDH, UK.
Noradrenaline, Levophed, 1mg/ml in saline solution.	Abbot Laboratories Ltd, UK.
Oleic acid: Approx. 95%,	Sigma Chemicals Co. UK
Penciclovir, batch no. WPB 1027.	Donated by Smithkline Beecham Pharmaceuticals, UK.
Propylene glycol	Sigma Chemicals Co. UK
Salicylic Acid	Sigma Chemicals Co. UK.
Sodium Acetate Anhydrous	AnalaR, BDH, UK.
Sodium Penciclovir sterile vials	Donated by Smithkline Beecham Pharmaceuticals, UK.
Sterile Human Serum Albumin Zenalb 4.5% solution	Bioproducts Lab, UK.
Sterile Ringer's solution	Baxter Healthcare Ltd, UK.
Vectavir cream: 1% w/w penciclovir.	Smithkline Beecham Pharmaceuticals, UK.

2.2 Microdialysis Probes.

The probes were produced in the laboratory. The individual fibres which originated from an artificial kidney unit (GFE-18, Gambro) were used. These had an outer diameter of 216μm, with a wall thickness of 8μm and a molecular cut-off of 2 KDa.

In initial studies, the probes were used as found, but to improve handling and probe rigidity, a length of wire was inserted into the lumen of the probe. The wire was stainless steel, with a diameter of 100μm (Goodfellow, Cambridge, UK).

The probe/probe+wire, was glued to a 10cm length of nylon portex tubing (OD: 1.02mm, ID: 0.58mm, Portex Ltd. UK.) using cyanoacrylate glue (Loctite Superglue). Probes were sterilised using ethylene oxide, at the University Hospital of Wales, Cardiff.

2.3 Microdialysis Equipment and Settings.

The perfusion flow rate was 0.94μl/min, 5μl/min or 6.6μl/min (stated in individual results), using syringe driver pumps (IVAC P3000). The probes were connected to the syringe in the pump using syringe extension tubing (Alaris, UK.). The perfusate was sterile Ringer's solution. Samples were collected every hour and frozen at -20^{0}C prior to analysis. In all studies, the length of membrane accessible to microdialysis was 3cm, unless otherwise stated.

2.4 *In Vitro* Studies.

In vitro studies were performed to evaluate the recovery of penciclovir. The studies were also used to determine the effects of various factors which may affect recovery, such as concentration of penciclovir in the *in vitro* bath solution, presence of protein in the perfusate and in the bath solution, the effects of sterilisation of the microdialysis probes, stirring of the bath solution, probe length, perfusion flow rate and the effects of temperature.

The *in vitro* experiments were performed in the laboratory at room temperature, unless when stated otherwise. The probes were placed in petri dishes with a 3cm length of probe exposed for microdialysis. The bath solution consisted of penciclovir dissolved in Ringer's solution and the perfusate was Ringer's solution only, unless otherwise stated. The dialysate was collected hourly for four hours, and 0.3ml of the bath solution was collected at each hourly sampling time point.

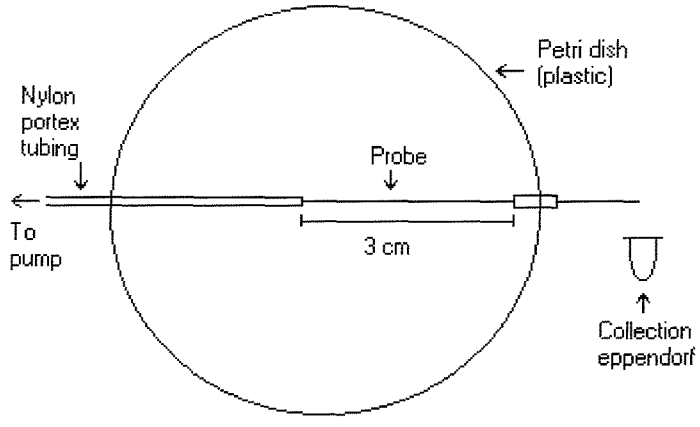


Figure 10 Schematic representation of the *in vitro* microdialysis system.

2.5 *In Vivo* Studies.

2.5.1 Volunteers.

All volunteers were healthy adults, male and female, age 18-65 years. The volunteers all gave written informed consent prior to participation and each study was approved by the Southampton and South West Hampshire Joint Research Ethics Committee. Volunteers were recruited by poster advertisements, email advertising and by personal communication.

On the day of the study, the volunteers applied EMLA cream under occlusive dressing (Tegaderm), to six areas of the forearm, at least 60 minutes before needle insertion. The insertion of the needles was well tolerated and painless. The areas of EMLA application were approximately 4cm long, and 3cm apart, in a thick strip.

2.5.2 Probe Insertion.

The probes were introduced into the skin of the volar forearm via a guide cannula (needle) (see Figure 11). The needle (23G, 30mm) was inserted under the skin, parallel to and as close to the surface as possible without puncturing the surface of the skin, for the entire length of the needle. The microdialysis probe was fed through the cannula in the opposite direction to needle insertion. The portex tubing of the probe was taped to the arm, and then attached to the syringe via the extension tubing. Perfusate was pumped through the probe, at a rate of 660μl/min to purge the extension tubing and probe with fluid, and free flow was ensured before the needle was removed. The flow was then reduced to the set flow rate for the study and the needle was gently withdrawn, leaving the probe in the intact skin.

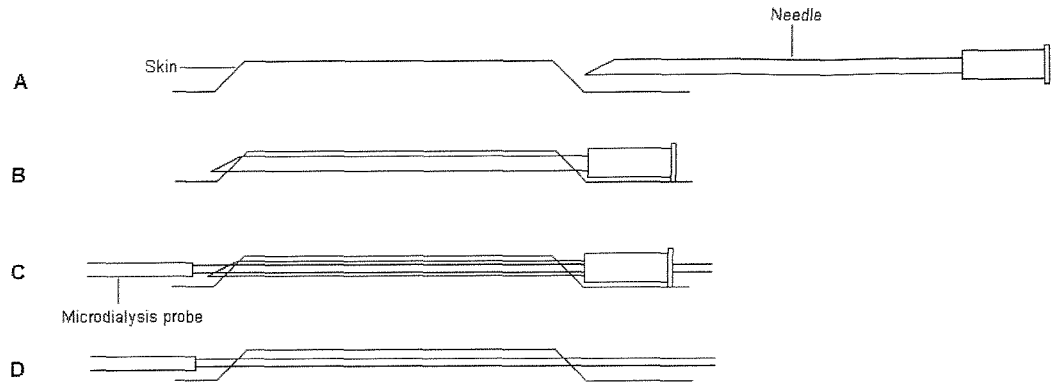


Figure 11 Schematic representation of probe insertion into the skin. The needle is positioned parallel to the surface of the skin (A), and then inserted as close to the skin surface as possible (B). The microdialysis probe is fed through the needle in the opposite direction to needle insertion (C). The needle is withdrawn leaving the probe in the skin (D).

The arm was then allowed to recover from the insertion trauma for 90 minutes before continuation of the study. During this recovery time the probes were perfused with Ringer's solution at a rate of either 5μl/min or 6.6μl/min, stated in the individual studies. The dialysate was collected during this time and was used as a pre-dose zero sample.

For percutaneous absorption studies, a drug well was applied to the skin directly over the site of probe insertion. The drug wells were composed of Comfeel self-adhesive ulcer dressing (Coloplast Ltd. UK.). This was cut into wells with the inner dimensions of 1.8 x 0.5 x 0.1cm, in which the drug formulation was placed. The outer dimensions were 2.8 x 2.5 cm. The topical formulation, when applied, was placed in, and filled to the surface of the well, ensuring good contact with the skin. The well was then covered with another solid piece of Comfeel dressing with the same outer dimensions, if occlusion was required.

For penciclovir delivery studies, the probes were perfused with Ringer's solution for the recovery time of 90 minutes. The syringe and extension tubing were then replaced with those containing the penciclovir solution for the duration of the study. The probe was purged with the penciclovir solution

at a rate of 660μl/min. to ensure complete replacement of the Ringer's solution with the penciclovir solution.

2.6 Penciclovir Solution for Delivery Studies.

A stock solution was made using a vial containing sterile sodium penciclovir. Each vial contained 215mg of penciclovir, all of which was dissolved in a 1 litre bag of sterile Ringer's solution; a 10ml aliquot was taken from the bag of Ringer's solution, and injected into the vial and mixed. The entire contents were transferred back into the bag of Ringer's solution and mixed well. This was repeated twice more, to ensure maximal removal of the penciclovir from the vial. This stock solution had a concentration of 0.215mg/ml.

For each working solution, a new 1 litre bag of Ringer's solution was used. 10ml was taken from the bag and discarded using a sterile syringe and needle. 10ml of the stock solution was then transferred into the new bag of Ringer's using a sterile 10ml syringe and needle, and was mixed thoroughly. This working solution had a concentration of 2.15μg/ml.

2.7 Noradrenaline Solutions.

In studies where noradrenaline was used to restrict the dermal blood supply, the noradrenaline was added to the perfusate at a concentration of 0.005mg/ml.

The noradrenaline in sterile solution at a concentration of 1mg/ml was diluted 1 in 10 with Ringer's solution and mixed well (0.1mg/ml). This solution was then further diluted 1 in 20 with Ringer's solution to give the final working solution of 0.005mg/ml. When perfused through the probes *in vivo*, this concentration resulted in a visible blanching of the skin of a radius of 5-6mm.

2.8 Ultrasound Imaging.

Probe depth and distance between the probes (in the dual probe study) were determined using a Dermascan C (20MHz ultrasound 2D and 3D scanner, (Cortex Technology, Denmark)). Each probe was scanned in three positions, at the centre and approximately 1cm either side of the centre. The mean of these three measurements was used for the reported values.

2.9 Analysis of Dialysates for Penciclovir.

The collection Eppendorfs were weighed prior to and after collection of the dialysates to accurately determine the volume of dialysate collected. The internal standard, aciclovir (with a concentration of 200ng in 10μl of double distilled water), was added to each sample, which were then mixed. The samples were transferred into appropriately labelled 10ml glass tubes, and were evaporated to dryness at 65^0C, under nitrogen. The residues were reconstituted in 100μl of mobile phase, mixed and transferred to labelled HPLC (High Pressure Liquid Chromatography) vials. Penciclovir concentrations were determined using a Waters HPLC system, (515 pump, 717 Plus autosampler and 2487 dual wavelength absorbance detector). Separation was achieved using a Prodigy 5 C8 column (250 x 4.6mm, 5 micron, Phenomenex, UK.) maintained at 28^0C in a waterbath. The mobile phase was 2% methanol, 98% 0.05M sodium acetate at pH 4.3 (adjusted using acetic acid glacial), at a flow rate of 1ml/min, with a run time of 20 minutes. Detection was at 254nm and peaks were quantified by height. Data were collected and integrated using the Waters Millennium[32] software. Retention times for aciclovir and penciclovir were 9.7 and 14.9 minutes, respectively. The limit of quantification was 0.3ng/ml, (limit of detection 0.1ng per sample).

For samples where albumin was used, the aciclovir internal standard was added as above. The protein was precipitated by the addition of 1ml of 100% ethanol, the samples were shaken for 10 minutes, followed by centrifugation for 10 minutes at 3000 rpm. The supernatants were transferred into 10ml glass tubes and then evaporated to dryness as above. The samples were then treated as above.

2.10 Analysis of Dialysates for Penciclovir and Salicylic Acid.

The perfusion flow rate for this study was 0.94μl/min, and so the volume of sample collected was 0.05ml on average. The samples were transferred directly into appropriately labelled HPLC vials. The penciclovir and salicylic acid concentrations were determined using the same HPLC system, column and water-bath temperature as described previously. The mobile phase was 5% methanol, 95% 0.05M sodium acetate at pH 6.5 (adjusted using acetic acid glacial), run at 1ml/min, with a run time of 25 minutes. Detection was at 254nm, and the peak heights were used for quantification. The retention times were 7.6 minutes for aciclovir, 11 minutes for penciclovir and 19 minutes for salicylic acid.

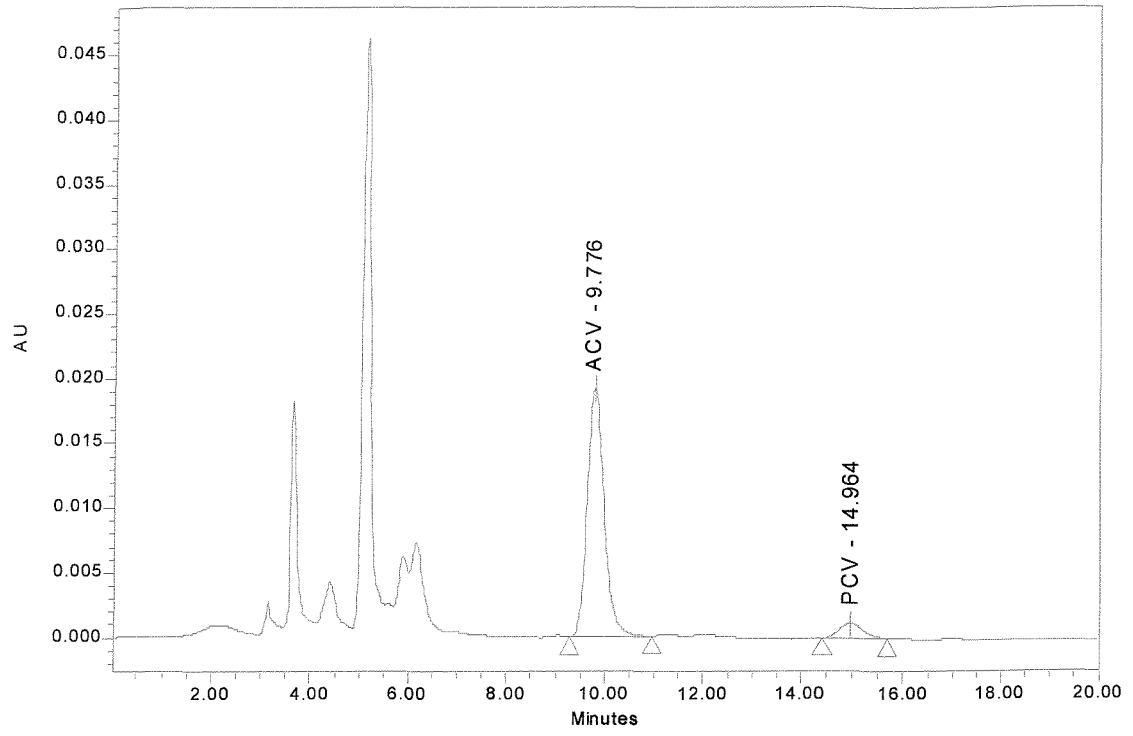


Figure 12 An example of a typical chromatogram of an *in vivo* dialysate sample showing the peaks for aciclovir (ACV) and penciclovir (PCV). The concentration of penciclovir represented here is 78ng/ml.

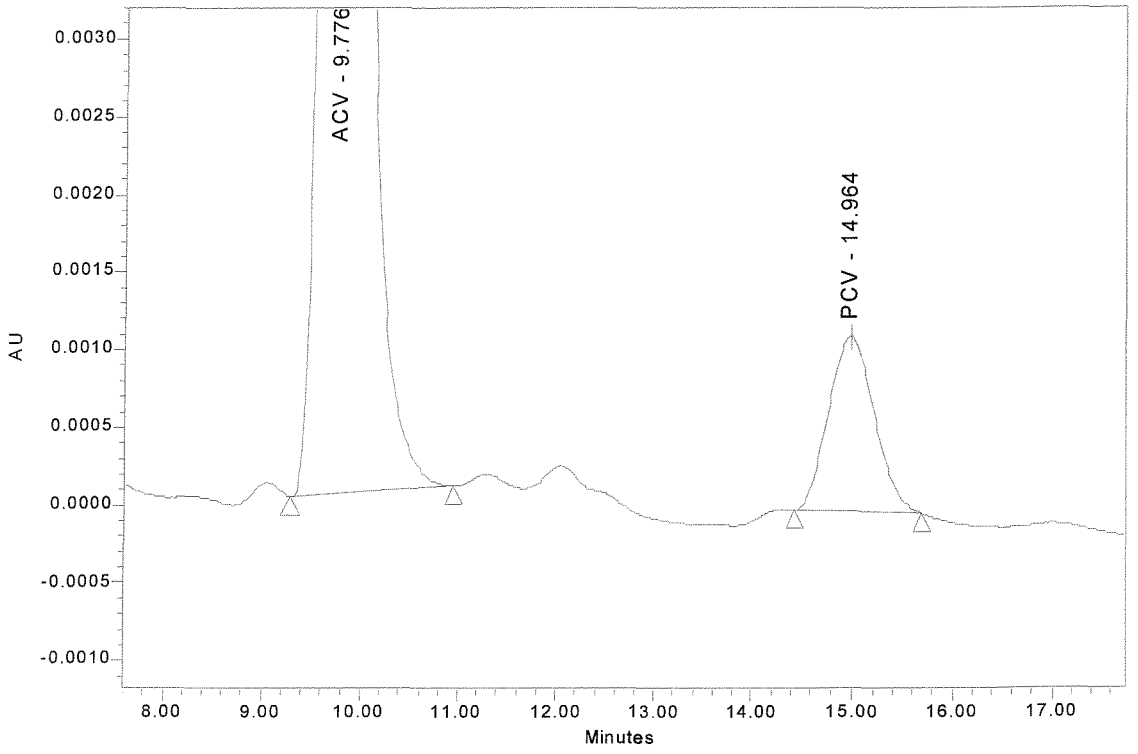


Figure 13 An enlargement of the previous chromatogram showing the integration of the peaks.

2.11 Determination of Penciclovir Concentrations.

A set of standard concentrations of penciclovir were analysed in duplicate with each set of samples. The standards were prepared by the addition of aciclovir internal standard (200ng in 10μl of water) and known amounts of penciclovir (0, 0.5, 2, 10, 100 and 2000ng in 10μl of water) to 300μl of Ringer's solution (or Ringer's/ albumin solution). The standards were treated in the same way as the samples prior to analysis. The ratios between the penciclovir peak heights and the aciclovir internal standard peak heights were plotted against the spiked concentrations of penciclovir. The slope of the line was used to determine the concentration of penciclovir in each of the samples.

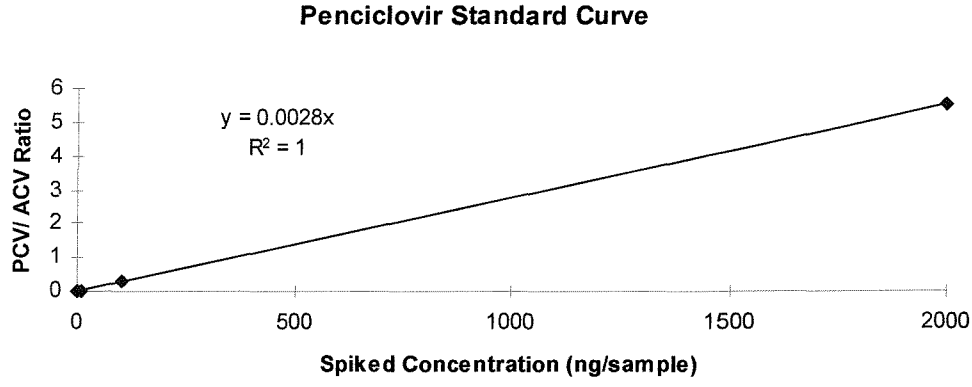


Figure 14 An example of a standard curve for penciclovir used for the determination of penciclovir concentration in the samples.

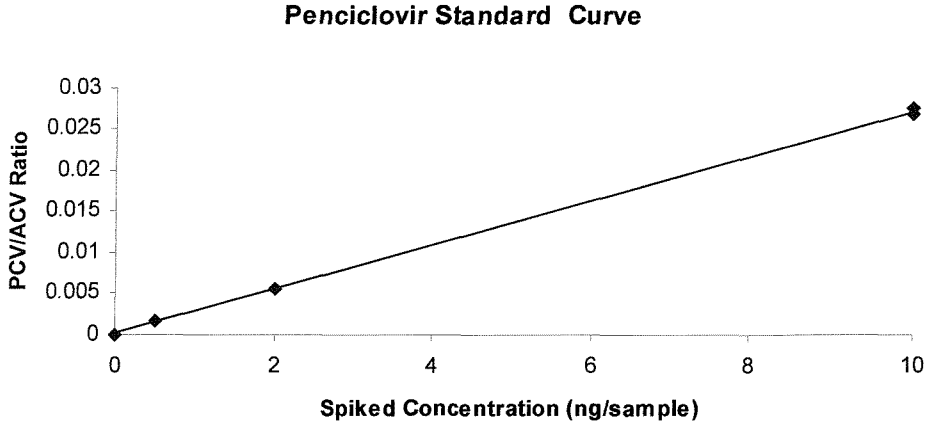


Figure 14a The standard curve showing the low standard concentration values.

2.12 *In Vitro* Absorption of Penciclovir Across Excised Rat Skin.

Full thickness rat skin was obtained from adult male Wistar rats, from the abdominal and dorsal regions of the animals. The rats were sacrificed by cervical dislocation, and the skin removed. The hair was clipped with electric clippers. The subcutaneous fat and muscle tissues were removed and the skin was frozen at -20^{0}C. It has been reported that there are no significant differences in the permeability between fresh and frozen skin for aciclovir (Cooper E.R. *et. al.* 1985) or water (Harrison S. *et al* 1984). Prior to the skin being mounted in the diffusion chambers, the skin was thawed at room temperature.

The diffusion chambers used were Ussing chambers. The skin was mounted between the two plastic chambers, with the dermal skin side facing the receptor chamber (see Figure 15). The diameter of the circle of skin available for diffusion was 13.5mm. The temperature of the receptor chamber was controlled by circulating water at 37^{0}C through the external water jacket. The receptor chamber was filled with 5ml of Ringer's solution (receptor fluid), which was oxygenated with 95% oxygen/ 5% carbon dioxide, this resulted in the circulation of the receptor fluid throughout the chamber. The receptor chambers were sealed from the atmosphere with parafilm to prevent evaporation of the Ringer's solution.

The drug formulations used were as described in each individual study. In each case, the formulation was applied into and filled the epidermal skin side chamber (donor chamber), approximately 1ml, and the formulation remained in contact with the skin throughout the study. The total volume of the receptor fluid was collected at 0, 6, 24 and 48 hours. The receptor fluid was collected into 6ml glass tubes and frozen at -20^{0}C prior to analysis.

The thickness of the skin was determined using calipers with a vernier scale.

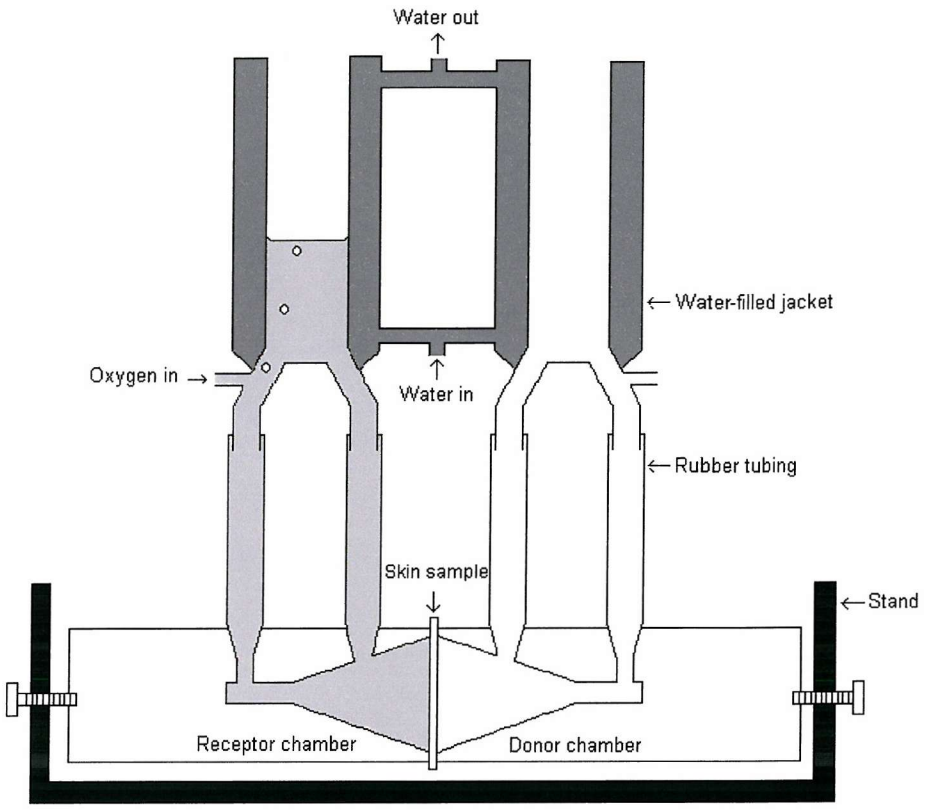


Figure 15 The Ussing Chamber.

2.13 Analysis of the Receptor Fluid for Penciclovir.

Of the total volume of receptor fluid collected, 0.25ml of each sample was used for analysis. This was transferred to 10ml glass tubes. The samples were then treated as described in section 2.9.

2.14 Statistical Analysis.

In vitro results were compared using an unpaired t-test assuming equal variances (homoscedastic). *In vivo* results were analysed using an unpaired t-test assuming unequal variances (heteroscedastic) comparing the area under the curve (AUC) of each probe or chamber. Significance was taken as being less than 0.05.

Chapter 3

Results of *In Vitro* Microdialysis Studies

3. Results of *In Vitro* Microdialysis Studies.

3.1 Aim.

A number of experiments were undertaken to determine the recovery and optimal conditions for the microdialysis of penciclovir.

3.2 Effects of Sterilisation using Ethylene Oxide.

3.2.1 Introduction.

Most of the *in vitro* experiments were conducted using non-sterile microdialysis probes, whereas all the *in vivo* studies used sterile probes. It was important to determine whether the sterilisation procedure altered the physicochemical properties of the membrane.

3.2.2 Method.

The microdialysis probes were mounted in petri dishes, as described in Experimental Methods (section 2.4). The length of fibre available for drug diffusion was 3cm, and this was perfused with Ringer's solution at a rate of 6.6μl/min. The perfusate was collected for one hour before the addition of the bath solution into the petri dish, and was used as a pre-dose zero sample. The bath solution consisted of penciclovir dissolved in Ringer's solution at a concentration of 10000ng/ml. The number of probes used was ten for each parameter.

The percentage recovery of penciclovir was calculated using the equation:

$$\text{recovery} = \frac{[\text{dialysate}]}{[\text{bath}]} \times 100 \qquad \textbf{Equation 2}$$

The recovery was calculated using the measured bath concentrations. The graph was plotted using the average recovery of each time point of the probes.

3.2.3 Results and Discussion.

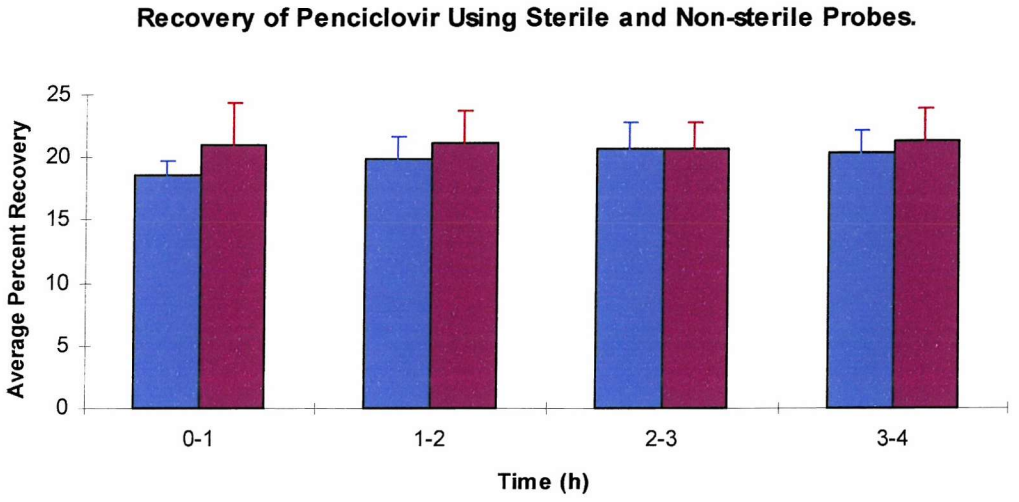


Figure 16 Average percent recovery of penciclovir, + one standard deviation, between sterilised (■) and non-sterile (■) microdialysis probes (n = 20).

Figure 16 shows that there was a slight reduction in penciclovir recovery with sterilised microdialysis probes. However, the decrease in recovery was not significantly different from the recovery seen with non-sterile probes ($p = 0.187$). This is in agreement with Hegeman L. *et al* (1995), that ethylene oxide sterilisation does not alter probe properties.

3.3 Effect of Albumin in the Perfusate Solution.

3.3.1 Introduction.

Protein-binding of compounds can play an important role in microdialysis recovery. The recovery of a highly protein-bound compound can be enhanced by the addition of albumin to the perfusion fluid (Groth L. and Jorgensen A. 1997 and Carnheim C. and Stahle L. 1991). Although penciclovir is less that 20% protein bound (Boyd M. *et al* 1988 and Pugh M. and Benet L. 1993), albumin was added to the perfusate to determine if this had any effect on the recovery, as this perfusion fluid had been used in previous microdialysis studies (Walter, E. 1998).

3.3.2 Method.

The *in vitro* microdialysis experimental design was as already described, but in this study, the perfusion fluid for half of the probes consisted of 50% Ringers solution and 50% human serum albumin (final albumin concentration of 2.25%), and recovery was compared to probes perfused with Ringers solution alone. The perfusion flow rate was 6.6μl/min. The bath solution was 10000ng/ml penciclovir dissolved in Ringer's solution. The number of probes used for each parameter was nine. The recovery was calculated with equation 2, and using the measured bath penciclovir concentrations.

3.3.3 Results and Discussion.

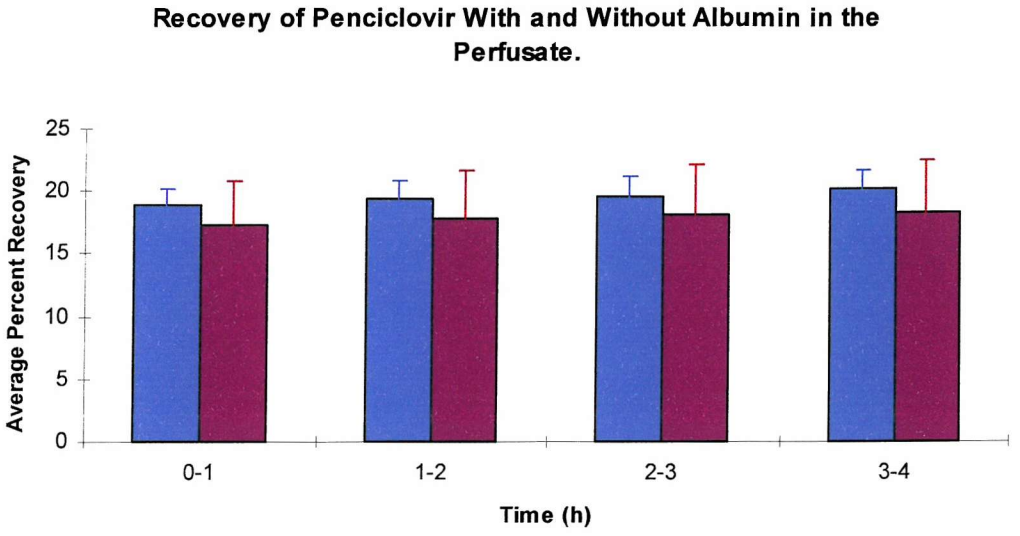


Figure 17 Average recovery of penciclovir, + one standard deviation, comparing perfusate with albumin (■) and without albumin (■) (n = 18).

The results show that penciclovir recovery *in vitro* was slightly higher when albumin was added to the perfusate, but this was not significantly different from recovery in probes where albumin was not added tot he perfusate (p= 0.287). The advantage of not using albumin in the perfusion fluid is that the samples are 'cleaner', and do not require the precipitation step to remove the albumin prior to sample analysis.

3.4 Effect of Albumin in the Bath Solution.

3.4.1 Introduction.

The results from the experiment of albumin in the perfusate (see Section 3.3) demonstrated that the recovery of penciclovir was slightly enhanced over that of the perfusate without albumin. The presence of albumin in the bath solution may have the effect that it could bind the penciclovir and retains it in the bath solution. This could result in a decrease in recovery of penciclovir into the perfusion fluid. Alternatively, it is possible that the protein could interact with the probe membrane, if the albumin binds to the pores of the probe it could reduce penciclovir recovery.

3.4.2 Method.

The probes were mounted in petri dishes as previously described. The probes were perfused with Ringer's solution at a rate of 6.6μl/min. The bath solutions were composed of 10000ng/ml penciclovir dissolved in either 50% Ringers and 50% albumin solution (final albumin concentration of 2.25%) or Ringer's solution alone. The number of probes used was twelve for each parameter. The recovery was calculated using equation 2 and using the measured bath concentration of penciclovir.

3.4.3 Results and Discussion.

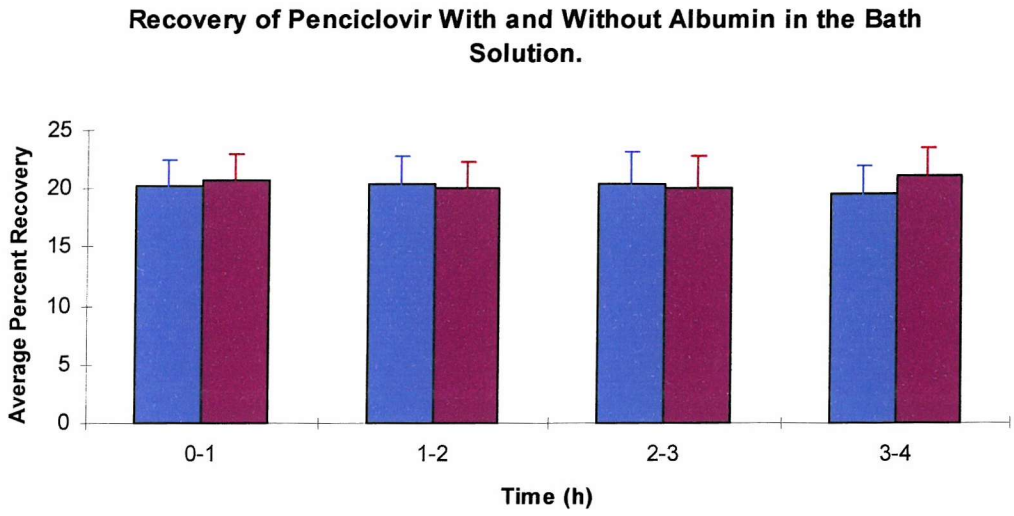


Figure 18 Average penciclovir recovery, + one standard deviation, when the bath solution consisted of Ringer's solution (■) compared to Ringer's and albumin solution (■) (n = 24).

Figure 18 demonstrates that there was no significant difference in the recovery of penciclovir when there was albumin present in the bath solution (p= 0.692). This indicates that the effect of protein-binding of penciclovir was negligible, which suggests that binding of endogenous proteins to the probes *in vivo* would not have an effect on penciclovir recovery. The data also suggests that if there were any interactions between the protein and the probe membrane, then these did not significantly affect the ability of the drug to diffuse into the perfusate.

3.5 Delivery of Penciclovir.

3.5.1 Introduction.

Microdialysis is a useful tool for the measurement of extracellular concentrations of many compounds. However, it can also be used for the delivery of many substances into the tissue being investigated. It is possible that during *in vivo* delivery experiments, the area immediately around the probe may become saturated with penciclovir as it leaves the probe. To determine if diffusion away from the probe affects delivery *in vitro*, the bath solution was stirred continuously throughout the experiment using a magnetic stirring bar.

3.5.2 Method.

The probes were mounted in petri dishes. Half of the petri dishes were placed on a magnetic stirring stand, and small stirring bars were placed in the solution. The bath solution was stirred continuously throughout the study. The perfusate consisted of a solution of penciclovir with a concentration of 2000ng/ml in Ringer's solution, and was perfused at a rate of 6.6μl/min. The bath solution was Ringer's solution. The percentage delivery of penciclovir was calculated using the measured initial perfusate concentrations and with the equation:

$$\text{delivery} = \frac{[\text{perfusate}] - [\text{dialysate}]}{[\text{perfusate}]} \times 100 \qquad \textbf{Equation 3}$$

The number of probes used was seven for each parameter. The measured perfusate concentration was used to calculate penciclovir delivery.

3.5.3 Results and Discussion.

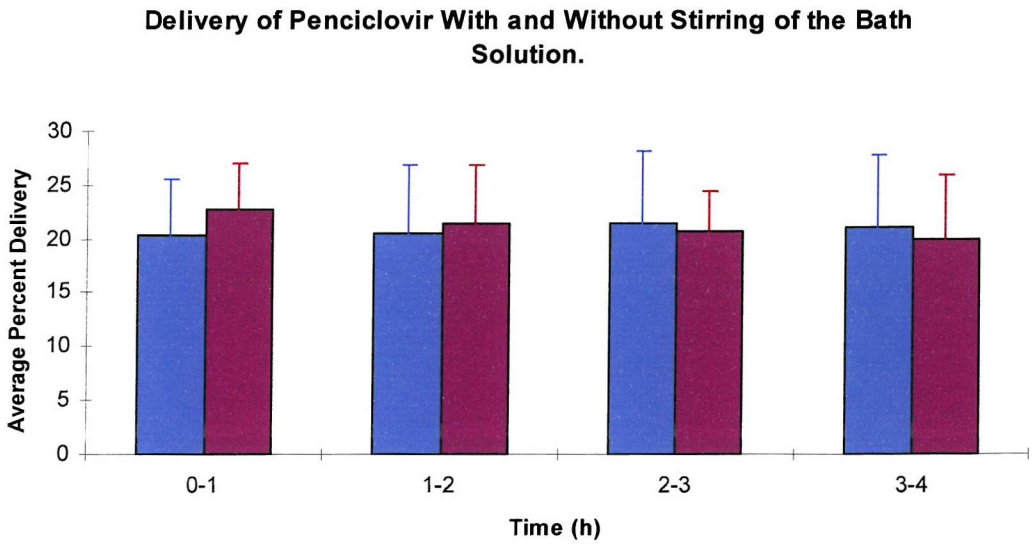


Figure 19 Average delivery of penciclovir, + one standard deviation, comparing situations of the bath solution being stirred (■) and not stirred (■) (n =14).

This study demonstrated that stirring of the bath solution caused no significant alteration in *in vitro* penciclovir delivery (p= 0.90). Although this shows that there was no accumulation of penciclovir around the microdialysis probe *in vitro*, this may not also be the case *in vivo*. This is due to the different media through which the drug has to diffuse. In the *in vitro* situation, it was a simple aqueous medium, whereas *in vivo* there is the more complex medium of the skin.

3.6 Effects of Presence of Wire in the Lumen of the Probe.

3.6.1 Introduction.

This experiment was performed to determine whether the addition of wire to the microdialysis probe resulted in any change in penciclovir recovery. The wire was inserted into the lumen of the probes to enhance rigidity, which assisted in the insertion of the probes into the skin, and to maintain patency of the probes during *in vivo* studies. Often the probes without wire would bend and become blocked, preventing the flow of perfusate. It could be expected that the recovery would be altered, as the lumen of the probe was reduced in the presence of the wire (diameter 100μm). The wire may also affect the speed of perfusate flow, as the pumps were set at the same rate, but had a smaller volume through which to flow.

3.6.2 Method.

The probes were mounted in petri dishes as described previously. Half of the probes contained wire and half did not, nine probes of each. The probes were perfused with Ringer's solution at a rate of 5μl/min. The bath solution was 2000ng/ml of penciclovir in Ringer's solution. The recovery was determined using the measured bath concentrations and equation 2.

3.6.3 Results and Discussion.

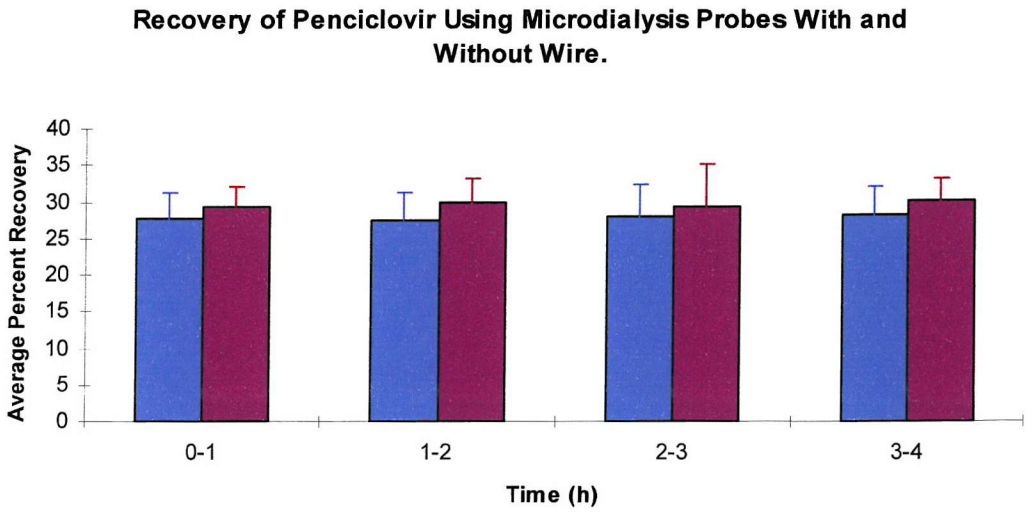


Figure 20 Average penciclovir recovery, + one standard deviation, in probes that contain wire (■) as compared to probes without wire (■) (n = 18).

Figure 20 shows that the presence of wire in the microdialysis probe resulted in a slight decrease in penciclovir recovery but that this reduction in recovery was not significant (p= 0.28). It was also seen that the volume of dialysate collected was not significantly decreased in the probes containing wire (average volume with wire was 0.29ml ± 0.03, and without wire was 0.29ml ± 0.02, p= 0.62). The results indicate that the speed of the perfusate may be increased, as the same volume is traveling along a much reduced lumen. The inner cross-sectional area of the probe was 31416μm^2 and the cross-sectional area of the wire was 7854μm^2, resulting in the reduction of the inner-probe volume by a quarter. However, this did not significantly affect the recovery of penciclovir *in vitro* and so would not be predicted to alter the *in vivo* recovery.

3.7 Effect of Concentration of Penciclovir on Recovery.

3.7.1 Introduction.

It has been reported that the recovery of a drug using microdialysis is independent of the concentration of the drug external to the perfusate (Lonnroth P. *et al* 1987), and that the recovery is merely a percentage of the external concentration.

3.7.2 Method.

The microdialysis probes were mounted in baths containing varying concentrations of penciclovir in Ringer's solution. The probes were perfused with Ringer's solution at a rate of 5μl/min. The probes contained wire in the lumen. The number of probes used for each bath concentration was six. The recovery of penciclovir was calculated from measured bath concentrations, using equation 2.

3.7.3 Results and Discussion.

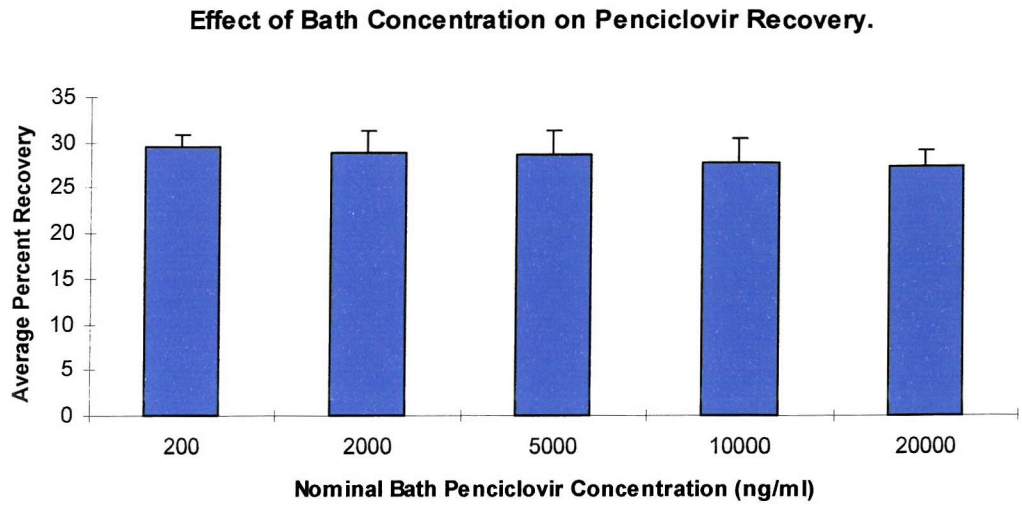


Figure 21 Average percentage of penciclovir recovery, + one standard deviation, when the microdialysis probes were placed in baths containing varying concentrations of penciclovir.

Figure 21 shows that the recovery of penciclovir decreased slightly as the external bath concentration increased. The average recovery of penciclovir when the bath concentration was 200ng/ml was 29.5 ± 1.4%, but this was significantly reduced to 27.8 ± 2.6% when the bath concentration was 10000ng/ml ($p < 0.01$), and was further reduced to 27.3 ± 1.8% when the bath concentration was 20000ng/ml ($p < 0.001$). There were no significant differences between any of the other recoveries.

The decrease in recovery with increased external penciclovir concentrations may be due to interaction of the drug molecules with the microdialysis membrane. At higher concentrations, there may be an increased likelihood of such interactions, which may then block the pores in the probe membrane, and reduce diffusion of the compound into the perfusate.

3.8 Comparison Between Recovery and Delivery of Penciclovir.

3.8.1 Introduction.

It has been speculated that, for many compounds the recovery and delivery via the microdialysis probe are equal (Zhao Y. *et al* 1995). This would be expected with moderate concentrations of the compound, as the diffusional characteristics across the microdialysis membrane would be similar for movement in both directions. The driving force for diffusion is a concentration gradient, and unless limited by drug-membrane interactions, the recovery of penciclovir into the perfusate would be expected to be comparable to delivery from the perfusate into the tissue.

3.8.2 Methods.

The data were derived from the studies of probe sterility (recovery) and the effects of stirring the bath solution (delivery). The data used was from the non-sterilised probes, and from the unstirred bath solution. The recovery was calculated using equation 2, and the percentage delivery was determined using equation 3.

3.8.3 Results and Discussion.

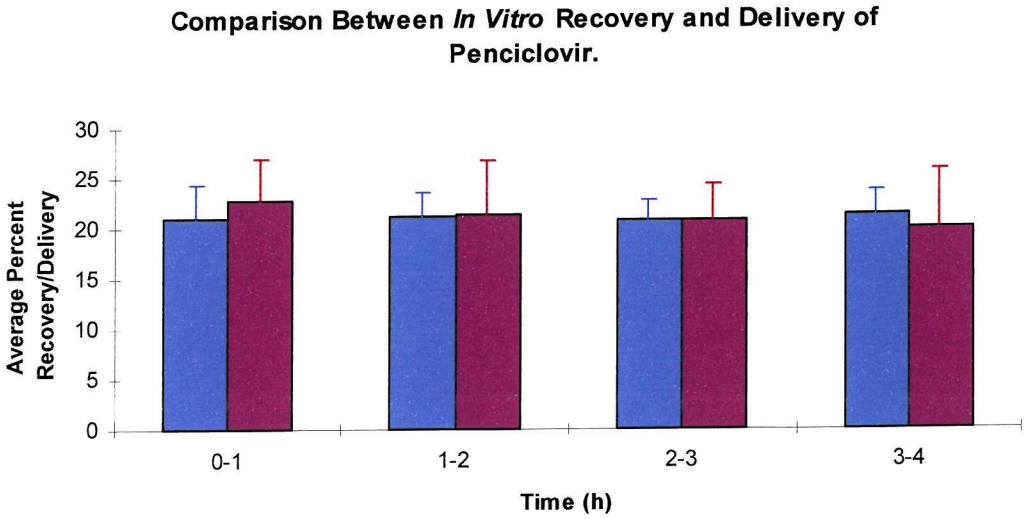


Figure 22 Comparison between the recovery (■) and delivery (■) of penciclovir, + one standard deviation, using *in vitro* microdialysis (n = 17).

These data demonstrate that the percentage recovery and delivery of penciclovir *in vitro* were not statistically different (p = 0.95).

3.9 The Syringe Adaptor.

3.9.1 Introduction.

The IVAC P3000 pumps used in this project are designed for the administration of drugs to human patients. The lowest flow rate that can be achieved with these pumps is 1.6μl/min. It has been well documented that percentage recovery/delivery of a compound in microdialysis is greater with lower flow rates. In order to attain lower flow rates, syringe modification was necessary. The pumps are designed to hold a 10ml syringe, and therefore a sleeve was constructed with the dimensions of a 10ml syringe, but which houses a 1ml syringe. In this situation, the pump drives the 1ml syringe at a rate designed to deliver at a certain rate assuming a 10ml syringe is being used. This has the effect of pumping the perfusate at a much reduced rate.

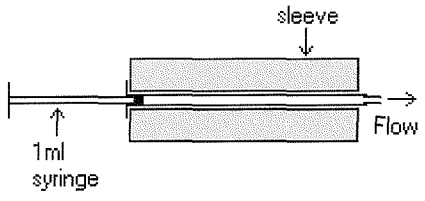


Figure 23 Cross-sectional diagram of the syringe adaptor.

3.9.2 Method.

The pumps were set at different nominal flow rates and Ringer's solution was pumped through microdialysis probes, which were mounted in baths containing Ringer's solution. The dialysate was collected over 4 hours and weighed to determine the actual rate being achieved. The number of probes used for each flow rate was six.

3.9.3 Results and Discussion.

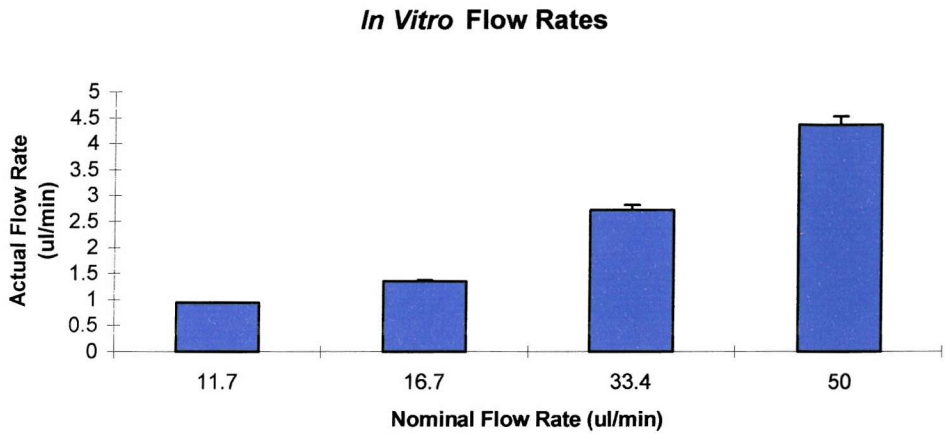


Figure 24 The actual flow rate achieved (+ one standard deviation) when using the syringe adaptors, compared to the nominal flow rate selected on the pump (n = 24).

The results of this study demonstrated the syringe adaptors were extremely effective at reducing the flow rate of the perfusion fluid through the microdialysis probes. It is desirable to use a very slow flow rate to maximise the recovery of penciclovir, especially during *in vivo* studies. However, the extent to which the flow rate can be reduced is limited by the fact that a suitable volume of dialysate is required for analysis of penciclovir concentrations. The flow rate set at 11.7μl/min on the syringe driver gave an actual flow rate of 0.94μl/min. This provides a very slow flow rate, but still yields sufficient volume of sample for analysis; approximately 50μl.

3.10 Effect of Flow Rate on Penciclovir Delivery.

3.10.1 Introduction.

An *in vivo* study was planned in which penciclovir would be delivered directly into the skin using dermal microdialysis. The diffusional characteristics of the drug and the effects of dermal blood flow could then be assessed using recovery via a second probe situated close to the delivery probe.

The aim of this study was to identify a rate of perfusate flow, using the syringe adaptors, that would result in the greatest percentage delivery of penciclovir across the microdialysis membrane but still yield a sufficient volume for sample analysis. This could then be utilised *in vivo* to maximise penciclovir delivery into the tissue.

3.10.2 Method.

The microdialysis probes were mounted in baths containing Ringer's solution, and were perfused with a solution of penciclovir in Ringer's solution (2000ng/ml). The pumps were set a varying nominal flow rates, and the syringe adaptors were used to house 1ml syringes for the delivery of the perfusate. The perfusate was collected every 20 minutes for 4 hours. The number of probes used for each flow rate was six. The percentage delivery of penciclovir was calculated from measured initial perfusate concentrations, using equation 3.

3.10.3 Results and Discussion.

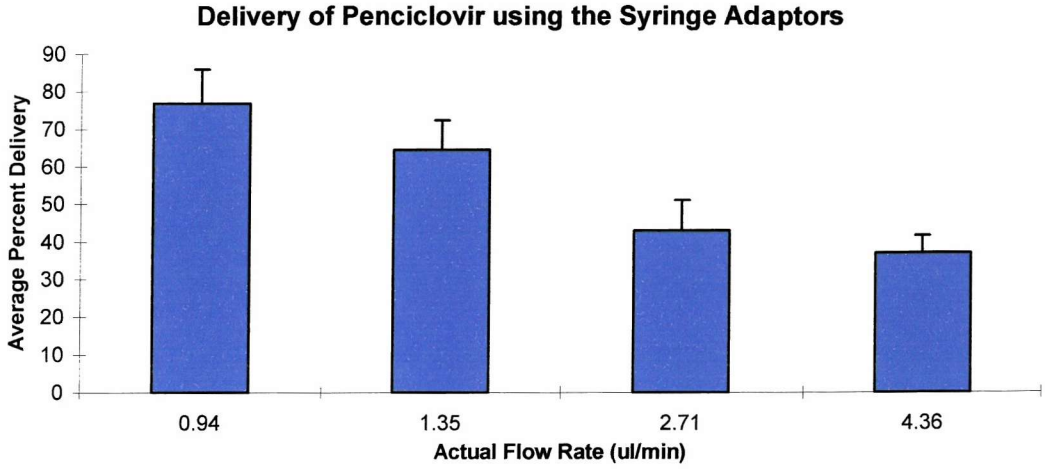


Figure 25 Average delivery of penciclovir (+ one standard deviation) with varying perfusion flow rates.

Figure 25 shows that as the perfusion flow rate increased, the delivery of penciclovir decreased. The slower flow rates allowed more time for the diffusion of the compound out of the microdialysis probe. The lowest flow of 0.94μl/min gave an average delivery of 76.8 ± 9.1%, whereas the highest flow rate of 4.36μl/min gave an average delivery of 36.9 ± 4.6% delivery. It had already been determined that the flow rate of 0.94μl/min. gave sufficient volume of sample of HPLC analysis (see Section 3.9).

3.11 Effect of Flow Rate on Penciclovir Recovery.

3.11.1 Introduction.

The *in vitro* studies began using perfusion flow rates of 6.6μl/min. but it was decided to reduce this to 5μl/min. in later studies to improve penciclovir recovery. These experiments were conducted without the syringe adaptors. The comparison between the recovery for these two flow rates is demonstrated here.

3.11.2 Method.

These data were derived from the *in vitro* studies of probe sterility and presence of wire in the probes. The 6.6μl/min. recoveries are from non-sterile probe data, and the 5μl/min. from probes without wire. The number of probes was nine of each parameter. The recoveries were calculated using equation 2.

3.11.3 Results and Discussion.

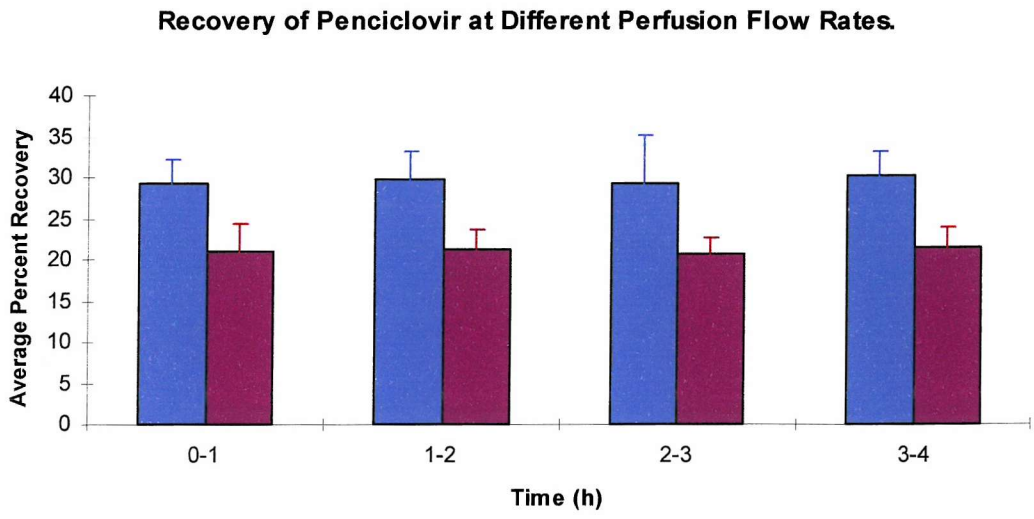


Figure 26 The average penciclovir recovery, + one standard deviation, when the perfusion flow rate was 6.6μl/min. (■) and at 5μl/min. (■). (n = 18).

The recovery of penciclovir *in vitro* was significantly increased from 21.5 ± 2.1% to 29.7 ± 3.3% using perfusion flow rates of 6.6μl/min. and 5μl/min., respectively ($p < 0.001$).

3.12 Effect of Probe Length on Penciclovir Recovery.

3.12.1 Introduction.

It has been speculated that the recovery of a substance using microdialysis increases if the length of probe available for diffusion is increased. An increase in length of probe would create a greater surface area for movement of penciclovir across the membrane and so an increase in the recovery of the compound into the perfusate.

3.12.2 Method.

The probes were mounted in petri dishes, and were positioned to expose 1, 2 and 3cm of the microdialysis membrane to the bath solution. The study was performed at two different flow rates, 6.6μl/min and 0.94μl/min. The probes were perfused with Ringer's solution. The bath solution was 2000ng/ml of penciclovir dissolved in Ringer's solution. The number of probes used for each length and flow rate was six. The recoveries were calculated using the measured bath concentrations and equation 2.

3.12.3 Results and Discussion.

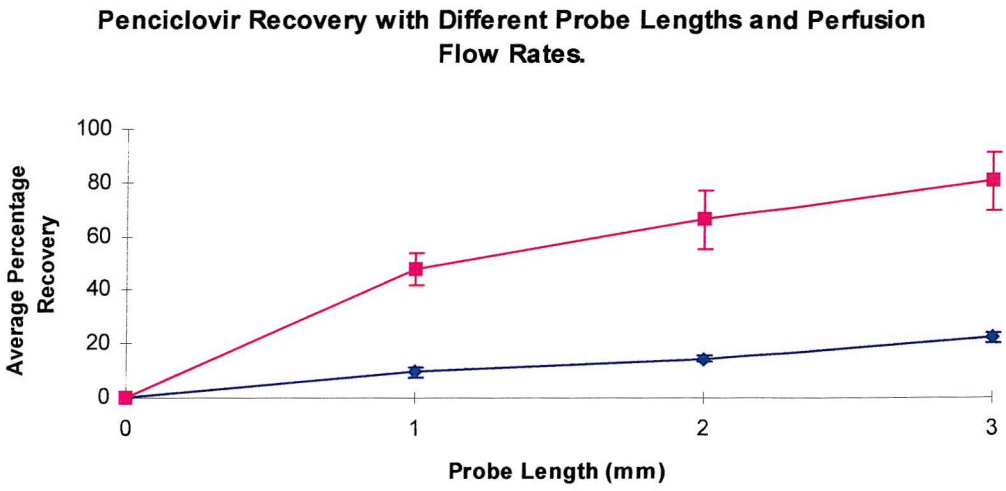


Figure 27 The average recovery (± one standard deviation) of penciclovir using 1, 2 and 3cm probe lengths, and at two different flow rates, 6.6µl/min. (—■—) and 0.94µl/min. (—■—) (n = 36).

Figure 27 demonstrates that as probe length increases, so does the recovery of penciclovir at two different flow rates. These data indicate that the use of very low perfusion rates and longer microdialysis probes should improve the recovery of penciclovir.

3.13 Effect of Temperature on the Recovery of Penciciovir.

3.13.1 Introduction.

Microdialysis performed at an increased temperature is likely to result in an increase in penciclovir recovery, due to an increase in the rate of diffusion of the drug molecules into the perfusate. The increased temperature would also cause increased movement of the drug molecules in the bath solution, ensuring that the area immediately around the microdialysis probe would not become depleted of drug.

3.13.2 Method.

The microdialysis probes were mounted in petri dishes containing Ringer's solution, and were perfused with a solution of penciclovir in Ringer's solution (2000ng/ml). The baths were either held at room temperature (19^0C) or at 32^0C, in a warm room. The probes were perfused with Ringer's solution at a rate of 5μl/min. The probes contained wire in the lumen. The number of probes used for each bath temperature was six. The recovery of penciclovir was calculated from measured bath concentrations, using equation 2.

3.13.3 Results and Discussion.

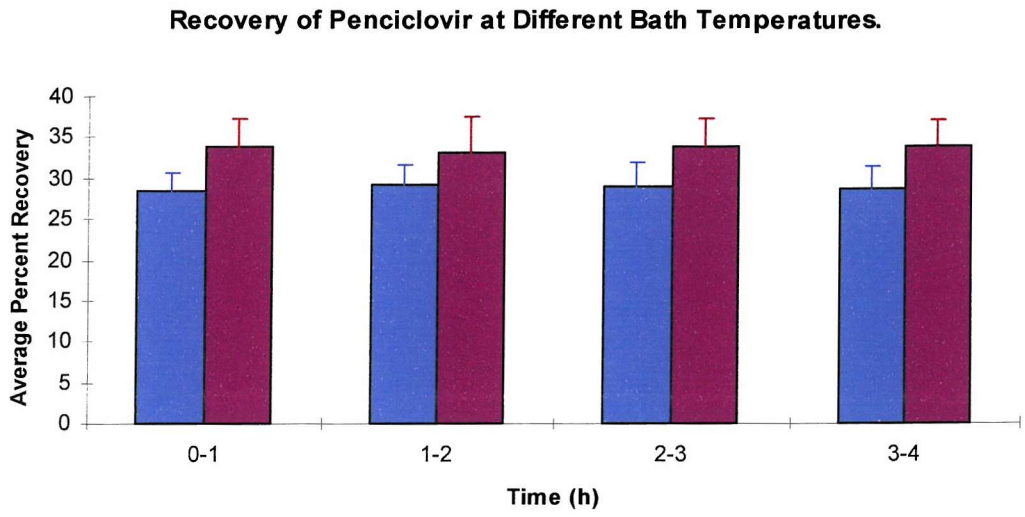


Figure 28 Average recovery of penciclovir when the *in vitro* system is held at 19^{0}C (■) and at 32^{0}C (■) (n = 12).

Figure 28 shows that the recovery of penciclovir was increased when the bath was held at 32^{0}C. The recovery at the higher temperature exhibited a 14-18% increase over the recovery at room temperature. This was highly significant, $p < 0.001$. This would be an advantage when performing microdialysis *in vivo*, as the skin is maintained at a temperature of 37^{0}C, which is likely to promote penciclovir recovery further.

3.14 Inter-Probe Variability.

As the results show, there is some variability between different probes, in the recoveries and deliveries of penciclovir via the microdialysis probes. However, there was very little variability between the recovery or delivery at different time points in the same probe. The inter-probe variability is likely to derive from its manufacture, both in the production of the individual fibres of the kidney dialysis capsule, from which the probes are obtained, and in the in-house manufacture of the probes for dermal microdialysis.

In the production of the renal dialysis capsules, the fibres are formed from cellulose which is spun to form long, narrow semi-permeable tubes. The inherent nature of this method of manufacture produces fibres that are not uniform. The pores are not of a consistent size, the molecular cut-off of 2KDa represents the largest possible pore size, but actually indicates that most of the pores are smaller than this. Also, some parts of the fibres may have larger pores than others, so allowing increased diffusion of substances.

In the production of the probes for use in dermal microdialysis, it may be possible that the insertion of the wire into the lumen of the probe may snag on the fibre walls and enlarge the pores or produce new holes, although great care was taken to discard any probes that were suspected to have been damaged.

It is unlikely that small fluctuations in the ambient temperature of the room contributed to the variability, as there were variations in probe performance seen between probes used on the same day, at the same time.

These factors affecting variability in microdialysis are ultimately carried over to studies performed *in vivo*. It is impossible to determine whether these factors contribute significantly to variability *in vivo*, but should be taken into account when describing variance in the recoveries and deliveries of compounds in human skin.

3.15 Summary of *In Vitro* Microdialysis Results.

The recovery of penciclovir was not significantly affected by the sterilisation procedure of the probes, the presence of albumin in either the perfusate or the bath solution or the presence of wire in the lumen of the probes. The recovery and delivery of penciclovir were equal using this type of probe. Stirring of the bath solution did not alter penciclovir delivery, indicating that, *in vitro*, the drug does not form a concentration gradient around the probe. This suggests that, for *in vitro* recovery studies, the area around the probe was not depleted of penciclovir.

The recovery of penciclovir was affected by the concentration of the drug in the external bath solution. However, significant decreases in recovery were only observed at the highest drug concentrations.

The recovery of penciclovir was directly proportional to the probe length, with increased recovery seen as the probe length increased. The increased length gave a greater surface area available to drug diffusion and a greater duration for equilibration, and so increased the amount of penciclovir that could enter the perfusate.

The recovery rate was inversely proportional to the perfusion flow. As the flow rate decreased, more time was available for the diffusion of the drug across the probe membrane, thus resulting in higher concentrations of penciclovir in the perfusate.

The temperature at which the *in vitro* system was maintained affected penciclovir recovery, with significantly higher recovery observed at an increased ambient temperature.

Chapter 4

Results of Transdermal Absorption of Penciclovir *In Vivo*

4. Results of Transdermal Absorption of Penciclovir *In Vivo*.

4.1 Effect of Delayed Application of Penciclovir.

4.1.1 Aim.

To investigate the shape of the absorption curve at different times of penciclovir application.

4.1.2 Introduction.

Previous work conducted in this department (see Section 1.8) showed that, following topical application of penciclovir (Vectavir cream), the absorption profile was an almost parabolic shape, with maximum concentrations seen at 1 to 5 hours after application. Given the long reservoir of the drug on the skin, prolonged absorption would be expected rather than a peak concentration followed by a decrease in absorption. It was proposed that the presence of the EMLA anaesthetic cream on the skin may facilitate the absorption of penciclovir. The EMLA cream was occluded on the skin for over an hour prior to penciclovir application, which may have enhanced penciclovir absorption through increased skin hydration. One of the effects of EMLA cream, besides local anaesthesia, is vasoconstriction of the dermal blood vessels. This could have the effect of decreased removal of the drug into the systemic circulation, and so improve penciclovir recovery via the microdialysis probe. The decrease in penciclovir absorption seen after three hours of drug application could be due to the loss of the vasoconstriction effect of the EMLA cream after 2 to 3 hours.

It was of interest to investigate whether application 4 to 5 hours after EMLA cream removal produced the same absorption profile. Allowing this length of time prior to penciclovir application should allow time for any skin

hydration and vasoconstriction due to the EMLA cream, to return to normal levels, thereby eliminating these potential absorption enhancing effects.

4.1.3 Method.

In this study the microdialysis probes were inserted using 21G needles as guide cannulae. The perfusate was 2.25% albumin and 0.005mg/ml noradrenaline in Ringer's solution, perfused at a rate of 6.6 μl/min. Penciclovir cream (Vectavir) was applied in drug wells onto the skin surface overlying the inserted probes at one hour, and 4 or 5 hours after EMLA cream removal. In each volunteer, a total of six probes were inserted into the skin of the forearm. In all subjects Vectavir cream was applied to the skin above 3 of the probes at one hour after EMLA cream removal. In two subjects penciclovir cream was applied to the remaining 3 probe areas at 4 hours after EMLA cream removal, and in the other two subjects the Vectavir cream was applied to the skin of the remaining 3 probe areas after 5 hours of removing the EMLA cream. The drug wells were occluded after penciclovir application. The dialysate was collected hourly for seven hours from the time of penciclovir application at one hour (4 subjects; 3 male, 1 female, aged 23-36 years. 19 probes).

4.1.4 Results and Discussion.

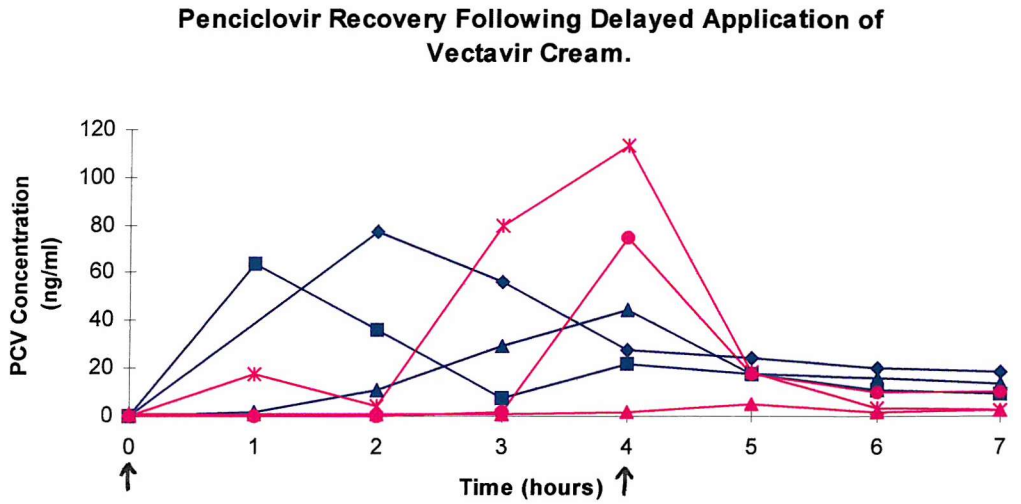


Figure 29 Penciclovir concentrations recovered in the dialysates following Vectavir cream application to the skin at time 1 hour (—■—) and 4 hours (—■—), indicated by the arrows, after removal of the EMLA cream. The symbols represent the individual probes used (subject male, aged 23, number of probes: 6).

Figure 29 appears to follow the same profile as seen in the previous unpublished work (Walter E. 1998), with the penciclovir concentrations reaching a peak at 1 to 4 hours (blue data lines). However, the graph also shows that in the case of the delayed application of penciclovir, the compound was detected in dialysate samples collected before the drug was applied to the skin surface, notably at hours 1 and 3.

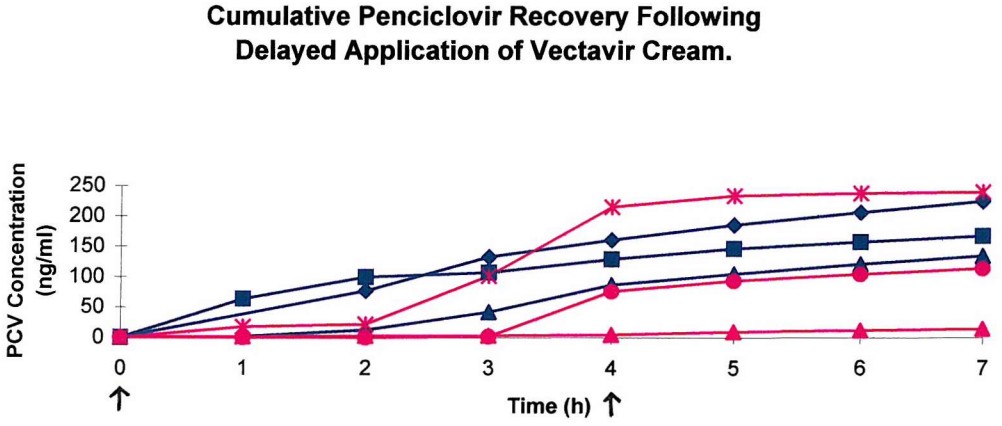


Figure 29a Cumulative recovery of penciclovir following application of Vectavir cream to the skin at time 1 hour (—■—) and 4 hours (—■—), indicated by the arrows, after removal of the EMLA cream (subject male, aged 23, number of probes: 6).

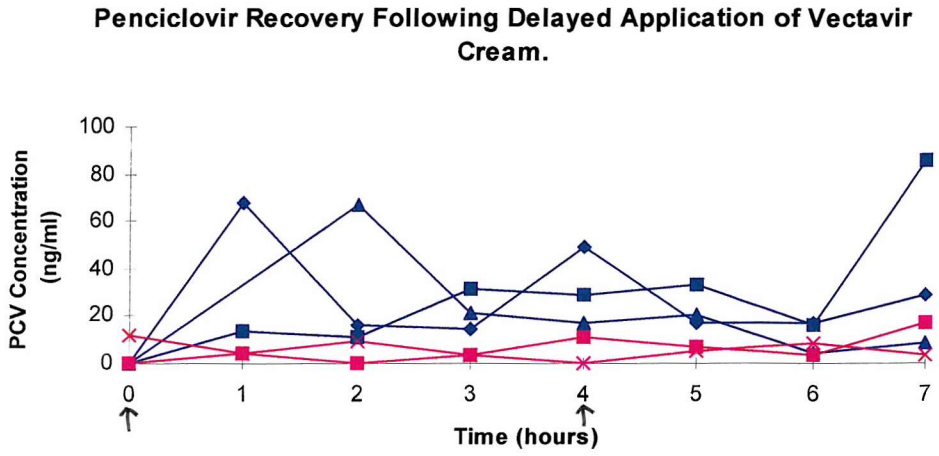


Figure 30 Penciclovir concentrations recovered in the dialysates following Vectavir cream application to the skin at time 1 hour (—■—) and 4 hours (—■—) after removal of EMLA cream (subject female, aged 27, number of probes: 5).

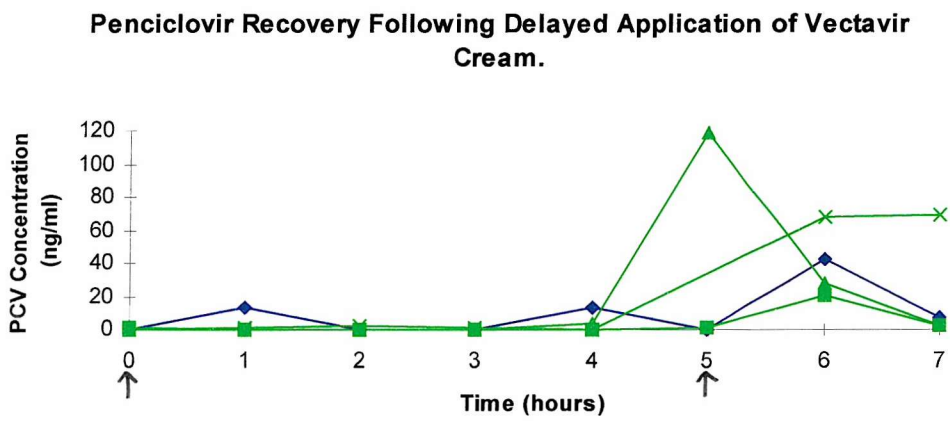


Figure 31 Penciclovir concentrations recovered in the dialysates following Vectavir cream application to the skin at time 1 hour (—■—) and 5 hours (—■—) after removal of EMLA cream (subject male, aged 25, number of probes: 4).

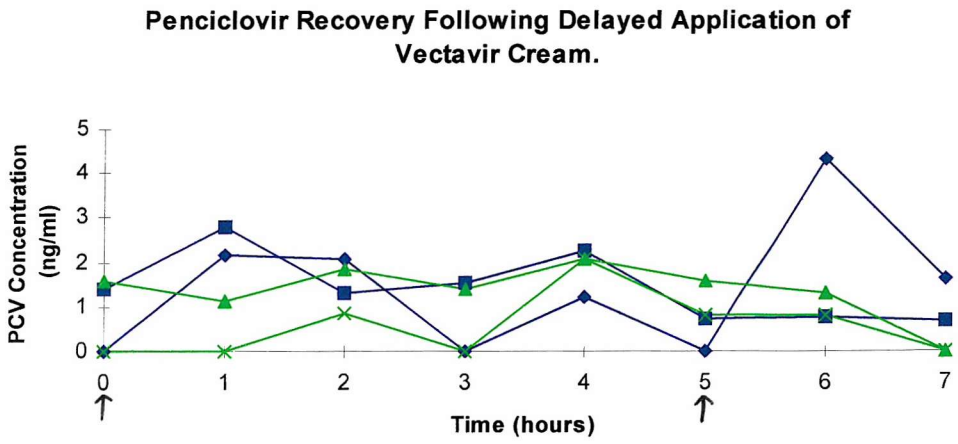


Figure 32 Penciclovir concentrations recovered in the dialysates following Vectavir cream application to the skin at time 1 hour (—■—) and 5 hours (—■—) after removal of EMLA cream (subject male, aged 36, number of probes: 4).

Figures 30 to 32 do not demonstrate the peak in penciclovir detection that was seen in Figure 29 They show no differences in penciclovir concentration between immediate and delayed application of the Vectavir cream, and they also indicate large variability, both between different sites in the same subject, and between different subjects (note the scales of the y-axes). It is also shown that, in some cases, there was penciclovir detection in samples that were collected before the Vectavir cream was applied to the skin.

Table 2 shows the average of the total area under the curve (AUC) values for penciclovir recovery in each of the probes.

Time Penciclovir Applied (h)	Number of probes	Total AUC (ng.h/ml) ± one standard deviation
1	9	140 ± 81
4	5	87 ± 92
5	5	66 ± 73

Table 2 Summary of total AUC's for penciclovir recovery with delayed application to the skin. The results are means ± one standard deviation.

These results showed large variability both between subjects and within the same individual, as already mentioned. The concentrations of penciclovir were much lower than expected from the previous unpublished work and contamination of the samples with penciclovir appears to be a major problem. There were peaks in the HPLC traces at the same retention time as penciclovir in many of the samples that were collected before the Vectavir cream was applied. The AUC's reported in the table include these pre-dose values.

There were occasionally large amounts of penciclovir detected (several hundred to thousands of nanograms per sample) that appear to be inconsistent erroneous peaks (not shown in the figures). Generally, the normal concentrations of penciclovir detected were in the tens of nanograms per sample. These large peaks are likely to be contamination of the samples during the experimental procedure. There were also small amounts of what is thought to be penciclovir in nearly all of the samples collected before penciclovir was applied to the skin. This contamination may be derived from analytical contamination, as contamination from the Vectavir cream would be several hundred times greater, or the peaks detected may be a substance other than penciclovir that is derived from the skin of the subjects, but which coincidentally has the same retention time as penciclovir.

The data obtained were very different from previous studies and the concentrations detected were much lower. Therefore, a series of studies was undertaken to identify these differences and to resolve the difficulties encountered in this preliminary study.

4.2 Effect of Occlusion of Percutaneous Absorption of Penciclovir.

4.2.1 Aim.

To demonstrate the effect of occlusion of the penciclovir formulation (Vectavir cream) on the absorption of the drug through the skin.

4.2.2 Introduction.

It has been documented that occlusion can enhance the penetration of a topically applied compound into the skin (see Section 1.2). It is possible that there would be enhanced absorption from a formulation of penciclovir that forms an occlusive coating over the cold sore after application. The actual benefit of occlusion of penciclovir cream therefore needs to be determined in order to assess the value of such an 'occlusive' formulation.

4.2.3 Method.

The microdialysis probes were inserted using 21G needles as guide cannulae. The perfusate was 2.25% albumin in Ringer's solution, perfused at a rate of 6.6μl/min. The penciclovir cream was applied one hour after probe insertion. In each subject, three of the wells were occluded and three remained uncovered (5 subjects; 4 male, 1 female, aged 18-29 years. 27 probes).

4.2.4 Results and Discussion.

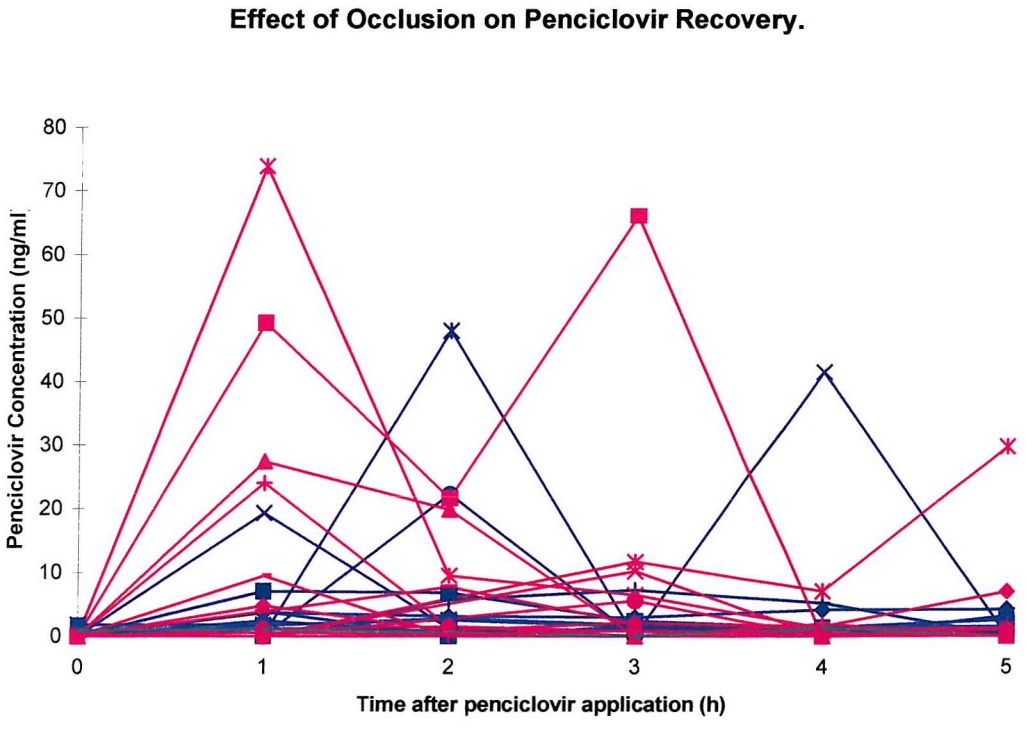


Figure 33 Penciclovir recovery when the formulation was either left unoccluded (—■—) or was occluded (—■—) on the skin surface.

Status	Number of probes	Total AUC (ng.h/ml) ± one standard deviation
Occluded	13	17 ± 14
Unoccluded	14	31 ± 41

Table 3 Summary of total mean AUC ± one standard deviation, for penciclovir recovery with and without occlusion of the formulation.

Consistent with previous findings, these results also demonstrate large variations. There was no apparent difference in recovery between the formulation that was occluded and that which remained uncovered ($p = 0.27$). The amounts and frequency of contamination in samples collected prior to application of the Vectavir cream (time zero in Figure 33) were negligible

compared with the first study. However, the concentrations of penciclovir detected were very low in comparison to the previous unpublished data generated in this department (Walter E. 1998).

The results of both of these studies were interesting in that, not only were the absorption profiles not parabolic, but the concentrations of penciclovir recovered were much lower than expected. There were also problems with contamination.

The presence of penciclovir in the pre-dose samples puts doubt upon the results reported for penciclovir recovery following topical application. The values were similar in both cases, it may be that the peaks were contamination or the unknown substance in all cases, but equally, they may be the true reflection of penciclovir absorption. It is difficult to determine which is true. These peaks were not seen in the zero standard samples. It is interesting to note that these peaks were larger and more consistently present in the dialysates from certain volunteers. This may indicate that these peaks were not penciclovir, but a substance that was being dialysed from the skin of these individuals. There was less contamination seen in the occlusion study, which may reflect a greater level of competency acquired in performing the microdialysis technique. However, the concentrations of penciclovir recovered were surprisingly low compared the previous work produced from this department.

4.3 Effects of Dermal Vasoconstriction.

4.3.1 Aim.

To investigate if dermal vasoconstriction local to the microdialysis probes, enhances penciclovir recovery into the perfusate following topical application.

4.3.2 Introduction.

The presence of noradrenaline in the perfusate has a profound effect on the dermal microcirculation local to the microdialysis probe. Noradrenaline acts on the smooth muscle of the blood vessels, and causes them to contract. This results in vasoconstriction. During the microdialysis study, the noradrenaline diffuses out of the perfusate into the surrounding tissue and causes local vasoconstriction of the blood vessels. This is likely to result in a decrease in the clearance of absorbed compounds into the systemic circulation as the sink effect of the dermis is compromised. This can be advantageous for microdialysis recovery, as the dermis then maintains a higher concentration of the compound of interest and so increasing the recovery into the perfusate.

4.3.3 Method.

The microdialysis probes were inserted using 23G needles as guide cannulae. The perfusate was 2.25% albumin, 0.005mg/ml noradrenaline in Ringer's solution in each probe, perfused at a rate of 6.6μl/min. Vectavir cream was applied 45 minutes after probe insertion. All of the drug wells were occluded. (1 subject; male, aged 26 years. 4 probes).

4.3.4 Results and Discussion.

Data from two of the probes were lost due to analytical failure, but it is clear to see from Figure 34 that the recovery concentrations were very small, with the majority of the concentrations being lower than 10ng/ml in an hourly collection.

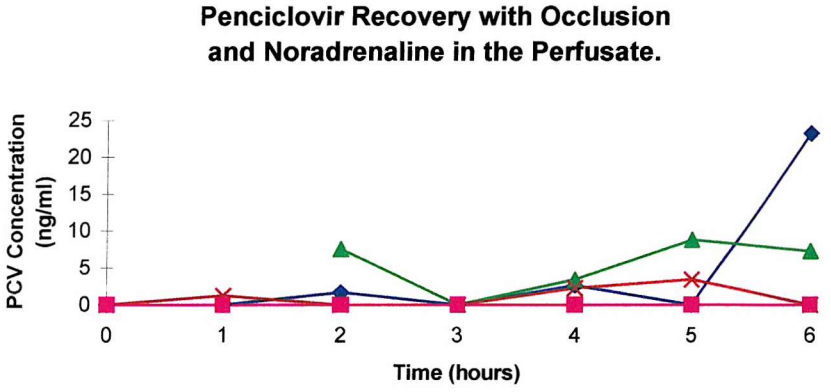


Figure 34 Penciclovir recovery through 4 probes in one subject, with vasoconstriction and occlusion. The probes were all studied under identical conditions.

A comparison between these limited data and the results shown in section 4.2 indicate that the low concentrations found in that study (compared with the unpublished work by Walter E. 1998) were not due to the absence of noradrenaline in the perfusate.

4.4 Effect of Manner of EMLA Cream Removal.

4.4.1 Aim.

This study was performed to investigate whether the way in which the EMLA cream was wiped off the skin altered the permeability of the skin, and so the extent of penciclovir absorption.

4.4.2 Introduction.

In the previous studies from this department the EMLA cream was removed from the skin using a coarse paper towel, whereas in the current studies a softer tissue was used. It was suspected that the barrier function of the stratum corneum may have been impaired due to abrasion from wiping with the coarser tissue. Wiping the skin with an alcohol swab was also under investigation, as this was performed in some of the previous studies, although not consistently. Alcohol wipes may have had an effect on the absorption of penciclovir due to possible delipidisation effects, which could enhance penciclovir absorption by reducing the lipid content of the stratum corneum. Reduction of stratum corneum lipids appears to reduce the barrier function of the stratum corneum and so improve drug penetration (Elias P. *et al* 1981 and Rogers J. *et al* 1996).

4.4.3 Method.

The microdialysis probes were inserted using 23G needles. The perfusion fluid comprised of 2.25% albumin, 0.005mg/ml noradrenaline in Ringer's solution, perfused at a rate of 6.6μl/min. In each subject, on two sites of the arm, the EMLA cream was removed using the coarse paper towel, two sites using the soft tissue and two sites using soft tissue followed by wiping with an alcohol swab. The skin was allowed to recover for 90 minutes

following needle insertion. The drug wells were all occluded. (2 subjects; male, aged 21 and 33. 12 probes).

4.4.4 Results and Discussion.

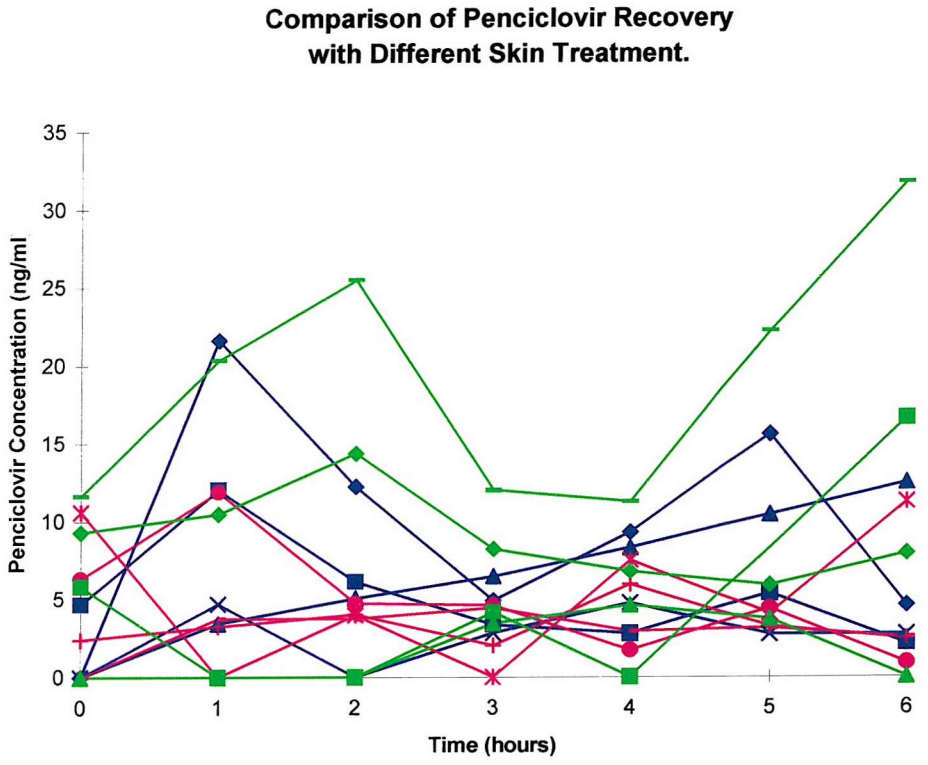


Figure 35 The recovery of penciclovir following topical application of Vectavir cream, after the EMLA cream was wiped from the skin using coarse tissue (—■—), soft tissue (—■—) and soft tissue followed by alcohol swab (—■—).

Status	Number of probes	Total AUC (ng.h/ml) ± one standard deviation
Coarse tissue	4	39 ± 21
Soft tissue	4	24 ± 5
Alcohol swab	4	51 ± 45

Table 4 Summary of total mean AUC ± one standard deviation, for penciclovir recovery following different methods of wiping the EMLA cream from the skin.

The data in this table may give the impression that the recovery of penciclovir is least following wiping of the skin with the soft tissue. This is meant to represent the skin with the smallest amount of physical barrier perturbation. The absorption appeared to increase after wiping with the coarse tissue, and more so following wiping with the alcohol swab, mirroring the suspected decrease in barrier function. However, the variability was large and there was also contamination of the pre-dose samples, which are included in the values of AUC.

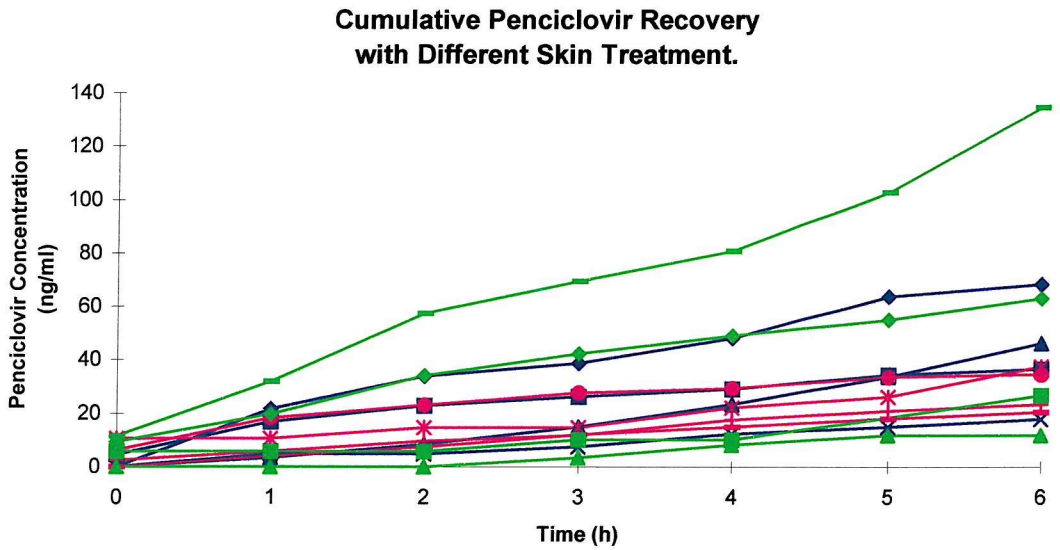


Figure 35a Cumulative recovery of penciclovir following topical application of Vectavir cream, after EMLA cream was wiped from the skin using coarse tissue (—■—), soft tissue (—■—) and soft tissue followed by alcohol swab (—■—).

It seems likely that abrasion of the skin and possible delipidisation with alcohol could produce a very minor effect on the transdermal absorption of penciclovir. The optimum method of EMLA cream removal, to ensure maintenance of the intact stratum corneum, was to wipe the skin with soft tissue only.

4.5 Effect of Probe Depth on Penciclovir Recovery.

4.5.1 Aim.

To determine whether the depth of the microdialysis probe in the skin has an effect on the recovery of penciclovir.

4.5.2 Introduction.

The depth that the probe is positioned in the skin may have an effect of recovery of penciclovir. It might be expected that the more superficial the probe is placed, the less distance the drug has to diffuse from the skin surface to the probe, resulting in increased levels of penciclovir in the upper layers, and so a greater recovery. Alternatively, if the appendageal route (via the hair follicles and the sweat ducts) is an important absorption pathway for penciclovir, it may be that the drug diffuses into the skin across the follicular epithelium of the ducts at the root of the appendage, usually located in the dermis. Therefore, the recovery concentration of penciclovir may be higher, the deeper in the tissue the probes are placed.

4.5.3 Method.

The microdialysis probes were inserted using 23G needles, intentionally positioned at different depths in the skin. The actual depth was determined at the end of the study using the Dermascan C ultrasound scanner. The perfusion fluid comprised of 0.005mg/ml noradrenaline in Ringer's solution, perfused at a rate of 5μl/min. The probes contained wire in the lumen. (3 subjects; 1 male, 2 female, aged 19-29. 15 probes).

4.5.4 Results and Discussion.

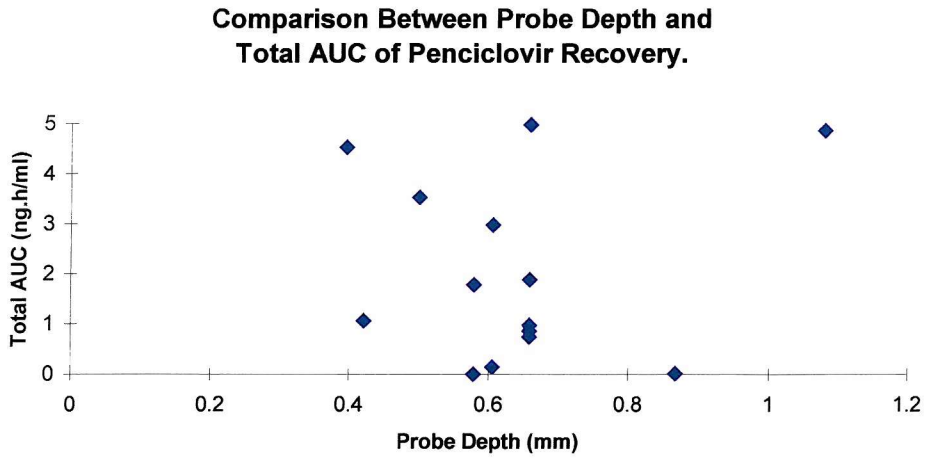


Figure 36 The total AUC of penciclovir recovery for probes placed at different depths in the skin. Number of probes: 15.

Figure 36 shows that there is no linear relationship between penciclovir recovery and depth of the probe in the skin (R^2 = 0.0055). The concentration of penciclovir recovered was independent of the depth of the probe. It is also important to note the very low concentrations of penciclovir recovered. These values represent the total amount of penciclovir collected over a period of five hours and are negligible compared with the previous unpublished data. It was not possible to identify a dominant route of penciclovir absorption from these results.

4.6 The Effect of EMLA Cream on Penciclovir Absorption.

4.6.1 Aim.

To investigate whether the use of EMLA cream for local anaesthesia affects the absorption of penciclovir.

4.6.2 Introduction.

It was thought that the use of EMLA cream may influence the absorption of penciclovir, due to the effects of hydration and vasoconstriction of the skin. Both of these parameters may promote the transdermal absorption and microdialysis recovery of penciclovir; hydration by altering the properties of the stratum corneum, so allowing the more free movement of the drug in this layer (see Section 1.2) and local dermal vasoconstriction by reducing the removal of penciclovir from the dermis into the systemic circulation (see Section 4.3).

4.6.3 Method.

The microdialysis probes were inserted using 23G needles. Three of the sites on the arm were anaesthetised using EMLA cream, and three had no anaesthesia. The perfusion fluid comprised of Ringer's solution, perfused at a rate of 5μl/min. The probes contained wire in the lumen. (1 subject; female, aged 29. 5 probes).

4.6.4 Results and Discussion.

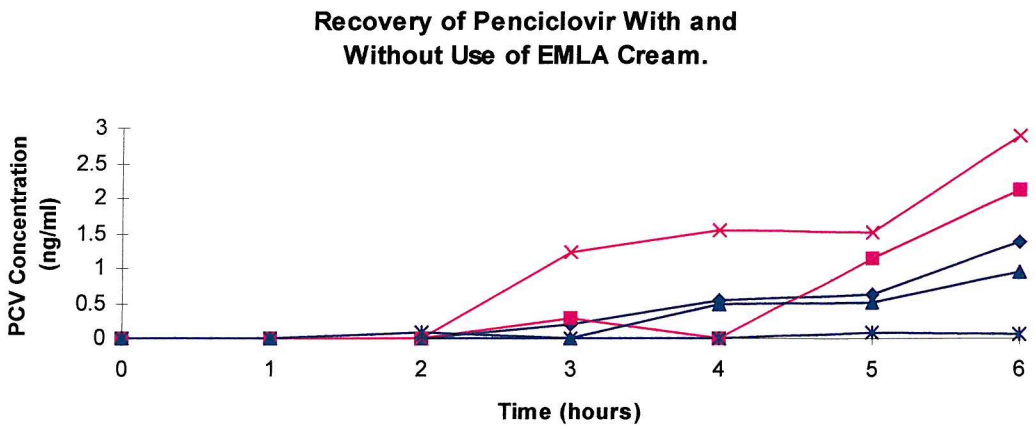


Figure 37 Penciclovir recovery in the situation of EMLA being used (—■—) and where EMLA was not used (—■—). One subject, 5 probes.

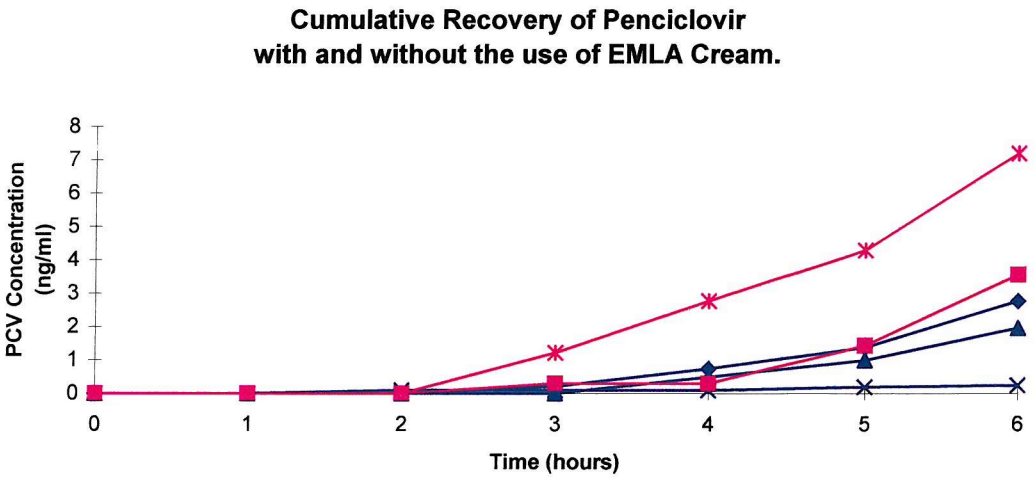


Figure 37a Cumulative penciclovir recovery in the situation of EMLA being used (—■—) and where EMLA was not used (—■—). One subject, 5 probes.

The data from one of the probes was lost due to probe failure during the study. The results seen in Figures 37 and 37a indicate that, although the penciclovir recovery was slightly increased when local anaesthesia was not used, the use EMLA cream prior to probe insertion did not significantly alter penciclovir recovery, and absorption through the skin ($p = 0.38$).

4.7 Comparison of the Results in this Project with Previous Data.

It is clear to see from these results that the concentrations of penciclovir that have been recovered via the microdialysis probes were very small compared to the results obtained by Walter E. (1998).

There are several differences in the methods used between the work by Walter E. and those described in this section. One of these differences is that the time left for recovery of the skin after probe insertion was 30-45 minutes in the previous work, and 45-60 minutes in the most recent studies. It is possible that this would influence the microdialysis recovery of penciclovir, as it has been suggested that the skin trauma generated by the insertion of the needle may affect the diffusion characteristics of compounds within the skin (Groth L. and Serup J. 1998). It has been recommended that allowing at least 90 minutes following probe insertion before commencing the experiment would enable the traumatic effects of the inserted needle to return as close to baseline as possible.

It was also found that the needles that were used to insert the probes had a larger bore (21G) than those used by other microdialysis investigators (23G). It may be that the needle alters the barrier by stretching the stratum corneum while it is in position. The cells may have been pulled apart and the barrier damaged and so may have allowed increased penetration of the drug. Using a larger bore needle to insert the probes may also have resulted in an increased skin trauma than a narrower bore needle. Subsequent studies described in this project used 23G needles (as given in the methods section).

The subjects of the previous study were investigated in alphabetical order (see Table 1, Section 1.8). It is apparent that, although the recovery concentrations of penciclovir were high in the first subjects studied, the concentrations gradually decreased as the experiments were performed. This may be a reflection of an increased capability to perform the microdialysis technique.

There were a number of practical problems during the early experiments, and those performed by Walter E. (1998) that may have contributed to possible contamination of the probes with penciclovir. These

experiments were performed before wire was introduced into the probes to maintain rigidity. The probes without wire were extremely prone to kinking at the points of entry and exit into and from the skin. This caused a very much reduced, or even no, flow, which resulted in no movement of the perfusate, and so allowed increased diffusion of penciclovir into the probe. This may have contributed to the high concentrations seen in some samples of dialysate. The other outcome of greatly reduced flow was the drying out of the probe. These studies were performed using albumin in the perfusate, which, when slightly dried, became sticky. The dryness and the stickiness then prevented the flow of the perfusate once the kink had been straightened. The problem was resolved by the dampening of the probes with wet cotton wool, as it entered and exited the skin. This action gave great scope for contamination between probes and the possibility of accidental contamination with the penciclovir cream, especially when the drug wells were unoccluded. The use of wire in the lumen of the probes resolved the problem of the kinking of the probe. Probes with wire never blocked or dried out and there was no requirement for the dampening or straightening. This greatly reduced the risk of contamination as there was no need to interfere with the probes throughout the experiment, except for the changing of the collection vials. This procedure could be performed without any contact between the investigator and the probe.

A further difference noted was that the drug wells used in the studies performed by Walter E. (1998) were comprised of a rigid plastic sheet, cut into the appropriately shaped well. They were adhered to the arm of the subject using double-sided sticky tape. The wells were so rigid that they were not able to adopt the curved shape of the arm. The edges and corners of the wells would adhere less well, and would tend to be raised above the skin. In contrast, the Comfeel wells, used in all of the current studies, were much more flexible and remained in good contact with the skin throughout the experiment. Indeed, they were often difficult to remove at the end of the study due to good adherence, and, once removed, the layer of Vectavir cream on the skin was seen to have remained in the well defined area.

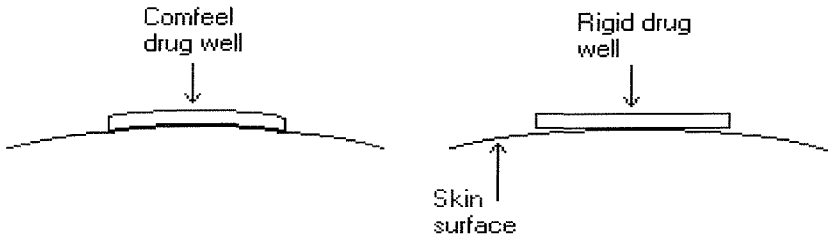


Figure 38 Schematic representation of the drug wells adhering to the skin surface.

It is clear from figure 38 that when a rigid well was used, there would be great potential for the creeping of the penciclovir formulation under the edges of the drug well. If this was to occur, the risk of contamination of the probe as it entered or exited the skin would be enormous.

However, it is difficult to explain why noradrenaline had such a marked effect on penciclovir recovery in the previous studies, if the main source of penciclovir was due to contamination when using the rigid drug wells.

A colleague (C. Morgan) performed more studies to try to determine the reasons for the huge differences in penciclovir recovery. The effect of size of needle bore was investigated but showed no difference between recovery following probe insertion using 21G and 23G needles. The length of time allowed for skin recovery from trauma generated by needle insertion was studied. When the Vectavir cream was applied immediately after probe insertion, greater recoveries were seen than when the formulation was applied 90 minutes after insertion. However, the concentrations of penciclovir recovered were not as high as those previously reported.

Stripping of the stratum corneum using cellophane tape was investigated, and showed much increased recoveries of penciclovir. This clearly demonstrated that the stratum corneum is the main barrier to penciclovir absorption. This was expected as penciclovir is a hydrophilic compound, and the stratum corneum presents a relatively impermeable lipophilic barrier.

It is difficult to explain why the results from previous studies and more recent experiments are so different. There have been many improvements on the microdialysis technique, which should allow more accurate and

realistic measurements of the concentration of penciclovir being absorbed through the skin. These improvements include the Comfeel drug wells that adhere so well to the skin, thus preventing possible contamination of the probe due to creeping of the formulation under the well. The presence of wire in the lumen of the probe ensures a constant, even flow of the perfusate. The reduction in perfusion flow rate results in increased recovery of the compound into the probe (see Section 3.11). The use of the narrower bore needle (23G) to insert the probes may reduce the trauma of the skin, and the length of time before commencement of the study being increased to 90 minutes allows more time for the skin to recover following this trauma. The perfusate no longer contained albumin, as it was found not to be required (see Section 3.3), which then also reduced the number of steps in sample preparation and so limited the possibility of loss of penciclovir from the sample during albumin removal.

The results shown in this section also demonstrate that, as time elapsed and the number of studies performed increased, the concentration of penciclovir being recovered decreased. This is likely to arise from greater skill and competence at performing the microdialysis technique, and the increased awareness of the possible sources of contamination. This opinion is extended to those studies performed by Walter E. (1998), and the very high concentrations of penciclovir seen in those early experiments are thought to be due to contamination. The fact that these studies are not reproducible by two investigators supports this theory.

The results also demonstrate that penciclovir does not appear to readily penetrate intact human skin. It is important to recognise that the microdialysis sampling in these studies was performed with the probe being situated in the dermis, below the upper dermal capillary plexus. It may be that the penciclovir is penetrating the skin to some extent, but is being removed by the systemic circulation. Even with the use of noradrenaline to reduce the blood flow, there may be some residual capillary perfusion which could remove the drug from the sampling area of the probe.

The target site of penciclovir is the basal layer of the epidermis, above the upper capillary plexus, and the microdialysis probe. It is possible that the

drug may penetrate the skin to a sufficient degree as to attain therapeutic concentrations at this site. Direct measurements of the drug concentration in each layer of the skin can be determined by full-thickness skin biopsies. However, this technique is highly invasive, allows only one measurement per skin site and can cause permanent scarring. The use of the microdialysis technique is designed to overcome these problems, but it can only be used for drug concentration measurements in one, fixed, layer of the skin.

Estimation of the Transdermal Absorption of Penciclovir.

If 100% of the penciclovir dose that was applied to the skin was absorbed, only a small proportion of this would encounter the microdialysis probe and be available for dialysis from the tissue.

The volume of the formulation applied into the drug wells was 0.09ml (see page 56 for the dimensions of the drug wells). The concentration of penciclovir in the formulation was 5 mg/ml (0.5%), resulting in an amount of 0.45mg of penciclovir being applied to the skin.

The width of the drug wells was 5000μm, whereas the diameter of the probe in the skin was 216μm. The amount of penciclovir available for dialysis, assuming 100% absorption would be:

$$\frac{216}{5000} \times 0.45 \quad \text{which equals 0.019mg.}$$

In contrast to this, the total concentration of penciclovir recovered in the dialysate in one of the probes from Figure 37 (page 107) was 2.72ng of a six hour period. This is the recovery of penciclovir assuming the maximum recovery of 25% estimated from the *in vitro* recovery data. This amount represents only 0.014% of the applied available dose.

4.8 Summary of Transdermal Penciclovir Absorption.

The results of this chapter indicate that penciclovir in the form of Vectavir cream may not readily penetrate intact human skin.

The concentration of penciclovir required to inhibit HSV-1 viral replication *in vitro* is 0.4μg/ml (Boyd M. *et al* 1987). This indicates that this concentration is necessary in the basal layer of the epidermis to eliminate herpes-virus infection.

The microdialysis technique, performed in the dermis, below the basal layer and the upper dermal capillary plexus, showed that recoveries of penciclovir ranged from 0-120ng/ml of perfusate per hour, with the majority of the recoveries being at the lower end of this range. These concentrations, although only a percentage of the actual tissue concentrations, were much lower than the concentration of the drug required for inhibition of viral replication. It is possible that some absorption of penciclovir may have occurred but that the dermal blood supply was sufficient, even under vasoconstrictive conditions, to effectively clear the drug from the tissue. This would prevent further diffusion of the drug into the dermis and result in very low penciclovir concentrations in this skin layer.

In order to deliver a greater concentration of penciclovir to its site of action, it may be necessary to modify the vehicle in which the drug is applied to the skin. The use of penetration enhancers may be advisable to promote the transdermal absorption of penciclovir.

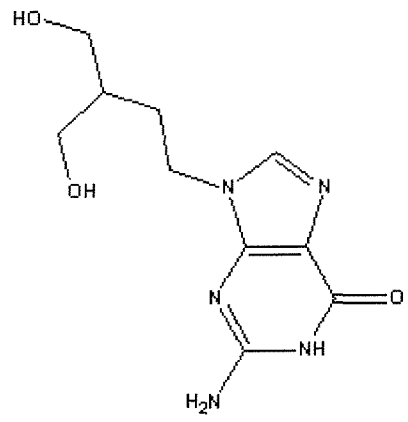
Chapter 5

The Use of Salicylic Acid as a Penetration Enhancer for the Transdermal Absorption of Penciclovir

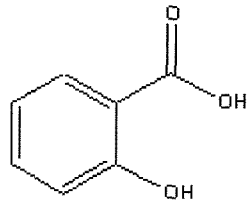
5. The Use of Salicylic Acid as a Penetration Enhancer for the Transdermal Absorption of Penciclovir.

5.1 Introduction.

It proposed that the co-application of salicylic acid with penciclovir may enhance the percutaneous penetration of the antiviral compound. The rationale behind this is that opposing charges of the two molecules may result in an ionic attraction, and the possible formation of a lipid soluble ion pair. Although the resultant molecule would be larger than penciclovir alone, it is likely to be a more neutral and lipophilic compound and may more readily penetrate the stratum corneum.



Penciclovir
MW: 253 Da



Salicylic Acid
MW: 138 Da

Figure 39 The molecular structures of penciclovir and salicylic acid.

Penciclovir is a polar compound and its main barrier to percutaneous penetration is the stratum corneum. It is likely that in the aqueous environment of the viable epidermis and dermis, the polar molecule will move freely within the tissue.

The studies outlined in Chapter 4 indicated that the transdermal absorption of penciclovir may be poor, and that the concentrations found in the basal epidermis may not be sufficient to inhibit herpes-virus replication. In order to improve antiviral efficacy in the treatment of herpes labialis using topical penciclovir, it is necessary to promote the absorption of increased amounts of the drug across the stratum corneum. The formation of a neutral ion pair would offer improved chances of realising this aim. In this way, the ion pair would carry the penciclovir through the stratum corneum and into the epidermis and dermis below. Upon reaching the aqueous regions of the viable epidermis (and dermis), the lipophilic ion pair is likely to dissociate into the two initial molecules, enabling penciclovir to act on virally infected cells in the basal epidermis.

Salicylic acid is used in many topical formulations for a variety of dermatological applications in varying concentrations. It is used in the treatment of hyperkeratotic skin disorders at a concentration of 2%. Hyperkeratoses are characterised by a thickening of the stratum corneum. The keratolytic action of salicylic acid is used to combat this. Such conditions as warts and calluses can be treated with salicylic acid in concentrations ranging from 11-50%, but the keratolytic effects can often cause cutaneous irritation at these high concentrations. Salicylic acid has previously been used in the topical treatment of acne, but recently has been thought to have dubious value in the treatment of this condition. However, many of these formulations containing salicylic acid at a concentration of 2% still have a role in the treatment of seborrhoeic dermatitis. Salicylic acid in low concentrations (1.46%) is used to treat some fungal skin infections, particularly tinea (a condition caused by dermatophytes; fungi which grow in soil and on animals. The infection is confined to the stratum corneum). Salicylic acid can also be used in conjunction with coal-tar to treat psoriasis, and occasionally for chronic atopic eczema (British National Formulary 1998).

5.2 *In Vitro* Studies of Penciclovir Recovery, with Salicylic Acid.

5.2.1 Introduction.

In order to assess whether the presence of salicylic acid would influence the recovery of penciclovir via cutaneous microdialysis, an *in vitro* study was performed.

5.2.2 Method.

The *in vitro* method used was as described in section 2.4. The perfusate consisted of Ringer's solution, and was perfused through the microdialysis probes at a rate of 0.94μl/min. The bath solution comprised either 0.5μg/ml penciclovir or 0.5μg/ml penciclovir plus 2μg/ml salicylic acid in Ringer's solution. The number of probes was eight for each parameter. The recovery was determined from the measured bath concentrations and calculated using equation 2.

5.2.3 Results and Discussion.

The *in vitro* study showed that the recovery of penciclovir via the microdialysis probes was slightly reduced when it was combined with salicylic acid, as can be seen in Figure 40. This is possibly due to the larger size of the combined molecule, the ion-pair, which may reduce diffusion across the microdialysis membrane. The differences in recovery were not significant ($p = 0.6$). The nature of the microdialysis membrane may also affect the recovery of the ion pair. The membrane was composed of cellulose and was hydrophilic in nature. The ion-pair may not be able to diffuse as easily through the membrane as the penciclovir molecule alone.

The recovery reflects not only that of the penciclovir molecules involved in the formation of the ion-pair, but also the free molecules in the bath solution.

The *in vitro* recovery of salicylic acid at the flow rate of 0.94μl/min was 100%.

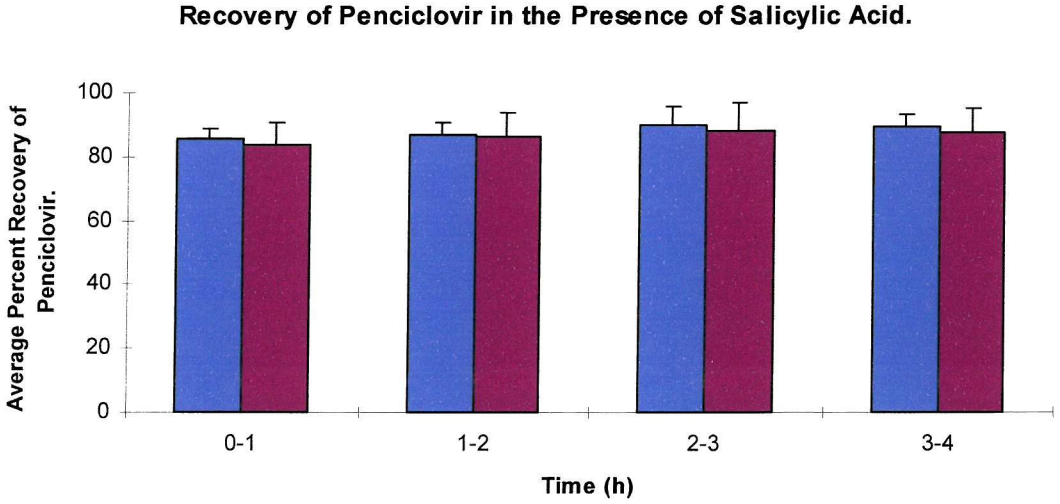


Figure 40 A comparison of the average percent recovery, + one standard deviation, of penciclovir (■) and the recovery of penciclovir when salicylic acid was present in the bath solution (■) (n = 16).

5.3 *In Vivo* Absorption of Penciclovir in Propylene Glycol and Water with Salicylic Acid.

5.3.1 Introduction.

The vehicle of propylene glycol/water was selected for this study to represent the simplest possible formulation, having an aqueous nature and the presence of a penetration enhancer, to promote transdermal penetration.

5.3.2 Topical Penciclovir and Salicylic Acid Formulations.

The drug solutions comprised of 0.5% (w/v) penciclovir dissolved in 50% propylene glycol and water, and 0.5% (w/v) penciclovir plus 2% (w/v) salicylic acid dissolved in 50% propylene glycol and water. The pH of the penciclovir solution was 5, and the penciclovir and salicylic acid solution was pH 3.

5.3.3 Method.

Microdialysis probes were inserted into the skin of the dorsal forearm as previously described in Section 2.5, and were perfused with 0.005mg/ml noradrenaline in Ringer's solution at a rate of 0.94μl/min. This flow rate was selected so as to improve recovery of penciclovir from the surrounding tissue into the perfusate (see Section 3.10). The arm was allowed to recover from the trauma of probe insertion for 90 min. The probes contained wire in the lumen. The drug solutions were then applied to the arm in drug wells, 60μl in each well, formed from Comfeel:Plus ulcer dressing, having an inner diameter of 1.8 x 0.5cm. All of the drug wells, were occluded with a second piece of the ulcer dressing. (2 subjects; male, aged 20 and 22 years. 6 probes).

5.3.4 Results and Discussion.

The propylene glycol/ water formulation had a very low viscosity and problems were encountered with regard to the leaking of the drug formulation from under the drug patches, between the patch and the skin, and causing contamination of the microdialysis probes as they entered and exited the skin. In one of the subjects all of the patches leaked and no data was recoverable. In the other subject, the patches appeared to be adhered to the skin for three hours after the formulation was applied to the patches, but began to leak after this time. For this reason the data retrieved only represents the first three hours of the study, as contamination occurred after this time.

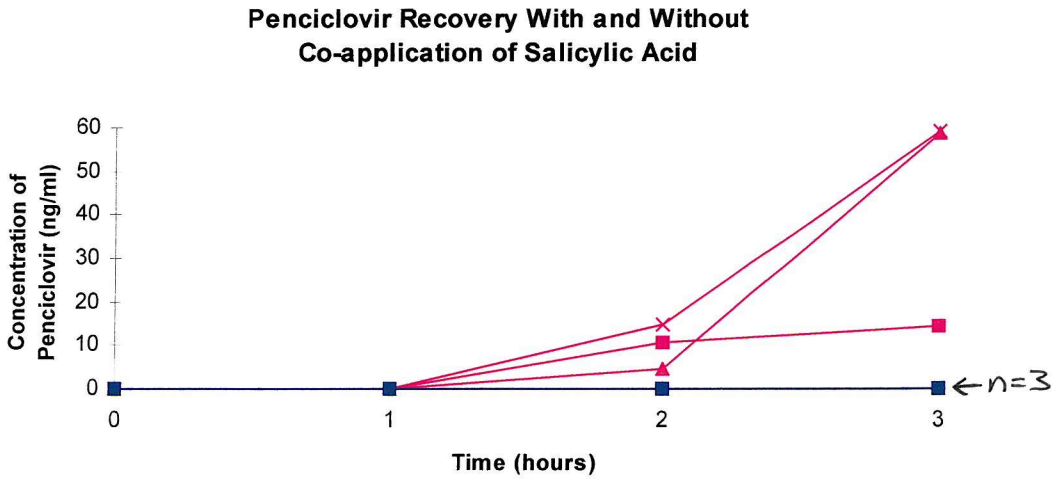


Figure 41 Comparison between penciclovir recovery when applied to the skin alone (—■—) and with salicylic acid (—■—) (1 subjects, 6 probes).

Although it appeared that the co-application of salicylic acid resulted in an increase in the transdermal absorption of penciclovir, the results could not be considered to be significant as the results were obtained from only one subject. Penciclovir was not detected in the dialysate when applied to the skin alone in the formulation.

This formulation was considered to be ineffective for use in the current study design, as the risk of formulation leakage and drug contamination of the probes was too great.

5.4 *In Vivo* Absorption of Penciclovir with Salicylic Acid in a 2% Carboxymethylcellulose Formulation.

5.4.1 Introduction.

The very liquid nature of the drug solutions consisting of 50% propylene glycol and water prompted the requirement of a thickening agent for the topical formulations. Carboxymethylcellulose is commonly used for this purpose. The aim was to prevent the leaking of the solutions under the drug patches and into direct contact with the microdialysis probes, and so more accurately assess the transdermal absorption of penciclovir when applied to the skin with salicylic acid.

5.4.2 Topical Penciclovir and Salicylic Acid Formulations.

The concentrations of the drug were 0.5% penciclovir and 2% salicylic acid. The drugs, (1% (w/v) penciclovir and 4% (w/v) salicylic acid) were dissolved in 100% propylene glycol. A 4% (w/v) solution of carboxymethylcellulose in water was also prepared. The carboxymethylcellulose solution was then transferred into the drug plus propylene glycol solutions. This resulted in formulations comprising 0.5% penciclovir and 0.5% penciclovir plus 2% salicylic acid in a 2% carboxymethylcellulose solution in 50% propylene glycol. The pH of the penciclovir solution was 5. The pH of the penciclovir and salicylic acid formulation was 3, and was adjusted to 5 using ammonium hydroxide.

5.4.3 Method.

Microdialysis probes were inserted into to the skin of the forearm as previously described. The perfusate consisted of 0.005mg/ml noradrenaline in Ringer's solution and the flow rate was set at 0.94μl/min. The probes

contained wire in the lumen. The skin was allowed to recover for 90 minutes after probe insertion before application of the drug formulations. 60μl of the drug formulations were placed in each drug well, the formulation was spread to cover the entire available skin surface. The drug patches were all occluded. (1 subject; male, aged 20 years, 3 probes for each drug formulation)

5.4.4 Results and Discussion.

The results of this study revealed that penciclovir and salicylic acid were not detected in any of the samples. It was expected that salicylic acid would penetrate the skin from this formulation, as work performed by a colleague in this department (W. Keene) demonstrated that this was so. It is possible that the viscosity of the drug formulations was too great, and the drug could not move through it to reach the surface of the skin. This does not however explain why no drug was detected at all, as drug present at the skin surface immediately following application should have penetrated the skin.

There was no evidence of the creeping of the drug solution. The drug patches appeared well adhered to the skin, and were removed with some degree of difficulty at the end of the experiment. The drug formulation remained in the shape of the well on the skin, following patch removal. This indicated that a thickened solution minimises contamination of the microdialysis probes through direct contact with the drug solutions.

5.5 *In Vivo* Absorption of Penciclovir with Salicylic Acid in a 1% Carboxymethylcellulose Formulation.

5.5.1 Introduction.

The two experiments described in Sections 5.3 and 5.4 indicated that the drug formulations required a higher viscosity than the 50% propylene glycol: water mix to prevent leaking of the solution under the patch, but also that the formulation must be fluid enough to allow diffusion of the compounds within the formulation. This would then allow the drugs to come into direct contact with the skin, for partitioning into the stratum corneum. Preparation of the drugs in a 1% carboxymethylcellulose solution fitted both of these criteria.

To investigate the possible effects of the pH of the vehicle on the penetration of penciclovir, a penciclovir formulation should be applied to the skin at pH 5 and another penciclovir formulation should be applied at the same pH as the penciclovir plus salicylic acid formulation i.e. pH 3. If the absorption of penciclovir was shown to be greater from the vehicle containing salicylic acid it would be necessary to show that this effect was due to the presence of the salicylic acid and not an artefactual effect due to the difference in pH of the formulation.

5.5.2 Topical Penciclovir and Salicylic Acid Formulations.

The drug solutions were prepared as for the 2% carboxymethylcellulose solutions, but with an initial carboxymethylcellulose solution of 2%, resulting in a 1% formulation following addition to the propylene glycol containing the penciclovir and salicylic acid. The final drug concentrations were 0.5% penciclovir and 2% salicylic acid. Three drug formulations were produced, two consisting of 0.5% penciclovir and one comprising 0.5% penciclovir plus 2% salicylic acid. One of the penciclovir formulations was applied to the skin at pH 5, and one at pH 3 (adjusted with acetic acid glacial), the penciclovir and salicylic acid formulation was pH 3.

5.5.3 Modification of the Drug Wells.

Preliminary studies were performed prior to this *in vivo* experiment to determine whether the drug solutions in 1% carboxymethylcellulose were likely to leak from the patches. These indicated that when the occlusive covering was applied over the patch and drug solution, the formulation that was in contact with the occlusive layer was attracted up towards the covering and away from the skin. The solution also appeared to be creeping between the surface of the patch and the occlusive cover. These phenomena resulted in the drug formulation being held away from the skin surface, so the drug was unavailable for absorption. To combat this problem it was thought that if the occlusive covering could be held away from the drug solution then it would remain in the well and so in direct contact with the skin.

A ridge of the Comfeel ulcer dressing was placed around the central well so as to elevate the area to which the occlusive covering was applied. It was then discovered that if the ridge was flush with the inner walls of the well, the drug solutions still crept up the sides and away from the skin. When the ridge was positioned at the outer edges of the drug patch, there was an increase in the overall rigidity of the patch. This resulted in the patch not being sufficiently pliable as to adopt the curvature of the arm and subsequently the edges of the patch lifted off the skin. This was not desirable, as this situation would allow movement of the drug formulation under the patch and may result in a high risk of contamination of the microdialysis probes as they entered and exited the skin. The ridge was then cut with a slightly larger inner diameter than the actual well, so that the edges were not flush (see Figure 42). The covering was applied to the ridge and the drug solution remained within the well, directly on the skin surface.

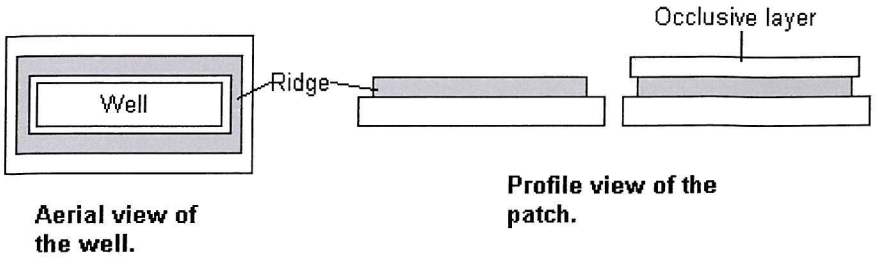


Figure 42 Diagram of the drug patch with the ridge of Comfeel around the central well to hold the occlusive layer.

5.5.4 Method.

The method used for probe insertion was as previously described. The perfusate consisted of 0.005mg/ml noradrenaline in Ringer's solution, perfused at a rate of 0.94μl/min through the probes. The probes contained wire in the lumen. The skin was allowed to recover for 90 minutes following probe insertion before application of the drug formulations.

In each well, 60μl of the drug solutions were placed and spread to cover the entire surface of the skin within the well. The patches were all occluded with a second piece of the Comfeel ulcer dressing. (3 subjects; female, aged 20-30 years, number of probes: 4 for each parameter).

5.5.5 Results and Discussion.

Penciclovir was not detected in any of the samples collected. The application of penciclovir in vehicles with different pH did not appear to influence penciclovir penetration.

It is possible that penciclovir could be interacting with the components of the vehicle, which may result in a decrease in the partitioning of the drug from the formulation into the skin. In this situation, an alteration of the vehicle would be required, as the formulation should be designed to promote partitioning of the drug into the stratum corneum.

Although the data have not been reported here, due to an inaccurate analytical method for salicylic acid, the approximate concentrations of salicylic acid recovered, following topical application of the three formulations, decreased in the following order:

propylene glycol/water > 1% carboxymethylcellulose/propylene glycol > 2% carboxymethylcellulose/propylene glycol.

This supports the theory that the compounds (both penciclovir and salicylic acid) may have formed interactions with the carboxymethylcellulose and were being held within the formulation.

5.6 *In Vivo* Absorption of Penciclovir with Salicylic Acid when Applied in Aquagel.

5.6.1 *Introduction.*

Since it appeared that the use of carboxymethylcellulose, used to thicken the vehicle, may have prevented the partitioning of the penciclovir into the skin, it became apparent that a different type of thickening agent was required.

Aquagel is an aqueous based gel which is generally used for lubrication purposes. It had been used previously as a vehicle in which to topically apply penciclovir with limited success (Morgan C., unpublished data). The thickening agent for this gel is carbomer.

5.6.2 Topical Penciclovir and Salicylic Acid Formulations.

Two drug formulations were produced in Aquagel, one consisting of 0.5% (w/w) penciclovir, and the second consisting of 0.5% (w/w) penciclovir plus 2% (w/w) salicylic acid. The pH of the penciclovir solution was 6, and the penciclovir and salicylic acid solution was 3, adjusted to 6 using ammonium hydroxide.

5.6.3 Method.

The microdialysis probes were inserted as previously described. The perfusion fluid consisted of 0.005mg/ml noradrenaline in Ringer's solution, and the flow rate was 0.94μl/min. The drug patches incorporated the ridges around the well, and the drug well had inner dimensions of 1.8 x 0.5cm. 60-80μl of the drug solutions were placed in each well, and was spread to cover the available skin. All of the patches were occluded. There was no evidence of creeping of the formulation under the patches, which were all still well

adhered to the skin at the end of the study. The formulation remained in the area to which it was applied following removal of the patches. (4 subjects; 3 male, 1 female, aged 20-25 years, 12 probes for each parameter).

5.6.4 Results and Discussion.

The results show that penciclovir was not detected in any of the samples. There was also no recovery of salicylic acid. A possible contributing factor to the lack of drug detected is that the formulations prepared in Aquagel did not contain propylene glycol. Propylene glycol promotes the release of drugs from the vehicle by altering the partitioning into the stratum corneum (Barry B. 1987). However, propylene glycol had been used in all of the previous studies, and penciclovir was not detected in significant amounts in any of them. The Aquagel may have interacted with the drugs and retained them in the vehicle rather than releasing them for partitioning into the skin.

5.7 Summary of the *In Vivo* Study of Penciclovir Absorption with Salicylic Acid.

There is some indication that salicylic acid acts as a penetration enhancer for the transdermal absorption of penciclovir *in vivo* (see Section 5.3). However, the data were derived from only one subject. The attempts to modify the topical formulation resulted in a decrease in the detection of both penciclovir and salicylic acid, providing evidence that the formulations selected were not favourable to the partitioning of these drugs into the skin.

In order to assess the full benefits of the co-application of salicylic acid with penciclovir, then a more traditional approach to the assessment of drug absorption was required, the use of diffusion cells.

5.8 *In Vitro* Absorption of Penciclovir with Salicylic Acid Across Excised Full-Thickness Rat Skin.

5.8.1 Introduction.

Excised rat skin is frequently used in *in vitro* studies to assess the percutaneous absorption of exogenous compounds (Wester R.C. and Maibach H.I. 1993). Experimental studies using laboratory rodents are often more convenient than using larger animals, such as the pig, due to the easier handling of the animals and reduced costs. However, absorption studies using rat skin may not be reliable predictors of human percutaneous absorption, and care should be taken when extrapolating the information to human exposure.

For many compounds the absorption across rat skin is greater than across human skin *in vitro*. The differences in absorption are not consistent between compounds and absorption across rat skin ranges from between 1 to 9 times greater than across human skin. These include benzyl acetate (Garnett A. *et al* 1994), mannitol and paraquat (Scott R.C. *et al* 1991), haloprogin, N-acetylcysteine, cortisone, testosterone and caffeine (Bartek M.J. *et al* 1972) and ethyl-1,3-hexanediol (Frantz S.W. *et al* 1995). Compounds that exhibit similar absorption across excised rat and human skin, include 2-nitro-p-phenyldiamine (Yourick J.J. and Bronaugh R.L. 2000) and coumarin (Beckley-Kartey S.A.J. *et al* 1997). Alternatively, some compounds such as 4,4-methylene-bis(2-chloroaniline) and methylenedianiline show greater absorption across excised human skin than across rat skin (Hotchkiss S.A.M. *et al* 1993).

There are some obvious structural differences between rat and human skin that may account for some of the differences in absorption of compounds. Generally, variations in permeability between rat and human skin are attributable to differences in skin thickness, the degree of hairiness of the skin and sweating. For example, the skin of the rat is abundant in hair follicles but lacks sweat glands (Al-Saidan S.M. *et al* 1998). The table below shows some of the reported parameters of the two types of skin.

Skin type	Stratum corneum thickness (μm)	Epidermal thickness (μm)	Whole skin thickness (mm)	Hair follicle density (per cm^2)
Rat (abdominal)[a]	18.4	32.1	2.09	289
Rat (dorsal)[b]	16.4	18.6	0.82	7930
Human (abdominal)[b]	19.2	25.5	>2.00	6.3

Table 5 Comparison between reported values of human and rat skin, showing the values for rat abdominal skin (female, Osborne-Mendel, age 10-20 weeks)[a] (Bronaugh R.L *et al* 1982) and for rat dorsal skin (male, Wistar, age 28 days)[b] (Scott R.C. *et al* 1991) and human abdominal skin (male (age 22-71) and female (age 13-75))[b] (Scott R.C. *et al* 1991).

It has been proposed that differences in absorption between human and rat skin may also arise from differences in the lipid region of the stratum corneum. The transition temperatures attributable for lipid melting (T2 and T3, see Section 1.1) (Barry B. 1991b), were found to be higher in human stratum corneum samples than in rat stratum corneum. This may imply that the lipid molecules in the human stratum corneum are more highly ordered and closely packed that those found in the stratum corneum of the rat. This would result in a more stable barrier, exhibiting greater impermeability in the human skin (Al-Saidan S.M. *et al* 1998).

Generally, although rat skin is often used for preliminary drug absorption studies, it cannot be considered to be a good model to predict absorption in humans. This is mainly due to the large number of hair follicles present in the skin, and possibly to the differences in skin thickness (Ritschel W.A. *et al* 1989).

In this study, rat skin was used to determine the effect of the formulation on the absorption of penciclovir across the skin, and the possibility of modification to enhance the absorption of the drug. The use of excised rat skin in Ussing diffusion chambers allowed a more reliable, reproducible and economic determination of the absorption a penciclovir, than *in vivo* human studies.

5.8.2 Topical Penciclovir and Salicylic Acid Formulations.

The formulations consisted of 0.5% (w/v) penciclovir dissolved in propylene glycol, and 0.5% (w/v) penciclovir plus 2% (w/v) salicylic acid dissolved in propylene glycol. The pH of the penciclovir formulation was 5, and the penciclovir : salicylic acid formulation was pH 3, which was adjusted to pH 5 with ammonium hydroxide solution.

5.8.3 Method.

The skin samples were mounted in the Ussing chambers, as described in section 2.12. The donor chambers were filled with the drug formulations (approximately 1ml in each). The total volume of receptor fluid was collected at 0, 6, 24 and 48 hours. The number of skin samples used was; abdominal skin: 9 penciclovir and 8 penciclovir plus salicylic acid, dorsal skin: 6 penciclovir and 6 penciclovir plus salicylic acid.

5.8.4 Results of Skin Thickness Measurements.

The thickness of the skin used for the *in vitro* absorption studies was determined using calipers with a vernier scale. Fifty samples of abdominal skin and fifty samples of dorsal skin were measured. The average thickness, ± one standard deviation, of the abdominal skin was 0.69 ± 0.15mm, and of the dorsal skin was 1.22 ± 0.21mm.

5.8.5 Results and Discussion.

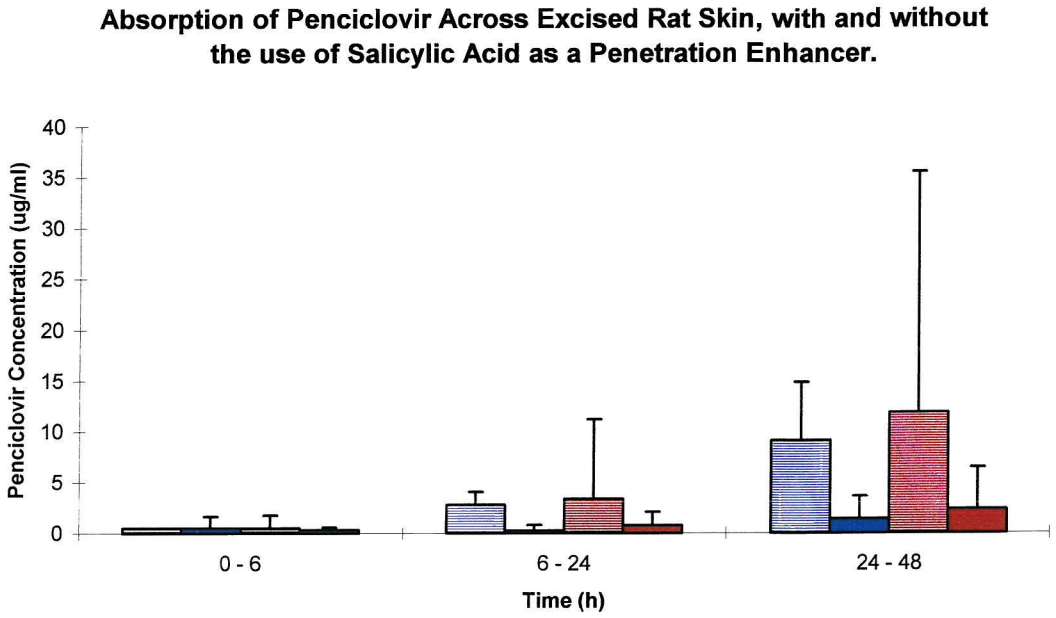


Figure 43 Comparison of the mean absorption of penciclovir, + one standard deviation, across excised rat skin when applied to the skin alone (■) and with salicylic acid (■). The striped columns represent absorption across the abdominal skin and the solid columns show absorption across the dorsal skin. Abdominal skin samples, 9 penciclovir and 8 penciclovir and salicylic acid, dorsal skin samples, 6 penciclovir and 6 penciclovir and salicylic acid.

It was apparent that there was a difference in absorption of penciclovir between the abdominal and the dorsal skin of the rat, with greater absorption seen across the thinner abdominal skin (see Figure 43). It is interesting to note that the degree of hairiness of the skin did not over-ride the effect of skin thickness for penciclovir absorption, since absorption was greater across the skin with the least number of hair follicles per cm^2 (see Table 5).

The results demonstrate that the transdermal penetration of penciclovir was slightly, but not significantly, enhanced when it was applied to the skin in conjunction with salicylic acid. The data exhibited a trend of a slight increase in penciclovir absorption in the presence of salicylic acid, and also showed the importance of the thickness of the skin. There was also a large degree of variation of absorption between skin samples of the same skin type.

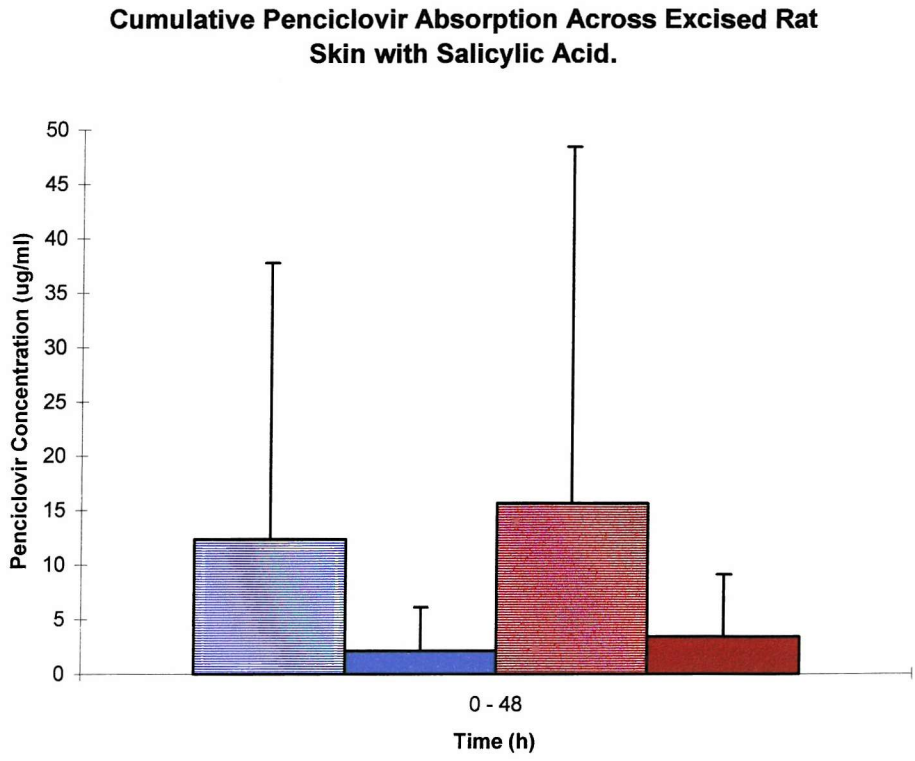


Figure 43a Cumulative penciclovir absorption across excised rat skin with salicylic acid, mean + one standard deviation. Penciclovir alone (■) and with salicylic acid (■). The striped columns represent absorption across the abdominal skin and the solid columns show absorption across the dorsal skin. Abdominal skin samples, 9 penciclovir and 8 penciclovir and salicylic acid, dorsal skin samples, 6 penciclovir and 6 penciclovir and salicylic acid.

These results indicated that an alternative penetration enhancer should be investigated for the promotion of the transdermal absorption of penciclovir, as salicylic acid did not enhance penetration of this drug to any significant degree.

Chapter 6

Effect of Oleic Acid on the Transdermal Penetration of Penciclovir

6. Effect of Oleic Acid on the Transdermal Penetration of Penciclovir.

6.1 Introduction.

Oleic acid is a mono-unsaturated fatty acid, which is found abundantly in nature, in most animal and vegetable fats, especially olive oil. The molecule consists an 18-carbon chain with a *cis*-double bond half way along its length.

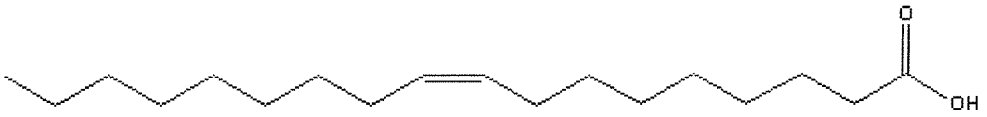


Figure 44 Structure of Oleic Acid.

Oleic acid is a known penetration enhancer for many compounds including aciclovir (Cooper E. *et al* 1985 and Loftsson T. *et al* 1989), salicylic acid (Golden G.M. *et al* 1987), cyanophenol (Mak V.H. *et al* 1990), hydrocortisone, nitroglycerin (Loftsson T. *et al* 1989), 5-fluorouracil, triamcinolone, acetonide, oestradiol (reviewed in Murakami T. *et al* 1998) and piroxicam (Francoeur M. L. *et al* 1990).

Fatty acids contribute a substantial proportion to the stratum corneum lipids, with a balance of saturated and unsaturated fatty acids. The majority of the stratum corneum fatty acids are saturated, and form closely packed lipid bilayers (Wiechers J.W. 1989). A change in the degree of saturation in the stratum corneum may influence the bilayer structure (Tanojo H. *et al* 1998). The barrier function of the stratum corneum is based on the molecular architecture of the lipids (Jiang S. *et al* 2000). Increases in the temperature or hydration of the stratum corneum can cause an increase in the fluidity of the stratum corneum lipids, and also induce changes in the permeability of the stratum corneum (Golden G.M. *et al* 1987 and Mak V.H. *et al* 1990b).

This suggests that there is a link between the fluidity of the stratum corneum lipids and transdermal flux.

Oleic acid partitions easily into the stratum corneum (Mak V.H. *et al* 1990) and induces structural disordering of the stratum corneum lipids (Tanojo H. *et al* 1998, Barry B. 1987, Taguchi K. *et al* 1999, Ongpipattanakul B. *et al* 1991, Takeuchi Y. *et al* 1993, Mak V.H. *et al* 1990, Mak V.H. *et al* 1990b, Francoeur M. L. *et al* 1990). A second response to the partitioning of oleic acid into the stratum corneum may be to allow more water to enter the tissue, which would result in an increase in the water volume between the lipid layers of the intercellular space (Barry B. 1991).

The presence of oleic acid in the stratum corneum results in a lowering of the lipid transition temperature (T2) of the stratum corneum, which induces an increase in the conformational freedom of the endogenous alkyl chains. This results in an increase in the fluidity of the stratum corneum lipids (Barry B. 1991b and Ongpipattanakul B. *et al* 1991). The decrease in the lipid transition temperature correlates with the amount of oleic acid that is taken up into the stratum corneum (Ongpipattanakul B. *et al* 1991).

The lipid disordering effects of oleic acid appear have a rapid onset with stratum corneum lipid changes seen after five minutes of application in rat skin (Takeuchi Y. *et al* 1993). When oleic acid was used to enhance the transdermal absorption of propylene glycol, the maximal flux of propylene glycol into the skin was seen after two hours, suggesting that the uptake of oleic acid and propylene glycol had approached saturation (Takeuchi Y. *et al* 1993 and 1995). The effects of oleic acid may be sustained over long periods, even after removal of the topical formulation (Mak V.H. *et al* 1990).

The uptake of oleic acid into the stratum corneum depends on the concentration of oleic acid in the topical formulation and on the duration of application to the skin (Jiang S. *et al* 2000, Takeuchi Y. *et al* 1993, Takeuchi Y. *et al* 1995, Mak V.H. *et al* 1990 and 1990b). Oleic acid uptake into human skin is linear with increasing concentration of oleic acid in the formulation, up to a concentration of 10% (Mak V.H. *et al* 1990b). The degree of lipid disordering also increases when the oleic acid concentration increases from 0.5% to 1%, but an increase in concentration from 1% to 10% does not result

in additional lipid disruption (Mak V.H. *et al* 1990b). Therefore, the lipid disordering effects of oleic acid reach a maximum (Mak V.H. *et al* 1990), despite the fact that oleic acid continues to be taken up into the stratum corneum. The maximum lipid disordering effects of oleic acid occur at concentrations of approximately 5-10ug/mg of stratum corneum. When oleic acid is present in the stratum corneum above this concentration, it is possible that the oleic acid forms a separate phase within this skin layer, and so does not further perturb the lipid domain ((Mak V.H. *et al* 1990b and Naik A. *et al* 1995). Further evidence for this proposal came from Ongpipattanakul B. *et al* (1991), who observed that oleic acid was disordered at temperatures when the stratum corneum lipids were ordered. This suggested that the oleic acid exists as a liquid within the stratum corneum lipids. It has since been observed that the lipid disordering effects of oleic acid are limited to the superficial layers of the stratum corneum, but that oleic acid present in liquid phase throughout all of the levels of the stratum corneum (Naik A. *et al* 1995).

The co-existence of ordered stratum corneum lipids and fluid oleic acid at physiological temps gave rise to the proposal of a phase-separation mechanism for the enhanced diffusion of polar compounds across the stratum corneum. The mechanism describes the formation of permeability defects at the liquid-solid interfaces within the lipid bilayers of the stratum corneum, which result in a decrease in the diffusional pathlength of the compound and/or a reduction in resistance to diffusion (see Figure 45) (Barry B. 1991b, Ongpipattanakul B. *et al* 1991 and Naik A. *et al* 1995).

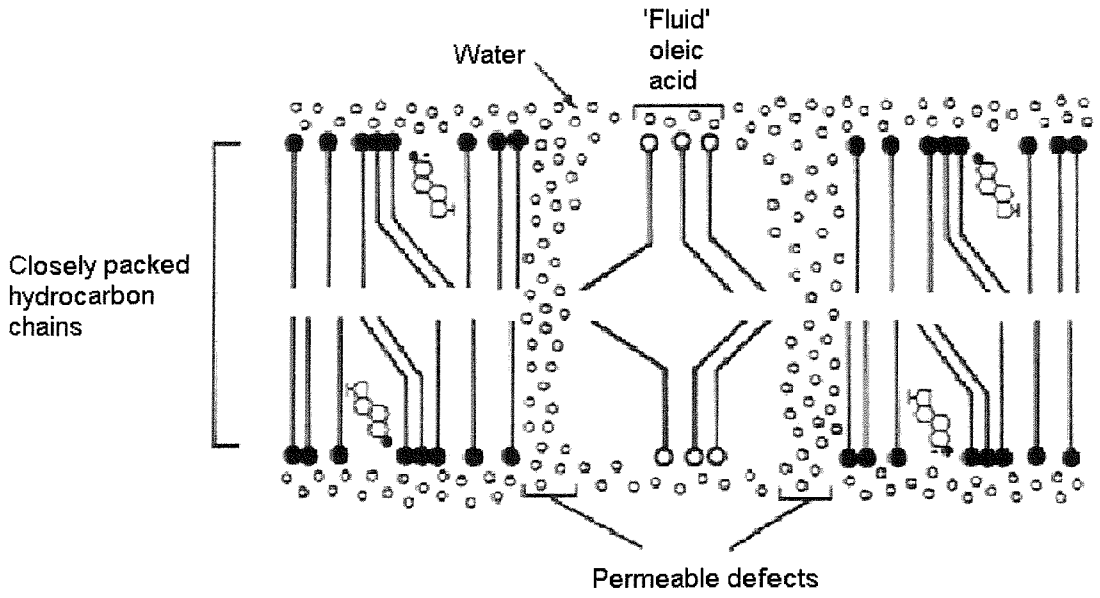


Figure 45 Schematic representation of the permeable defects formed in the stratum corneum lipid bilayers by oleic acid (Barry B. 1991b).

The unsaturated fatty acids with a *cis*-double bond result in a greater degree of lipid disordering and drug flux than their corresponding trans-unsaturated fatty acids or saturated fatty acids (Golden G.M. *et al* 1987 and Francoeur M.L. *et al* 1990). It has been reported that the maximum increase in fluidity induced in the stratum corneum lipids is seen when the *cis*-double bond is at the centre of the alkyl chain (Golden G.M. *et al* 1987 and Taguchi K. *et al* 1999). However, Tanojo H. *et al* (1997) reported that a range of octadecenoic acids with the *cis*-double bond in positions 6, 9, 11 and 13 all enhanced the absorption of p-aminobenzoic acid to a similar extent. They concluded that it was the presence of the *cis*-double bond, rather than its position, that accounted for the enhancement capabilities. This theory was supported by Takeuchi Y. *et al* (1998) who reported that *cis*-12-octadecenoic and *cis*-9-octadecenoic acids (oleic acid) caused the disordering of the stratum corneum lipids to a similar extent.

Oleic acid readily penetrates into the stratum corneum, but has limited movement into the aqueous regions of the viable epidermis and dermis (Taguchi K. *et al* 1999). *In vitro* diffusion studies showed that oleic acid did

not penetrate through human stratum corneum after 20 hours (reviewed in Tanojo H. *et al* 1999). Oleic acid applied to the skin causes mild irritation effects, comparable with other unsaturated fatty acids (Tanojo H. *et al* 1998 and Stillman M.A. *et al* 1975). A topical formulation of 0.16 M oleic acid in propylene glycol caused a two-fold increase in skin blood flow and three times more irritation than when propylene glycol was applied alone to the skin (Tanojo H. *et al* 1999). The increases in TEWL (transepidermal water loss) showed a similar duration to that of the irritation effects, indicating a possible link between the perturbation of the skin barrier and irritation by oleic acid.

Oleic acid is particularly effective as a penetration enhancer when used in conjunction with propylene glycol, with which it acts synergistically to facilitate the diffusion of compounds through the skin (Tanojo H. *et al* 1999, Taguchi K. *et al* 1999, Murakami T. *et al* 1998, Tanojo H. *et al* 1998, Takeuchi Y. *et al* 1995, Takeuchi Y. *et al* 1993 and Barry B. 1987).

Propylene glycol appears to increase transdermal absorption by the solvation of alphakeratin in the corneocytes of the stratum corneum (Takeuchi Y. *et al* 1993 and Barry B. 1987). This denaturation of the protein causes the loss of the secondary, tertiary and quaternary structures of the proteins, and may result in enhanced intracellular drug motility (Barry B. 1987 and Goldsmith L. 1978). Propylene glycol may also occupy hydrogen-bonding sites in the stratum corneum, thus preventing tissue bonding with the penetrating compound and so enhancing permeation (Barry B. 1987 and Bendas B. *et al* 1995). Propylene glycol may also aid the movement of oleic acid into the stratum corneum lipid domain by altering the lipid head group packing. Oleic acid appears to also promote the penetration of propylene glycol (Barry B. 1987, 1991 and Murakami T. *et al* 1998). Propylene glycol does not appear to disorder the stratum corneum lipids above that provided by water (see Section 1.2) (Barry B. 1987 and Jiang S. *et al* 2000) and was shown not to cause an increase in TEWL (Tanojo H. *et al* 1998).

6.2 *In Vitro* Absorption Across Excised Rat Skin of Penciclovir Combined with Oleic Acid in Propylene Glycol.

6.2.1 Introduction.

Oleic acid has been shown to enhance the absorption of aciclovir across human skin *in vitro* (Cooper E. *et al* 1985) and across hairless mouse skin *in vitro* (Loftsson T. *et al* 1989). There was an increase in absorption of 138-fold across hairless mouse skin when aciclovir was topically applied in propylene glycol and 2% oleic acid, compared to when applied in propylene glycol alone. Cooper E. *et al* (1985) demonstrated a 9-fold increase in aciclovir absorption when applied to excised human skin in propylene glycol and 1% oleic acid over a 24 hour period (aciclovir concentration of 0.25%), and an increase in absorption of 55-fold when the concentration of oleic acid in the propylene glycol was increased to 5%.

Penciclovir has a similar structure to that of aciclovir and would be expected to have comparable transdermal absorption characteristics (Boyd M. *et al* 1988). It was supposed that the absorption of penciclovir would be enhanced by the addition of oleic acid to the topical formulation.

6.2.2 Topical Formulation in Propylene Glycol.

Three topical formulations were produced, in 100% propylene glycol; penciclovir alone, penciclovir plus 1% oleic acid and penciclovir plus 5% oleic acid. The penciclovir was dissolved in propylene glycol at a concentration of 0.5% (w/v), and the oleic acid was added subsequently in concentrations of 1% and 5% (v/v). The three formulations were pH 5.

6.2.3 Method.

The skin samples were mounted in the Ussing chambers, as described in section 2.12. The donor chambers were filled with the drug formulations, approximately 1ml put in each chamber. The total volume of receptor fluid was collected at 0, 6, 24 and 48 hours. The number of skin samples was; abdominal skin: 7 penciclovir, 7 penciclovir plus 1% oleic acid and 7 penciclovir plus 5% oleic acid, dorsal skin: 8 penciclovir, 8 penciclovir plus 1% oleic acid and 7 penciclovir plus 5% oleic acid.

6.2.4 Results and Discussion.

Figure 46 shows the average concentrations of penciclovir detected in the receptor fluid.

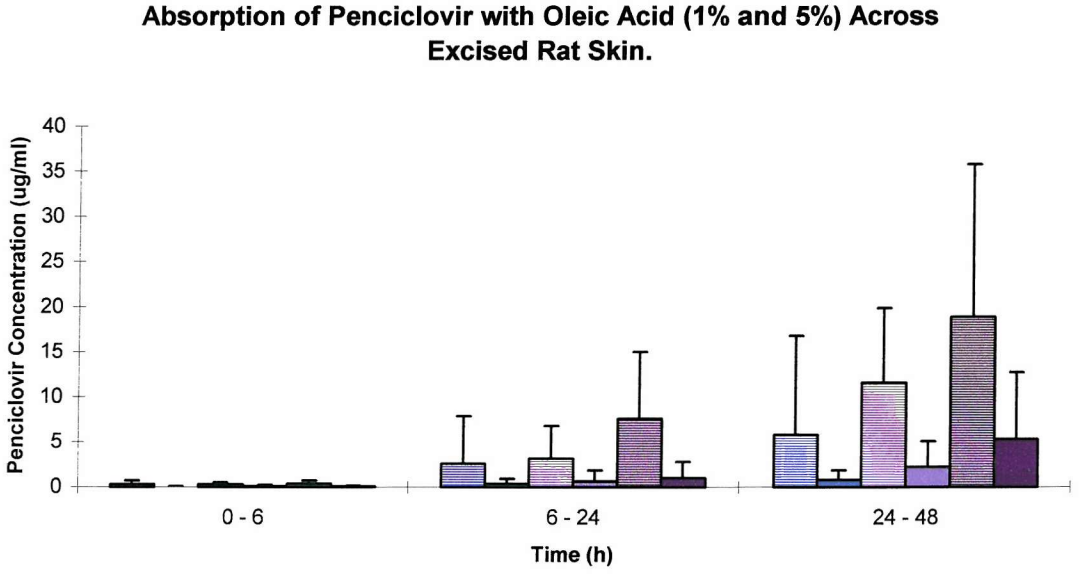


Figure 46 Absorption of penciclovir across excised rat skin, mean + one standard deviation. Penciclovir alone (■), penciclovir plus 1% oleic acid (■) and penciclovir plus 5% oleic acid (■). The striped columns show absorption across abdominal skin, solid columns represent absorption across dorsal skin. Formulation: 100% propylene glycol. Number of skin samples; seven for each formulation using abdominal skin, eight samples for penciclovir and penciclovir plus 1% oleic acid using dorsal skin and seven for penciclovir plus 5% oleic acid (dorsal skin).

The data show that the absorption of penciclovir across excised rat skin was enhanced by the presence of oleic acid in the formulation. This was

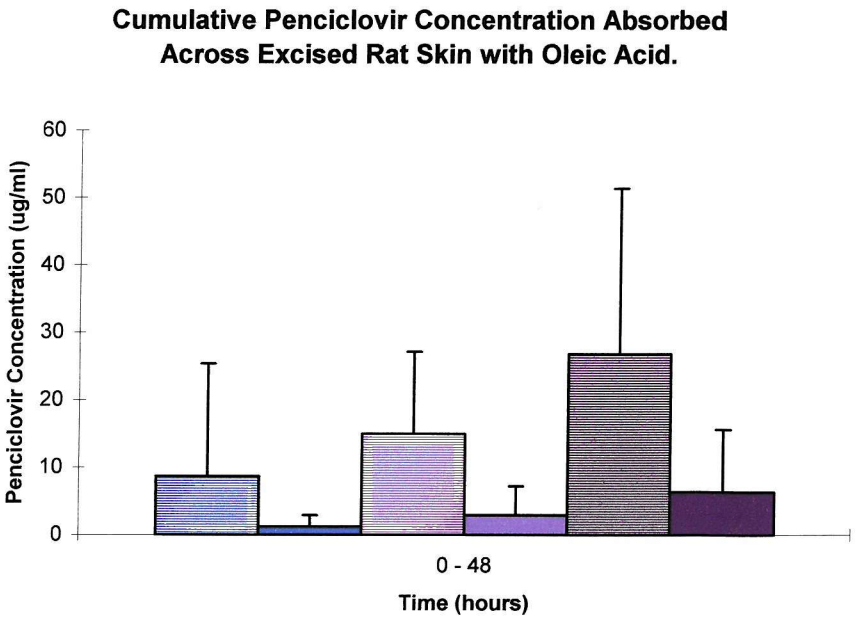


Figure 46a Cumulative penciclovir absorption across excised rat skin with oleic acid, mean + one standard deviation. Penciclovir alone (■), penciclovir plus 1% oleic acid (■) and penciclovir plus 5% oleic acid (■). The striped columns show absorption across abdominal skin, solid columns represent absorption across dorsal skin. Formulation: 100% propylene glycol. Number of skin samples; seven for each formulation using abdominal skin, eight samples for penciclovir and penciclovir plus 1% oleic acid using dorsal skin and seven for penciclovir plus 5% oleic acid (dorsal skin).

a concentration dependent enhancement, with increased penciclovir absorption associated with increasing concentrations of oleic acid in the vehicle. However, the increases in penciclovir absorption were not significant, ($p > 0.05$) and the results showed a high degree of variation between absorption across different skin samples, both from the same animal and from different animals.

In all cases, including when penciclovir was applied to the skin alone (i.e. without oleic acid), the rate of absorption increased with time. The amount absorbed per hour across the skin was greater with each successive collection time-point. The ratio of penciclovir absorption plus oleic acid, compared to absorption without oleic acid, also increased with time, for example, for the penciclovir plus 5% oleic acid formulation, the absorption was 1.3 times greater per hour than absorption from penciclovir alone in the first 6 hours, which then rose to a 3.3-fold increase per hour for the 24-48 hour time-point in abdominal skin. This observation was true when 1% oleic acid was used, and was apparent in both abdominal and dorsal skin. Parry G.E. *et al* (1992) demonstrated that aciclovir absorption across excised human skin showed a sharp rise after 48 hours and proposed that this may have been the result of drug or vehicle induced changes in the stratum corneum. The data suggests that the degree of partitioning of oleic acid from the vehicle into the skin may be continuous throughout the 48 hour period of study, thus causing increased oleic acid concentrations in the stratum corneum, and so promoting further penciclovir absorption.

The absorption was greater across the thinner abdominal skin, than across the dorsal skin. The dorsal skin of the rat exhibits a greater number of hair follicles per cm^2 than the abdominal skin (see Table 5, Section 5.8). However, the absorption of penciclovir across the dorsal skin was much less than across abdominal skin. This is likely to be related to the skin thickness, being 1.8 times greater in dorsal than abdominal skin. This indicates that the thickness of the layer of skin that the penciclovir has to cross is more important than the number of hair follicles per unit area of the skin, which suggests that the trans-follicular route is probably not a prominent pathway for penciclovir penetration.

These results are consistent with a study performed by Cooper E.R. *et al* (1985) who demonstrated *in vitro* absorption of aciclovir across human skin. The concentrations of aciclovir detected were lower than the penciclovir concentrations observed in this study, but this is probably due to the use of human skin by Cooper E.R. *et al* (1985).

6.3 Absorption Across Excised Rat Skin of Penciclovir Combined with Oleic Acid in an Aqueous Cream/ Propylene Glycol Formulation.

6.3.1 Introduction.

In the clinical use of topical penciclovir, the drug is applied to the lesion in a cream base. The study described in Section 6.2 showed absorption from a propylene glycol only vehicle. It was important to determine whether a similar degree of absorption would be seen from a cream based formulation.

6.3.2 Topical Formulation in Aqueous Cream.

Three topical formulations were produced; penciclovir, penciclovir plus 1% oleic acid and penciclovir plus 5% oleic acid. The penciclovir was dissolved in propylene glycol at a concentration of 1% (w/v). An equal amount of aqueous cream to propylene glycol (w/v) was added to the formulation, resulting in a penciclovir concentration of 0.5%. The formulations were warmed gently in a water-bath until the aqueous cream was molten and became completely mixed with the propylene glycol. The oleic acid was added subsequently, in concentrations of 1% and 5% (v/v). The three formulations were pH 5.

6.3.3 Method.

The skin samples were mounted in the Ussing chambers, as described in Section 2.12. The donor chambers were filled with the drug formulations while the formulations were still molten, approximately 1ml in each chamber. The formulations solidified once in place in the chambers. The total volume of receptor fluid was collected at 0, 6, 24 and 48 hours. The number of skin samples used were; abdominal skin: 6 of each formulation, dorsal skin: 10

penciclovir, 7 penciclovir plus 1% oleic acid and 8 penciclovir plus 5% oleic acid.

6.3.4 Results and Discussion.

Figure 47 shows the average concentration of penciclovir absorbed across excised rat skin from the aqueous cream/propylene glycol formulation.

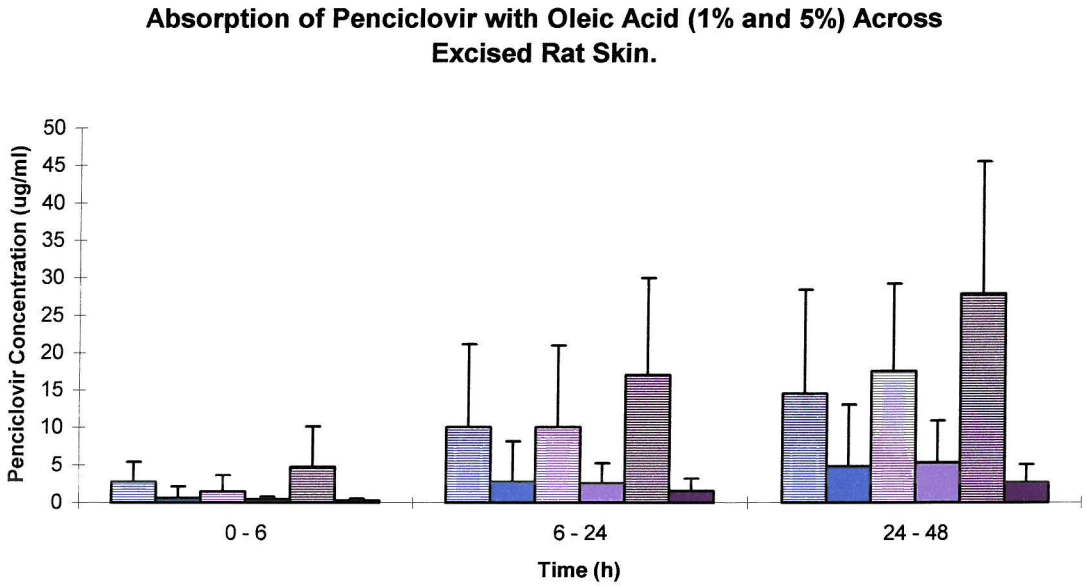


Figure 47 Absorption of penciclovir across excised rat skin, mean + one standard deviation. Penciclovir alone (■), penciclovir plus 1% oleic acid (■) and penciclovir plus 5% oleic acid (■). The striped columns show absorption across abdominal skin, solid columns represent dorsal skin. Formulation: 50% aqueous cream and propylene glycol. The number of skin samples were; six for each formulation for the abdominal skin, and ten samples for penciclovir, seven for penciclovir plus 1% oleic acid and eight for penciclovir plus 5% oleic acid for dorsal skin.

The first observation of these data is that the absorption of penciclovir across the skin was greater from the cream-based formulation than from the propylene glycol formulation. This was true for all three formulations and skin types with the exception of absorption across dorsal skin from the penciclovir plus 5% oleic acid formulation. This suggested that the aqueous

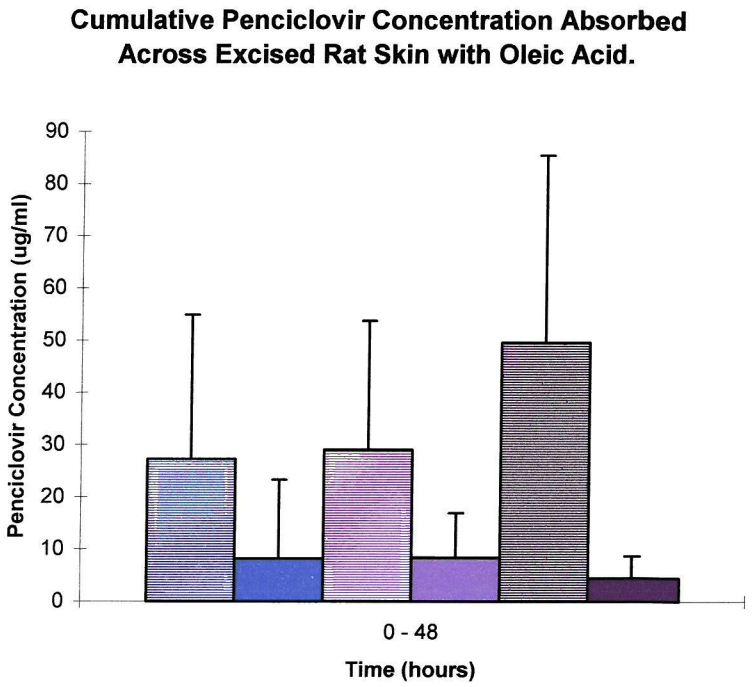


Figure 47a Cumulative penciclovir absorption across excised rat skin with oleic acid, mean + one standard deviation. Penciclovir alone (■), penciclovir plus 1% oleic acid (■) and penciclovir plus 5% oleic acid (■). The striped columns show absorption across abdominal skin, solid columns represent dorsal skin. Formulation: 50% aqueous cream and propylene glycol. The number of skin samples were; six for each formulation for the abdominal skin, and ten samples for penciclovir, seven for penciclovir plus 1% oleic acid and eight for penciclovir plus 5% oleic acid for dorsal skin.

cream/propylene glycol vehicle itself may promote transdermal absorption of penciclovir. It is possible that penciclovir was more soluble in this formulation, so increasing the fraction of drug available for partitioning into the stratum corneum. An increase in partitioning across the skin would be beneficial for the establishment of therapeutic concentrations in the basal layer of the epidermis, for the treatment of herpes virus infections.

However, the penetration enhancing effect of oleic acid appears to be reduced when using this cream-based formulation. In comparison to the absorption of penciclovir from the three formulations, the vehicle containing 1% oleic acid produced similar absorption to that from the penciclovir alone formulation. There was slightly greater absorption from the latter formulation in the first 6 hours of the study. The formulation containing 5% oleic acid did enhance the absorption of penciclovir across the abdominal skin by approximately 2-fold, but the enhancement remained constant throughout the study, and did not increase in degree of enhancement as was seen for the propylene glycol formulations. This indicates that the effect of oleic acid was not increasing, suggesting that the partitioning of the oleic acid from the vehicle may be reduced in degree or may have ceased entirely, resulting in a steady state level of oleic acid in the stratum corneum. This would result in a constant enhancement of penciclovir absorption, rather than the increasing enhancement that was observed from the propylene glycol formulation.

The penciclovir plus 5% oleic acid in the cream-based formulation did not enhance penciclovir absorption across dorsal skin. The absorption from the penciclovir alone formulation was approximately 2-fold greater than from the formulation containing 5% oleic acid. This was an unexpected result, as the absorption from the cream-based formulation containing 1% oleic acid gave similar rates of absorption to penciclovir alone across dorsal skin. Also, the 5% oleic acid formulation produced enhancement of penciclovir absorption across abdominal skin, so there was no reason to expect a reduction in absorption across the dorsal skin. Differences in the thickness of the skin and the stratum corneum, and degree of hairiness of the skin could not account for this reduction in penciclovir absorption. The skin used was comparable to the skin used in the other absorption studies, where penciclovir

absorption was shown to remain either constant (i.e. similar to absorption from the penciclovir alone formulation) or to increase due to the effect of oleic acid on the stratum corneum.

The formulation used on the dorsal skin was the same as was applied to the abdominal skin, which did show some degree of enhanced penciclovir absorption. This, too, cannot account for the decreased absorption.

The viscosity of the formulation may have contributed to a reduction in the partitioning of compounds into the skin, as the drug molecules may not have free movement through a very viscous cream (Wiechers J.W. 1989). This would cause a depletion of the compound from the layer closest to the skin, as the drug partitions from the vehicle into the stratum corneum, but there may be no movement of drug to replace the absorbed fraction. This phenomenon may have prevented the movement of the oleic acid to the skin surface and so result in a decrease in partitioning into the stratum corneum. However, the formulation used in this study was much less viscous than a cream, and was more like a thick lotion. If the viscosity of the formulation was having a hindering effect on the partitioning of oleic acid into the skin, this would also prevent movement of penciclovir into the stratum corneum, and decreased penciclovir absorption would be observed with time. This, however, was not the case, and with all three formulations the rate of absorption of penciclovir increased with time (with the exception of across dorsal skin from the penciclovir plus 5% oleic acid formulation). In light of this, it is unlikely that the viscosity of the formulation reduced the partitioning of oleic acid into the skin.

Chapter 7

The Effect of a Combination of the Penetration Enhancers, Oleic Acid and Salicylic Acid, on the Transdermal Absorption of Penciclovir

7. The Effect of a Combination of the Enhancers, Oleic Acid and Salicylic Acid, on the Transdermal Absorption of Penciclovir.

7.1 Introduction.

It was apparent, from the previous studies of penciclovir absorption across excised rat skin, that oleic acid caused the enhanced absorption of penciclovir, and that salicylic acid also caused a slight increase in its skin penetration. It was thought possible that if the two enhancers were applied together with penciclovir, then the stratum corneum lipid disrupting effects of oleic acid would promote the absorption of the penciclovir: salicylic acid ion-pair through the skin.

7.2 *In Vitro* Absorption of Penciclovir with Oleic Acid and Salicylic Acid across Excised Rat Skin.

7.2.1 Topical Formulation.

Two topical formulations were produced, in 100% propylene glycol; penciclovir alone, and penciclovir plus oleic acid and salicylic acid. The penciclovir and salicylic acid were dissolved in propylene glycol at a concentration of 0.5% penciclovir (w/v) and 2% salicylic acid (w/v), and the oleic acid was added subsequently in the concentration of 5% (v/v). The penciclovir formulation was pH 5, and the penciclovir: oleic acid: salicylic acid formulation was pH 3. This was adjusted to pH 5 with ammonium hydroxide solution.

7.2.2 Method.

The excised skin was mounted in the Ussing chambers as described in Section 2.12. The donor chambers were filled with the drug formulations, approximately 1ml in each chamber. The total volume of receptor fluid was collected at 0, 6, 24 and 48 hours. The number of skin samples used were; abdominal skin: 6 for both formulations, dorsal skin: 6 for each formulation.

7.2.3 Results and Discussion.

Figure 48 shows the average absorption of penciclovir across excised rat skin with oleic acid and salicylic acid.

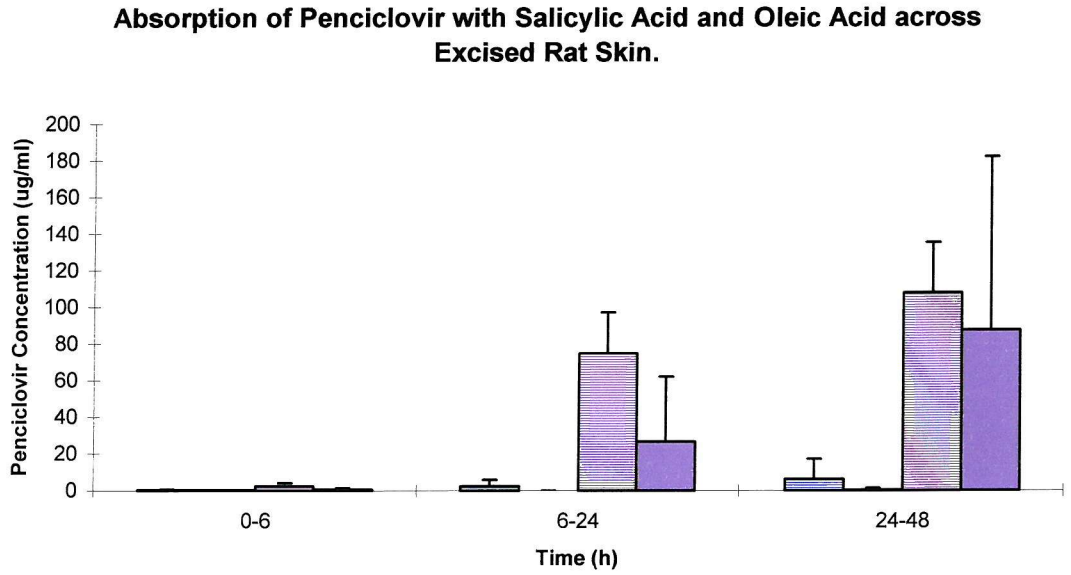


Figure 48 Absorption of penciclovir across excised rat skin, mean + one standard deviation. Penciclovir alone (■), penciclovir with oleic acid and salicylic acid (■). The striped columns show absorption across abdominal skin, solid columns represent absorption across dorsal skin. Formulation: propylene glycol. The number of skin samples; abdominal skin: 6 penciclovir and 6 penciclovir plus oleic acid and salicylic acid, dorsal skin: 6 for each formulation.

These data demonstrate that oleic acid plus salicylic acid when applied to the skin with penciclovir, greatly promoted penciclovir absorption across

excised rat skin, and that the two enhancers appear to act in a synergistic manner. The difference in absorption of penciclovir between the two formulations across abdominal skin was highly significant, $p < 0.001$. The absorption differences across dorsal skin between the formulations were also significant, $p = 0.05$. There was also a significant difference in penciclovir absorption from the penciclovir: oleic acid: salicylic acid formulation between abdominal skin and dorsal skin, $p = 0.05$.

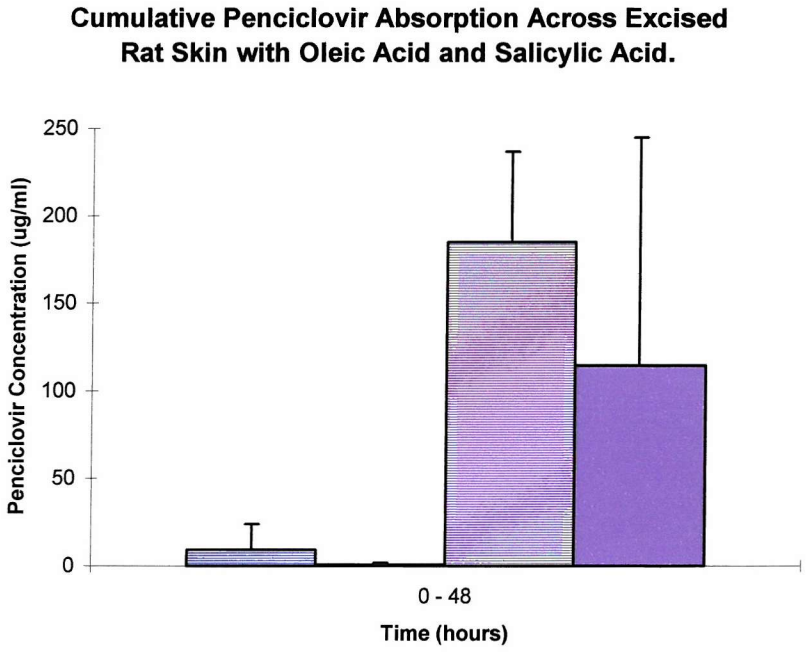


Figure 48a Cumulative absorption of penciclovir across excised rat skin with oleic acid and salicylic acid, mean + one standard deviation. Penciclovir alone (■), penciclovir with oleic acid and salicylic acid (■). The striped columns show absorption across abdominal skin, solid columns represent absorption across dorsal skin. Formulation: 100% propylene glycol. The number of skin samples; abdominal skin: 6 penciclovir and 6 penciclovir plus oleic acid and salicylic acid, dorsal skin: 6 for each formulation.

Due to the large enhancing effects of the combination of oleic acid and salicylic acid, it was decided that this formulation would be the most suitable vehicle to use in an *in vivo* study, to promote the absorption of penciclovir across intact human skin.

7.3 Summary of the Absorption of Penciclovir Across Excised Rat Skin.

Table 6 shows the summary of the average percent enhancement of penciclovir absorption found with each modified topical drug formulation.

Formulation	Skin type	0-6h	6-24h	24-48h
PCV + SA	Abdominal	-4.08	21.24	29.96
100% PG	Dorsal	-38.20	167.52	73.64
PCV + 1% OA	Abdominal	-13.33	22.32	99.68
100% PG	Dorsal	153.65	86.26	167.02
PCV + 5% OA	Abdominal	29.95	193.36	226.25
100% PG	Dorsal	171.37	184.16	543.14
PCV + 1% OA	Abdominal	-46.11	0.25	20.71
Aqueous cream	Dorsal	-34.65	-6.39	11.53
PCV + 5% OA	Abdominal	74.36	69.29	91.65
Aqueous cream	Dorsal	-62.33	-44.02	-45.21
PCV, OA + SA	Abdominal	505.22	2947.78**	1563.30**
100% PG	Dorsal	213.63	7462.70*	10612.22*

Table 6 Summary of the average percentage enhancement of penciclovir absorption from the modified formulations compared to absorption from the control formulation. Significance: * $p = 0.05$, ** $p < 0.001$.

The values in Table 6 represent a percent increase in absorption compared to control values. The control values were the amount of penciclovir absorbed from the appropriate topical formulation containing penciclovir alone, at the appropriate time points. Positive values in the table indicate that the absorption of penciclovir was enhanced by the modified

formulation. Negative values represent situations where absorption was greater from the formulation containing penciclovir alone.

The data in Table 6 demonstrates that, although most of the topical formulations studied did produce enhancement of penciclovir absorption across excised rat skin, most of the increases in absorption were not statistically significant, with the exception of the penciclovir: oleic acid: salicylic acid formulation.

7.4 *In Vivo* Absorption of Penciclovir with Oleic Acid and Salicylic Acid across Human Skin.

7.4.1 Introduction.

The results in Section 7.2 indicated that the formulation containing the enhancers oleic acid and salicylic acid would be the most suitable vehicle to use in which to topically apply penciclovir, to assess the absorption of penciclovir across human skin *in vivo*.

7.4.2 Topical Formulation in Aqueous Cream.

Two topical formulations were produced; penciclovir and penciclovir plus oleic acid and salicylic acid. The penciclovir and salicylic acid were dissolved in propylene glycol at concentrations of 1% (w/v) and 4% (w/v), respectively. An equal amount of aqueous cream to propylene glycol (w/v) was added to the formulation, resulting in a penciclovir concentration of 0.5% and a salicylic acid concentration of 2%. The formulations were warmed gently in a water-bath until the aqueous cream was molten and became completely mixed with the propylene glycol. The oleic acid was added subsequently, at a concentration of 5% (v/v). The penciclovir only formulation was pH 5, and the penciclovir: oleic acid: salicylic acid had a pH of 3. This was adjusted to pH 5 with ammonium hydroxide solution.

7.4.3 Method.

Six microdialysis probes were inserted into the skin as described in Section 2.5, using 23G needles as guide cannulae. The perfusate consisted Ringer's solution containing noradrenaline (0.005mg/ml), perfused at a rate of 0.94μl/min. The drug wells incorporated a ridge around the central well, as described in Section 5.5, having an inner diameter of 1.8 x 0.5cm. The drug

formulations were applied to the skin, filling each well (three for each formulation), 90 minutes after probe insertion. The drug wells were all occluded with a piece of the Comfeel ulcer dressing. (6 subjects; 3 male, 3 female, aged 20-28. Data available for 16 probes with each formulation).

7.4.4 Results and Discussion.

Figure 49 shows the average recovery of penciclovir through the microdialysis probes *in vivo*, following topical application with oleic acid and salicylic acid.

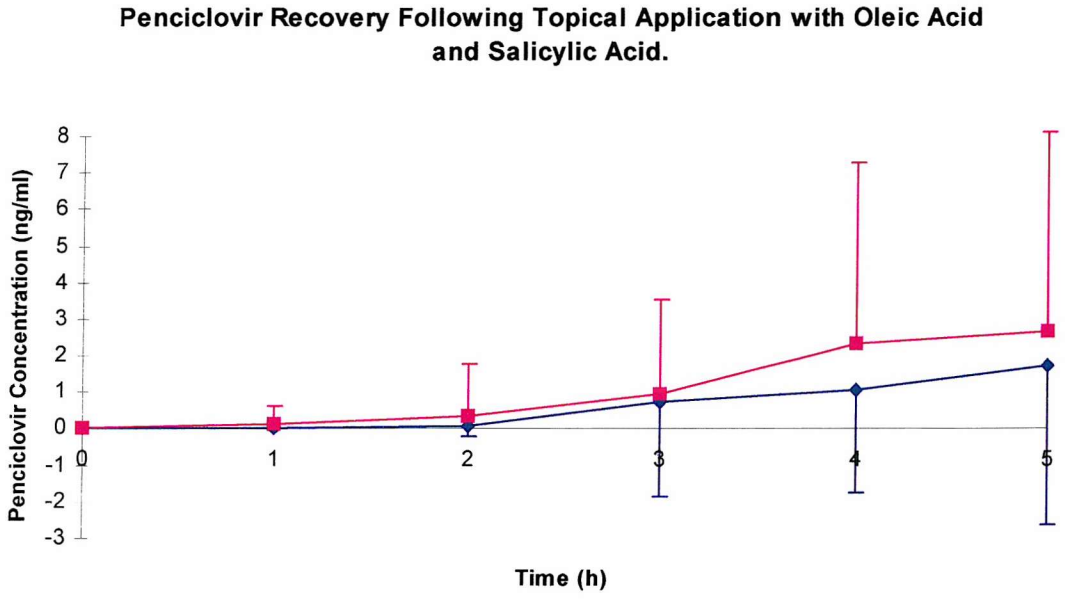


Figure 49 Comparison of mean penciclovir recovery, ± one standard deviation, when applied to the skin alone (—■—) and with oleic acid and salicylic acid (—■—). The results were the mean for 16 probes (6 subjects) for each formulation.

Although these data indicate that the presence of oleic acid and salicylic acid caused a slight increase in the recovery of penciclovir in the microdialysis probes, the results were highly variable and there was no significant difference in absorption of penciclovir across the skin from the two

different formulations (p = 0.46). It is also noteworthy that penciclovir was not detectable in 11 of the 16 probes for the penciclovir formulation and 12 of the 16 probes for the penciclovir: oleic acid: salicylic acid formulation. This means that penciclovir was detected in only 9 of the probes in total. The probes in which penciclovir was detected were from two of the subjects (both female). The remaining probe was from one other subject (male), but the concentrations of penciclovir detected were very low in the dialysate from this probe. There was no penciclovir detected in the remaining probes.

These results were not comparable to the concentrations observed in the *in vitro* study of penciclovir absorption across excised rat skin. A probable explanation is that the time course of the studies were different. However, this does not fully explain why penciclovir was not detected *in vivo* over this time course, as there were differences in penciclovir absorption due to the co-application of oleic acid and salicylic acid at 6 hours in the *in vitro* study (see Figure 50).

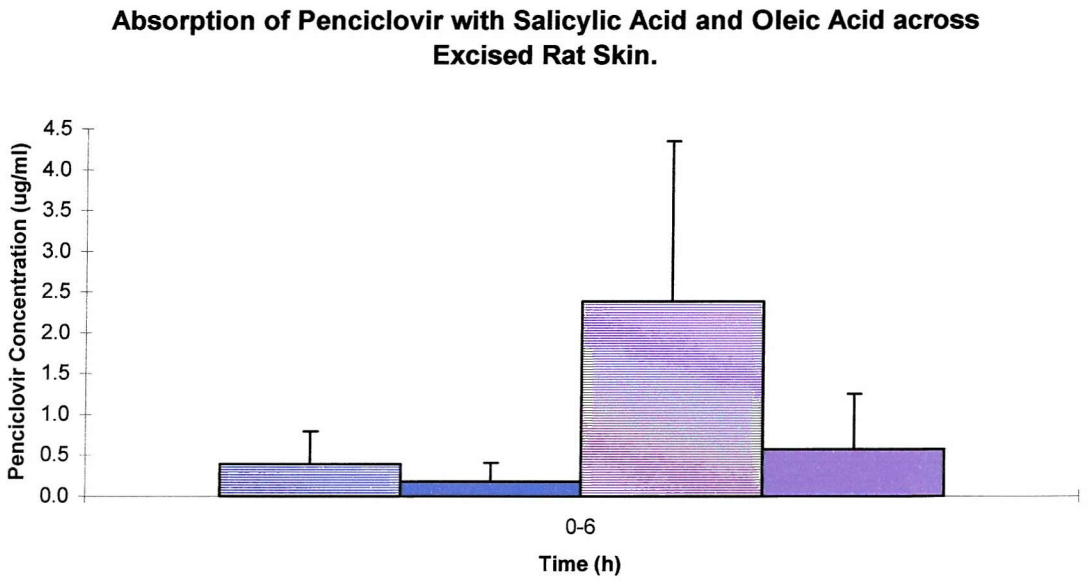


Figure 50 Absorption of penciclovir across excised rat skin, mean + one standard deviation. Penciclovir alone (■), penciclovir with oleic acid and salicylic acid (■). The striped columns show absorption across abdominal skin, solid columns represent absorption across dorsal skin. Formulation: 100% propylene glycol.

Figure 50 shows that there was a marked difference in absorption from the two formulations in the first six hours of the study. There was a 6-fold increase in penciclovir absorption across abdominal skin ($p < 0.05$) and a 3-fold increase across the dorsal skin (not significant), when oleic acid and salicylic acid were present in the topical formulation.

There are a number of factors that could account for the differences in penciclovir absorption between the human and the rat skin. The degree of hairiness of the skin may play an important role. The skin of the rat is densely covered in hair follicles compared to the skin of the human forearm (see Table 5). Diffusion down the hair follicles may contribute to the total absorption of penciclovir. An increase in the number of follicles would then result in an increase in the extent of absorption of the compound. However, it has already been suggested that the degree of hairiness of the rat skin was not as an important factor in penciclovir absorption as the thickness of the skin (see Section 6.2). It may become more important when comparing different types of skin, e.g. between rat and human skin, as the degree of hairiness of human skin is far less than that of rat skin. It may be that the penciclovir was using the transfollicular route of absorption in the rat skin, but that this was overshadowed by differences in the skin thickness. If this route predominates for penciclovir, then absorption across human skin would be expected to be very little.

Once the penciclovir has been absorbed into the skin, the effect of the dermal blood flow may become important. In the case of the excised rat skin studies, there was a complete absence of blood flow, however, in the *in vivo* human study, the dermal microcirculation could cause removal of penciclovir into the systemic circulation. This renders the drug unavailable for recovery by the microdialysis probe and so would not be detected in dialysate. The study used noradrenaline in the microdialysis perfusate to restrict the dermal blood flow in order to minimise this effect, but the noradrenaline does not reduce the blood flow to zero and there would still be some residual blood flow that could affect the dermal drug concentrations around the microdialysis probe.

It is possible that the effect of topically applied oleic acid could have caused mild skin irritation which may have caused an increase in dermal blood flow. If this were the case, then an increased dermal clearance of topically absorbed penciclovir would result in a decrease in recovery by microdialysis and very low or undetectable concentrations of the drug would be found in the dialysate. However, the noradrenaline present in the perfusate caused visible blanching of the skin, demonstrating dermal vasoconstriction. This blanching was still visible at the site of probe insertion at the end of the *in vivo* study, indicating that oleic acid did not cause a noticeable increase in dermal blood perfusion.

The thickness of the skin layer has been shown *in vitro* to have an effect on the transdermal absorption of penciclovir, with lower concentrations of the drug detected with thicker skin. This may reflect the time that the drug takes to cross the skin layer, with shorter diffusion times through thinner skin. Human skin is generally thicker than rat skin (see Table 5) and this may affect the concentrations of drug available for recovery in the dermis. However, the design of these experiments (*in vitro* and *in vivo*) was different. In the *in vitro* studies, the penciclovir had to cross the entire thickness of the skin and enter the receptor fluid, before the drug was considered to be absorbed. *In vivo*, a compound must only reach the upper dermal capillary plexus and enter the systemic circulation to be considered absorbed. This difference may reflect over 1mm in distance between the two experiments. In the case of *in vivo* microdialysis, the penciclovir concentration in the tissue was sampled in the dermis, below the upper capillary plexus. The average depth of the probes in the skin was 0.62 $\pm$ 0.14mm. This distance below the skin surface was comparable with the thickness of the abdominal skin used in the *in vitro* absorption studies. It was expected that penciclovir would be detected in the skin at this depth following topical application of the drug with the penetration enhancers oleic acid and salicylic acid.

In summary, the *in vivo* study did not produce the results that were expected from the *in vitro* experiments, i.e. that of enhanced penciclovir recovery by the microdialysis probes due to the increased transdermal absorption following topical application with oleic acid and salicylic acid.

However, it is possible that this topical formulation could have clinical benefit for the treatment of cold sores. The *in vitro* study using excised rat skin showed that over extended periods of time, up to 48 hours, the absorption of penciclovir was significantly increased from the modified formulation. If this were to be used clinically, the cream would be applied to the lesion every 2 hours for 5-7 days. In this situation, with repeated and extended application of the drug, it is likely that sufficient concentrations of penciclovir would penetrate the skin and reach therapeutic levels in the basal epidermis.

Chapter 8

Delivery and Recovery of Penciclovir *In Vivo* Using Microdialysis

8. Delivery and Recovery of Penciclovir *In Vivo* Using Microdialysis.

8.1 Dual Probe Study.

8.1.1 Aim.

To assess the diffusion of penciclovir in the skin following dermal delivery via microdialysis, and to determine the delivery of penciclovir *in vivo* with altered dermal blood flow.

8.1.2 Introduction.

This study was designed to investigate the influence of the dermal blood flow on the removal of penciclovir from the skin into the systemic circulation. It has been demonstrated that the transdermal delivery of penciclovir is too low and too variable for this to be an appropriate route of drug delivery for the investigation of this factor. To overcome this, and to allow maximal input of penciclovir into the tissue, the drug was delivered into the skin using the microdialysis technique.

This novel approach to assess the dermal handling of a compound involved the insertion of two probes close together in the skin. One probe in each pair was perfused with a solution of penciclovir (the delivery probe), the principle being, that the drug diffused out of this probe and into the tissue. The extent of diffusion of penciclovir through the skin was assessed by the second probe in each pair (the recovery probe), which was positioned at a variable distance from the delivery probe. This recovery probe was perfused with Ringer's solution, with or without noradrenaline to modify the blood flow. The presence of the noradrenaline should have the effect of restricting the dermal blood perfusion. It was thought that the reduction in blood flow would reduce the removal of penciclovir from the skin into the systemic circulation.

In turn this would cause an increase in the concentration of the drug in the skin, and may have the effect of allowing the compound to diffuse through the skin to a greater extent. It is likely that under normal skin conditions, i.e. without noradrenaline, the penciclovir would be cleared from the skin and so would not be detected at increasing distances from the delivery probe. The recovery of penciclovir in the second probe reflects the concentration in the skin at that distance from the delivery probe.

8.1.3 Method.

The probes were inserted using 23G needles as guide cannulae. In each subject three pairs of probes were inserted, positioned at varying distances apart. In order to deliver maximal penciclovir into the skin, the delivery probes were perfused at a rate of 0.94μl/min. *In vitro* studies demonstrated that the average penciclovir delivery at this flow rate was 76.8% (see Section 3.10). The uptake probes were perfused at a rate of 5μl/min in order to maintain consistency between this study and the previous *in vivo* studies with respect to recovery of penciclovir. The perfusate of the delivery probes consisted of 2.15μg/ml penciclovir in Ringer's solution. The recovery probes were perfused with Ringer's solution, or 0.005mg/ml noradrenaline in Ringer's solution.

Each volunteer participated on two occasions, in random order, separated by at least three weeks to allow time for the skin to recover from probe insertion. On one of the visits, the study was performed with the use of noradrenaline in the perfusate of the recovery probe, and on the other visit, noradrenaline was omitted from the perfusate. In all cases where noradrenaline was used, the extent of visible blanching of the skin encompassed the delivery probe.

The depths of the probes in the skin, and their distance apart from each other were determined using the Dermascan C ultrasound scanner at the end of each study. The probes contained wire in the lumen. (8 subjects; 4 male and 4 female, aged 19-26 years. Number of probes: 45 delivery and 45 recovery).

8.1.4 Results and Discussion.

8.1.4.1 Delivery of Penciclovir.

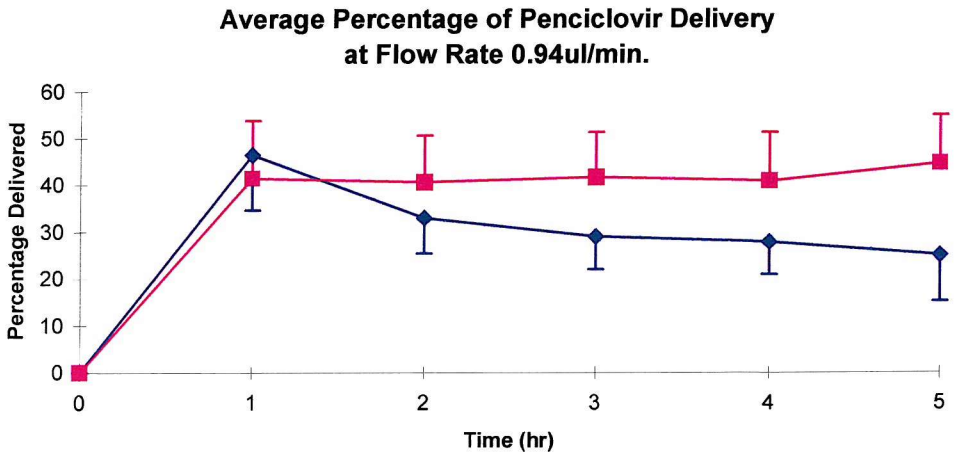


Figure 51 Average *in vivo* percentage delivery of penciclovir (± one standard deviation) when noradrenaline was present in the perfusate (—■—), and without noradrenaline (—■—).

The results demonstrate that the delivery of penciclovir was influenced by the dermal microcirculation, being significantly lower with a reduced skin blood flow ($p < 0.001$). The presence of noradrenaline reduced the flow of blood, via local vasoconstriction of the capillaries, and prevented clearance of penciclovir from the area immediately surrounding the microdialysis probe. This resulted in the accumulation of penciclovir in the skin. The increased concentration of penciclovir in the skin reduced the concentration gradient for penciclovir between the skin and the internal perfusate concentration. This would have had the effect of reducing the diffusion of drug across the microdialysis probe membrane, and so delivery out of the perfusate would be reduced. This is clearly seen Figure 51, where the initial average delivery is 46.4% with noradrenaline, but by three hours has dropped to an average of 28.9% ($p < 0.001$).

The presence of normal skin blood flow, however, had the effect of penciclovir removal from the vicinity of the probe. In this situation, the concentration gradient for the penciclovir across the probe membrane was maintained and the delivery of penciclovir was constant throughout the study.

It is interesting to note that when the penciclovir solution was first perfused through the microdialysis probe, the delivery into the skin was equal regardless of blood flow modification. This demonstrated that, initially, the concentration gradient across the microdialysis membrane and the sink effect of the dermis, were so great that the role of blood flow was negligible. It was only after the penciclovir concentration became more established in the skin that the concentration gradient declined, and the importance of systemic removal of penciclovir was observed.

8.1.4.2 *Recovery of Penciclovir.*

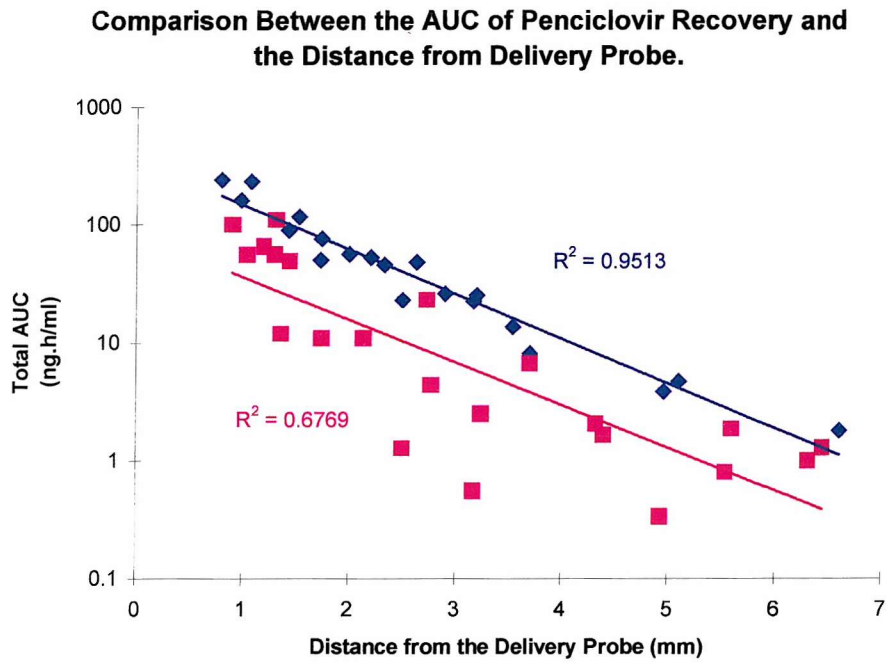


Figure 52 Total AUC of penciclovir recovery for 5 hours with distance from the delivery probe, where noradrenaline was used to restrict the dermal blood flow (■), and without noradrenaline (■).

Figure 52 shows that recovery of penciclovir with distance from the delivery probe demonstrated an exponential relationship. As was expected from the delivery data, the effect of noradrenaline on the local microcirculation produced higher concentrations of penciclovir in the skin. This resulted in a greater uptake of penciclovir in the recovery probe and in diffusion of the drug to greater distances from the delivery probe.

The use of noradrenaline also reduced the data scatter variability, whereas under normal conditions, the inter-individual variability was more enhanced. This is likely to be due to large variations in dermal blood flow between individuals, and may also be influenced by the ambient temperature of the room at the time of the study. Noradrenaline would be expected to reduce the dermal blood flow to a similar extent in all individuals, as it was

delivered at the same concentration and perfusion flow rate throughout the study. This is likely to explain the small variability in recovery.

It might be expected that the trend lines for the two sets of data would not be parallel because of active clearance of penciclovir in the presence of normal blood flow. Such clearance may result in a steeper gradient in the data with normal blood flow, as the drug may be removed from the tissue rapidly, and so not diffuse to greater distances from the delivery probe. However, this did not appear to be the case.

The two main factors influencing the recovery of penciclovir via the recovery probe, were the diffusion of the penciclovir through the skin, and the removal of the compound from the tissue by the systemic circulation. The diffusion of penciclovir in the skin should be constant, as it was the same molecule moving through the same type of tissue. Also, the blood flow was likely to be constant over the small area of the skin that was being studied. The one difference between the two sets of data was the presence of blood flow in the skin. If the blood continuously removed a constant amount of the drug, and the blood flow itself was constant, then in theory, it might expected that the concentration of penciclovir would be lower, but that the diffusion through the skin would be the same.

However, the removal of penciclovir by the dermal blood flow may not be a simple process. As the drug diffuses out of the probe and into the tissue there may be several processes taking place at once.

Firstly, there is simple diffusion of the drug through the tissue, down a concentration gradient, away from the probe.

Secondly, as the penciclovir diffuses through the tissue, it may come into contact with dermal capillaries. The drug may either enter the blood stream, or may remain in the tissue for further diffusion.

Thirdly, once the penciclovir has entered the blood stream, it could either be transported away from the site completely, or may diffuse out of the capillaries, back into the tissue. This diffusion from the blood into the skin may deliver the drug into the tissue at greater distances from the delivery probe, thus contributing to the concentration of penciclovir found at that

distance. The blood stream may also transport the drug back towards the delivery probe. Delivery of penciclovir from the capillaries into the tissue close to the delivery probe may not contribute substantially to the dermal drug concentration as the concentration gradient between the blood and the tissue may be small. It is likely that the tissue concentration would be higher than the blood concentration close to the delivery probe, resulting in further diffusion of the drug into the blood, and not delivery from the blood into the tissue.

In summary, a steeper gradient of penciclovir recovery with distance from the delivery probe in the presence of normal blood flow is likely to result from a constant removal of the drug from the site by the blood. However, if penciclovir is also being delivered to the tissue from the blood stream, then this may produce the parallel trend in penciclovir recovery seen in this study.

8.1.4.3 Concentration of Penciclovir Recovered per Hour.

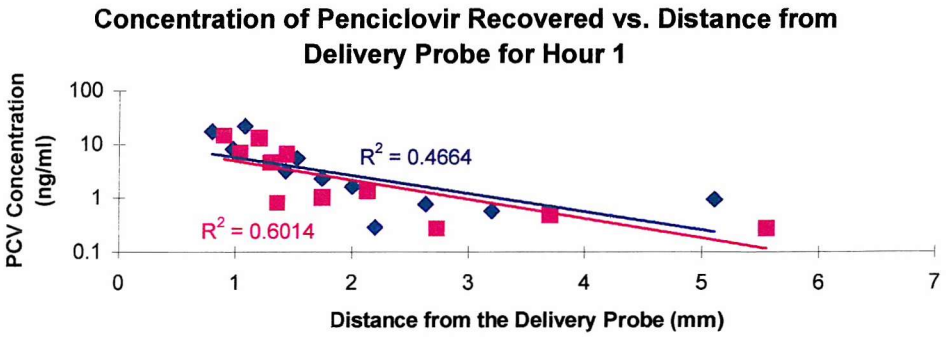


Figure 53 Concentration of penciclovir recovered for hour 1 with distance from the delivery probe, where noradrenaline was used to restrict the dermal blood flow (■), and without noradrenaline (■).

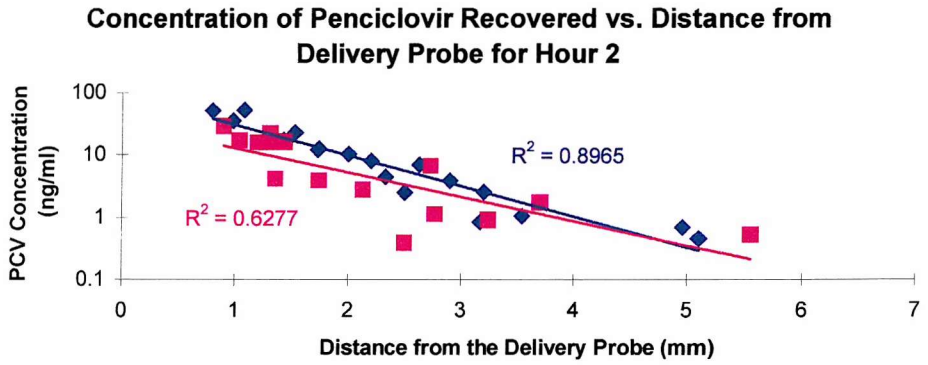


Figure 54 Concentration of penciclovir recovered for hour 2 with distance from the delivery probe, where noradrenaline was used to restrict the dermal blood flow (■), and without noradrenaline (■).

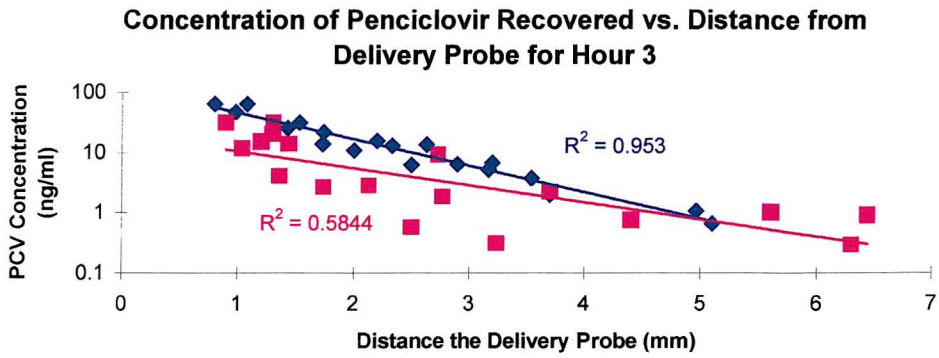


Figure 55 Concentration of penciclovir recovered for hour 3 with distance from the delivery probe, where noradrenaline was used to restrict the dermal blood flow (■), and without noradrenaline (■).

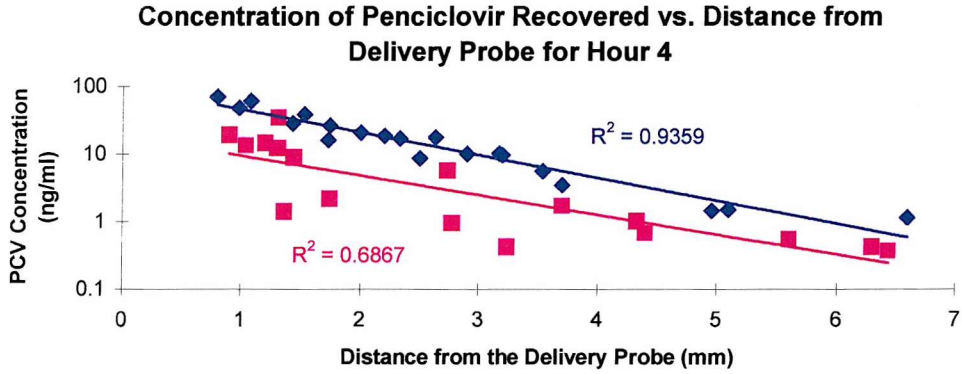


Figure 56 Concentration of penciclovir recovered for hour 4 with distance from the delivery probe, where noradrenaline was used to restrict the dermal blood flow (■), and without noradrenaline (■).

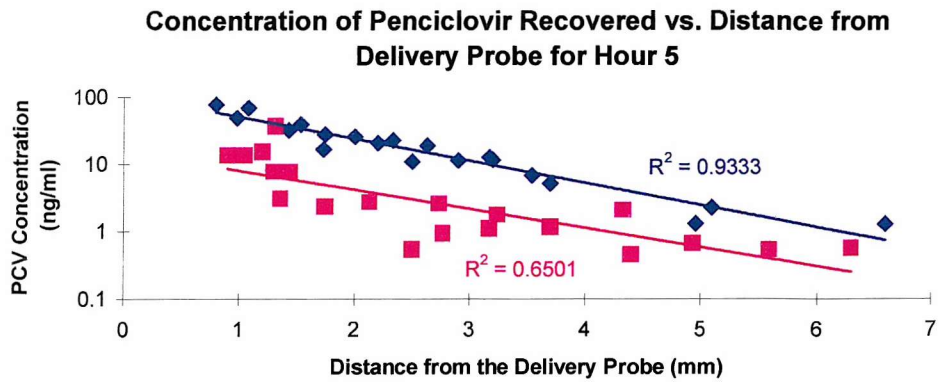


Figure 57 Concentration of penciclovir recovered for hour 5 with distance from the delivery probe, where noradrenaline was used to restrict the dermal blood flow (■), and without noradrenaline (■).

Figures 53 to 57 demonstrate that as the study progressed, the concentrations of penciclovir recovered increased, and also that the drug diffused further from the delivery probe with time.

When penciclovir was introduced into the skin via the delivery probe, it would have diffused through the tissue in all directions, and would not move exclusively towards the uptake probe; the result of this would be that the amount of penciclovir actually reaching the probe was a very small proportion of that which was initially delivered into the tissue.

It was thought unlikely that the physical presence of the recovery probe in the tissue would affect the diffusion of penciclovir to greater distances, beyond the recovery probe. A study showing the lateral diffusion of histamine in the skin, at sites remote from the initial intradermal histamine injection, demonstrated that the physical presence of the microdialysis probe did not affect histamine levels at more distant sites. However, histamine was only detectable up to 1mm from the injection site (Petersen L. *et al* 1997).

At hour one, the recovery of penciclovir was consistent with the extent of delivery; the concentrations recovered seem to be very similar, regardless of whether or not there is reduced dermal blood flow. The concentrations of penciclovir recovered in this first hour were all very low.

If the data points produced in the presence of normal blood flow (pink data) in Figures 53 to 55 up to a distance of approximately 3mm from the

delivery probe, are considered independently of the additional data at greater distances, they appear to exhibit the steeper gradient that is thought to be expected due to the removal of penciclovir via the blood stream. It may be that the data points at greater distances in these graphs are due to delivery of penciclovir from the blood capillaries into the tissue as described above.

The data points produced in the situation of reduced blood flow (blue data) seen at the greater distances, may be due to simple diffusion, as the drug is not likely to have been cleared from the site by the dermal blood flow to the same extent as the pink data. However, there would be some residual blood flow in this situation, which may contribute to the penciclovir concentrations at greater distances by delivery of the drug via the blood stream.

In Figures 56 and 57 the data points exhibit more parallel trends, which may reflect the establishment of an equilibrium between removal of penciclovir from the site, and delivery of the drug back into the tissue from the blood stream.

8.2 *In Vivo* Delivery of Penciclovir at Flow Rate 5μl/min.

8.2.1 Aim.

To determine the delivery of penciclovir using microdialysis *in vivo*, with and without the effects of noradrenaline, at 5μl/min.

8.2.2 Introduction.

The results of the dual probe study demonstrated that the delivery of penciclovir at 0.94μl/min was in the range of 16-56%. It was suspected that, perfusion at the flow rate of 5μl/min, would demonstrate a much lower recovery of penciclovir. The delivery of penciclovir has been shown to be equal to its recovery *in vitro*, and so *in vivo* delivery should be a good measure of penciclovir recovery at this flow rate. In order to assess the influence of blood flow, the *in vivo* delivery of penciclovir was determined with and without the use of noradrenaline in the perfusate to produce local vasoconstriction.

8.2.3 Method.

The probes were introduced into the skin using 23G needles as guide cannulae, and were perfused with 2.15μg/ml of penciclovir in Ringer's solution at a flow rate of 5μl/min. In each subject, three probes were perfused with the penciclovir solution only, and the perfusate of the remaining three probes consisted of penciclovir plus 0.005mg/ml noradrenaline. The probes contained wire in the lumen. (5 subjects; 3 male and 2 female, aged 19-24 years. Number of probes: 15 with noradrenaline and 15 without noradrenaline).

8.2.4 Results and Discussion.

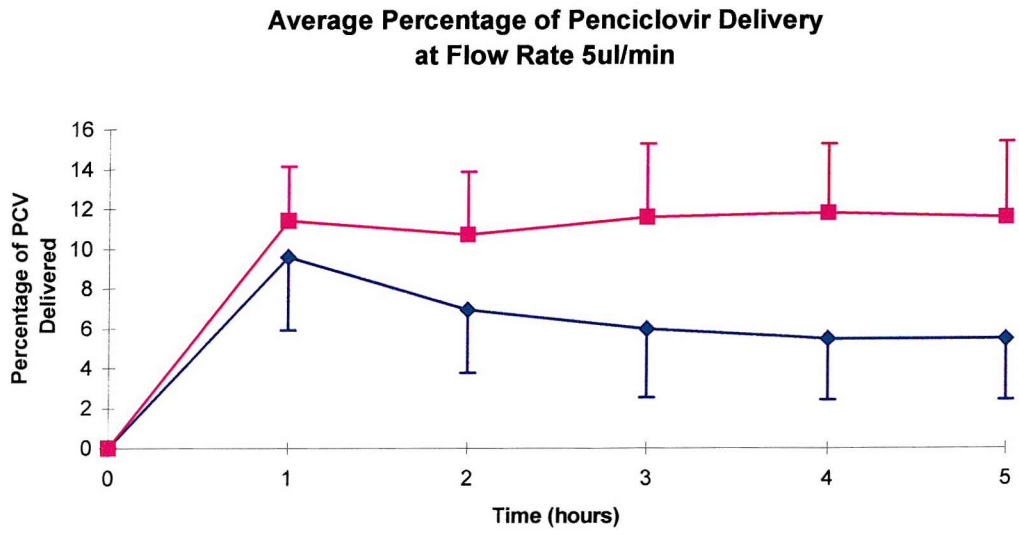


Figure 58 Average percentage delivery of penciclovir into the skin (± one standard deviation) with a perfusion flow rate of 5µl/min, with noradrenaline (—■—) and without noradrenaline (—■—).

Figure 58 shows that the delivery of penciclovir was greater without the action of noradrenaline on the dermal microcirculation, as was previously observed in Section 8.1. The blood flow in the skin cleared the area of drug, so maintaining the concentration gradient across the microdialysis membrane. With noradrenaline present, the drug remained in the immediate vicinity of the probe, the concentration gradient across the microdialysis membrane was reduced and there was less diffusion of penciclovir across the membrane, and out of the perfusate into the tissue. It is also clear to see that, at this flow rate, there was a very small percentage of penciclovir being delivered to the skin; 2-14%. It has been shown *in vitro* that the delivery and the recovery of penciclovir are equal, thus the *in vivo* delivery demonstrated here can be taken to represent the percentage recovery that is found *in vivo* at the same flow rate. The results indicate that, for the amount of penciclovir penetrating the stratum corneum and diffusing through the viable epidermis and dermis to reach the vicinity of the microdialysis probe, it is probable that a maximum of

only 16% of the absorbed drug was being sampled. If the dermal concentration itself is low, then 16% of that may be undetectable using the current method of penciclovir analysis.

It is interesting to note that in the dual probe study, the recovery probe was perfused at 5μl/min. These probes recovered appreciable concentrations of penciclovir, up to 6mm away from the delivery probe. If this concentration was only 16% of the actual dermal concentration, then at a lower flow rate it may have been possible to detect penciclovir in the skin at even greater distances from the delivery probe.

8.3 Comparison of Penciclovir Delivery *In Vivo* and *In Vitro* at Different Perfusion Flow Rates.

8.3.1 Introduction.

To demonstrate the differences in the delivery of penciclovir between *in vitro* and *in vivo* microdialysis, a summary graph was compiled to show the relevant delivery data.

8.3.2 Results and Discussion.

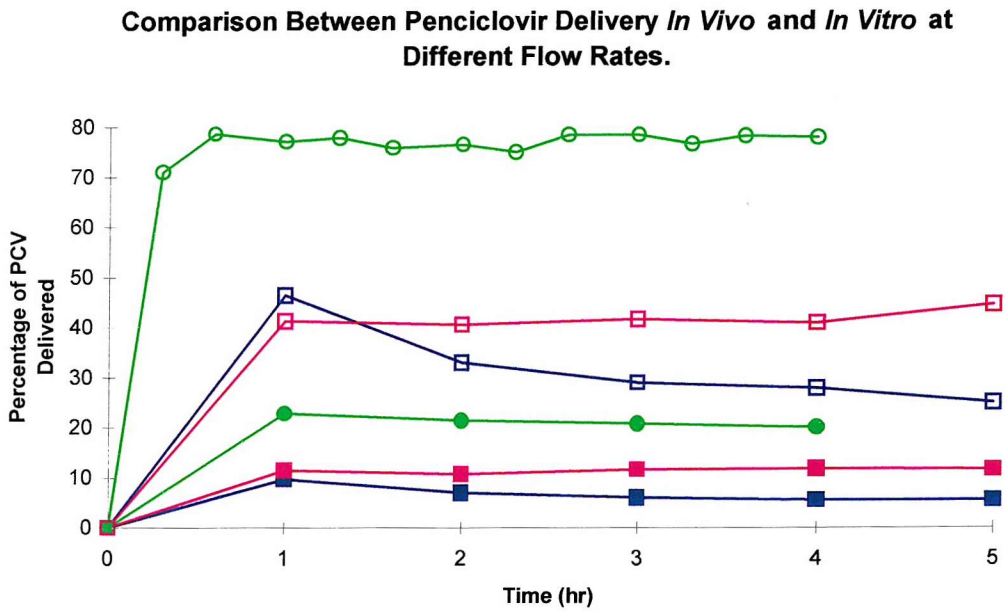


Figure 59 Comparison between *in vitro* and *in vivo* delivery of penciclovir. The solid symbols show delivery at a perfusion rate of 5µl/min. and the open symbols show delivery at 0.94µl/min. *In vitro* data shown by —●—, and *in vivo* data; with noradrenaline (—■—) and without noradrenaline (—■—).

Figure 59 shows the average penciclovir delivery at two perfusion flow rates, 0.94μl/min and 5μl/min, both comparing *in vitro* and *in vivo* data. The *in vivo* data also show the delivery with normal skin blood flow and in skin with reduced blood flow due to noradrenaline present in the perfusate.

It is clearly shown that delivery, both *in vitro* and *in vivo*, is much lower at the faster perfusion rate. The graph also demonstrates that at both flow rates, the *in vivo* delivery is approximately half of its respective *in vitro* delivery. However, it is unlikely that *in vivo* delivery and therefore recovery, can be estimated reliably from *in vitro* data, as this does not account for changes in dermal blood flow or the degree of variation in delivery, both within the same subject and between subjects.

8.4 Intra- and Inter-individual Variability.

The results obtained from the dual probe and the delivery studies demonstrated that there was substantial variability in the delivery and recovery of penciclovir *in vivo*. It is likely that fluctuations in the dermal blood flow were a major contributor to this variability, both between individuals and between different sites in the same subject. It was shown that when the dermal blood flow was restricted, the variations in penciclovir recovery and delivery were reduced, as the blood flow was likely to be reduced to a similar level in all individuals.

Variations in body temperature may produce changes in dermal blood flow, both in normal skin and in skin with restricted blood perfusion.

8.5 Summary of Dual Probe and Delivery Studies.

The results of these two studies describe several points of interest with regard to microdialysis and drug diffusion in the skin. Penciclovir diffused through the skin in an exponential manner with distance from its source, following direct dermal delivery. The dermal blood flow played a major role in determining tissue drug concentrations, with accumulation in the skin of the compound, in situations of restricted dermal perfusion. In skin with normal blood flow, penciclovir was constantly removed from the tissue into the systemic circulation.

When microdialysis was used to deliver penciclovir into the dermis, the flow rate of the microdialysis perfusion fluid greatly affected the amount of the drug that entered the tissue, with the greatest percentage delivery seen at lower flow rates. The delivery was also shown to be much greater in the *in vitro* situation than the *in vivo* one. This is likely to be due to the highly tortuous nature of the tissue, impeding the diffusion of the drug in the skin, as compared to the simple aqueous solution *in vitro*.

Chapter 9

Conclusions

9. Conclusions.

Transdermal absorption has come to be recognised as a realistic alternative route of drug delivery for potent compounds with a narrow therapeutic window, for those which are subject to substantial hepatic first-pass metabolism or those which are inadequately absorbed when administered orally. The pharmaceutical industry is continuously seeking to modify and improve topical drug formulations, so as to increase the transdermal absorption of the active component. One of the main problems is that drug absorption across the skin is highly variable, and differences in penetration can be seen not only between individuals, but also between different sites of the body within the same individual (Roberts M.S. 1997 and Wiechers J.W. 1989). In order to assess the penetration of possible candidate drugs for transdermal delivery, there is a need for the identification and development of a sampling technique which allows the direct measurement of drug concentrations. The technique must be accurate and reliable, but also practical and minimally invasive, so that *in vivo* drug concentrations can be assessed in human skin.

Cutaneous microdialysis is one such method for the measurement of dermal drug concentrations. This technique was investigated in this project to assess whether this sampling method would be suitable to replace existing methods of the measurement of transdermal drug absorption (see Section 1.3).

9.1 The Use of Microdialysis to Assess Dermal Penciclovir Concentrations.

In this thesis, the microdialysis technique was shown to have performed very well both *in vitro* and *in vivo*. The *in vitro* experimental design resulted in stable and reproducible data for recovery and delivery of penciclovir and allowed the development of this sampling method to achieve

maximum efficiencies of penciclovir recovery. In the *in vivo* studies the microdialysis probes were placed reproducibly in the dermal layer of the skin, and used to demonstrate the extent of percutaneous penetration of penciclovir by the measurement of dermal drug concentrations.

It was concluded from the transdermal absorption studies, presented in this thesis, that the very low concentrations of penciclovir detected in the dermis were due to inadequate skin penetration of the drug and/or efficient clearance of penciclovir by the dermal microcirculation, rather than a failure of the microdialysis technique. The dual probe study demonstrated that when penciclovir was present in the dermal layer, microdialysis effectively sampled the compound and measurable amounts were detected in the perfusate.

The microdialysis technique was very effective in the *in vivo* experimental study of delivery and diffusion of the drug in the dermis, and the influence of the dermal blood supply on dermal drug concentrations was very well illustrated. *In vitro* studies indicated that the recovery of penciclovir was equal to delivery. When this principle was applied *in vivo*, it was determined from the delivery of penciclovir, that drug recovery was low at the flow rate of 5μl/min. (see Section 8.2), but that recovery could be increased both *in vivo* and *in vitro* by a reduction in the perfusion flow rate.

The use of microdialysis for the measurement of *in vivo* concentrations of other compounds may have limitations. However, the problems of low microdialysis recovery associated with lipophilic and protein-bound compounds may be largely overcome by careful selection of the perfusate solution and the type of microdialysis membrane used (Carneheim C. and Stahle L 1991). It may be necessary to undertake standard *in vitro* studies, before assessing tissue concentrations *in vivo*, to characterise ideal microdialysis conditions for the sampling of a compound. However, predictions of *in vivo* performance may not be feasible from *in vitro* data due to the different nature of the media surrounding the probes.

Microdialysis can be considered an excellent technique for the assessment of the concentrations of transdermally absorbed compounds that exhibit good skin penetration. Currently, the use of microdialysis sampling for the measurement of skin drug concentrations is limited to experimental

research, and the technique requires standardisation for it to be accepted as a clinical method of drug concentration measurement. Microdialysis is the only method which allows the continuous sampling of drugs in the tissue with minimal trauma, and will prove to be a useful technique for the development of transdermal drug delivery systems.

In summary, dermal microdialysis is a reproducible and simple sampling technique, which is minimally invasive, that can be modified to suit the compound of interest and assess the transdermal absorption of many substances.

9.2 Factors Affecting Transdermal Absorption and Dermal Drug Concentrations.

The percutaneous penetration of a compound is dependent on several factors, including the structure of the skin, the characteristics of the penetrating compound, the vehicle in which the drug is topically applied and conditions of dosing, such as degree of skin hydration and site of application (see Section 1.2).

9.2.1 Skin Structure.

The lipophilic stratum corneum presents a relatively impermeable barrier to the transdermal absorption of polar molecules such as penciclovir. This type of drug may penetrate the skin via the intracellular route, and the skin appendages may have a role in penetration (see Section 1.2).

It may be possible to assess the importance of transdermal absorption via the hair follicles by the use of haired and unhaired skin in *in vitro* absorption studies. A comparison of aciclovir absorption across excised guinea pig skin (haired skin) and excised human skin (with few hair follicles), was performed by Freeman D. *et al* (1986). The results showed that when the drug was applied to the skin surface in an aqueous cream formulation the concentration of aciclovir detected in the receptor fluid for both skin types was

approximately 0.5μg/ml after 24 hours and after 48 hours was approximately 2μg/ml for human skin and approximately 1.5μg/ml for guinea pig skin. This suggested that the transfollicular route of absorption was not dominant for aciclovir absorption. Similar absorption characteristics may be expected for penciclovir, which is structurally similar to aciclovir (see Section 1.6). The extent of *in vivo* drug absorption via the sweat glands may be determined by the induction of sweating by a suitable agent, such as pilocarpine. Drug absorption would occur by diffusion through the sweat fluid. The compound may be able to gain access to the dermis via the lining epithelium of the ducts, thus avoiding the relatively impermeable stratum corneum (Tregear R. 1961, Kao J. *et al* 1988, and Schaefer H. and Redelmeier T. 1996).

9.2.2 Drug Characteristics.

There are some problems inherent in polar compounds, such as penciclovir, that may reduce their effectiveness as candidates for use in topical therapy. Polar compounds may not be able to partition across the neutral, lipophilic stratum corneum, thus rendering them unavailable for absorption across the skin (Barry B. 1991b). This may be overcome by diffusion along the transappendageal route, but this route may only provide a small portal of entry, due to the sparsity of the appendages in human skin (Schaefer H. and Redelmeier T 1996 and Tur E. *et al* 1991). The result of this may be that insufficient amounts of drug enter the tissue to have a pharmacological effect. Polar compounds may interact with the tissue, for example, forming hydrogen bonds, which can hinder the diffusion of the drug through the skin, and may also prevent the drug from having a pharmacological effect (Idson B. 1983, Pugh W. *et al* 1996 and Roberts M.S. *et al* 1996). If a polar compound does penetrate the stratum corneum and enter the viable epidermis, then upon diffusion to the dermis, the drug may be rapidly and almost completely cleared from the skin by the dermal microvasculature (Singh P. and Roberts M.S. 1994 and Roberts M.S. and Walters K.A. 1998). If the topically applied drug is intended for systemic administration, then this would be advantageous, but if the drug's site of

action is local in the skin then the compound may not be maintained at its target site in sufficient concentrations.

9.2.3 Selection of Vehicle.

The choice of vehicle is important in terms of achieving the maximum effect of the drug (Wiechers J.W. 1989). The properties of the vehicle can determine the extent of drug release into the skin. The important factors which influence drug release are the solubility of the drug in the vehicle and the partition coefficient between the vehicle and the skin (Idson B. 1983). Release is favoured by selecting a vehicle which has a low affinity for the penetrant, and so will have a large partition coefficient. The drug also needs to be soluble in the vehicle as only the soluble fraction of the drug is available for partitioning into the stratum corneum (Idson B 1983 and Wiechers J.W. 1989). It has been suggested that each compound selected for topical application would require an individual formulation based on its solubility characteristics (Idson B. 1983).

9.2.4 Effect of Blood Flow on Dermal Drug Concentrations.

A penetrating compound must enter the stratum corneum from the vehicle, diffuse through this layer and partition into the viable epidermis. Upon arrival at the upper dermal capillary plexus, the compound may be removed from the tissue by the skin microvasculature, thus rendering the drug unavailable for further diffusion through the dermis. Penciclovir is a polar compound and it is likely that its systemic clearance is very efficient. Clearance of penciclovir from the skin may affect the drug's pharmacological performance. The drug would not be retained at its site of action in the basal epidermis, and continuous transdermal absorption would be necessary to replenish the depleted supply. Although the basal layer of the epidermis is above the dermal blood supply, it is possible that a proportion of the absorbed compound would diffuse beyond the basal layer and encounter the dermal microvasculature. Systemic clearance may be enhanced with an increased blood supply, which is observed in the erythematous stage of herpes labialis

lesion progression (see Section 1.7). In an experimental *in vivo* situation, restriction of the blood supply with a vasoconstrictive agent, such as noradrenaline, could result in a decreased clearance of the drug and increased dermal concentrations (Singh P. and Roberts M.S. 1994 and Roberts M.S. and Walters K.A. 1998).

9.3 Clinical and Experimental Findings of Penciclovir Absorption.

9.3.1 Skin Structure.

The absence of detection of penciclovir in the dermis using *in vivo* microdialysis, following topical drug application, was thought to be attributable to inadequate skin penetration of the drug, and/or efficient clearance of penciclovir from the tissue into the systemic circulation. It should be noted that the *in vivo* microdialysis studies performed in this project were all conducted on normal, healthy skin. In the clinical trials on penciclovir cream for the topical treatment of herpes labialis, the concentrations of the drug in the skin were not measured, but instead, measurable landmarks in disease progression and lesion resolution were used as clinical endpoints to gauge the antiviral efficacy of the drug. The trials indicated that the topical application of penciclovir showed clinical benefit when applied in the early stages of disease progression (Spruance S.L. *et al* 1997). This indicates that sufficient penciclovir was able to penetrate the stratum corneum and reach the basal epidermis. It would be of interest to assess the concentrations of penciclovir absorbed across HSV infected human skin. Although, it was found that there were no differences in the *in vitro* absorption of aciclovir across guinea pig skin infected with HSV compared to absorption across uninfected skin, and it was observed that in the early stages of lesion development, the stratum corneum remains intact (Freeman D. *et al* 1986). A reliable indicator of drug concentrations at each skin layer would be a full thickness skin biopsy of the herpes labialis lesion. However, although this may be ideal in principle, in practice it may be difficult to recruit subjects to

such a trial, as the lesions usually occur on the face, and this sampling technique can cause permanent scarring.

Studies conducted in this department by a colleague (J. Botten, unpublished data) demonstrated that the *in vivo* transdermal absorption of sodium penciclovir could be increased by the induction of skin sweating. Topical application, or delivery by microdialysis, of pilocarpine caused an increase in sweating, and the recovery of penciclovir into the perfusate was enhanced, providing that the dermal blood vessels were vasoconstricted, due to the microdialysis delivery of noradrenaline. Diffusion of penciclovir down the sweat ducts appeared to be unhindered by the outward flow of the sweat. The recovery of penciclovir in the absence of noradrenaline was similar to that seen when pilocarpine was not used to induce sweating. This suggests that in conditions of normal skin blood flow, the drug was rapidly removed from the skin into the systemic circulation. Pilocarpine itself did not increase skin blood flow.

Although this study demonstrated that penciclovir could be delivered into the skin by this transappendageal route, there are doubts as to whether this information would lead to increased clinical benefit of topically applied penciclovir. The absorption of penciclovir across the lining epithelium of the sweat glands is likely to occur more slowly than the continued diffusion of the drug through the sweat fluid. This may result in a small amount of drug absorption into the epidermis, but the majority of penciclovir that enters the sweat gland is likely to enter the skin tissue at the dermal level, as this is the skin layer in which the base of the sweat duct is situated. This situation is ideal for the measurement of drug concentrations using microdialysis, as it is at this level in the skin that the probes are situated. The site of HSV infection, however, is in the basal epidermis, where drug concentrations may be low. Diffusion of penciclovir may occur up through the dermis into the epidermis, to reach the site of action, but in conditions of normal blood flow, it is unlikely that diffusion would extend beyond the upper dermal capillary plexus, as penciclovir appears to be efficiently cleared from the tissue by the vascular system.

The work on skin sweating was of relevance to the studies performed in this project, as it was undetermined whether the clearance of the drug through the vascular system was the main cause of the negligible recovery of penciclovir following topical application. The skin sweating study demonstrated that when the blood flow was unmodified the microdialysis recovery of penciclovir was significantly reduced. The transdermal absorption studies outlined in this thesis showed similar dermal penciclovir concentrations both with and without vasoconstriction, thus indicating that the main cause of low drug recovery by microdialysis was due to minimal absorption of penciclovir across the stratum corneum. The work performed using dual probes to show the *in vivo* delivery and diffusion of penciclovir in the dermal tissue and the effects of blood flow, demonstrated that removal of penciclovir by the dermal microcirculation was significant, indicating that large amounts of the compound could be effectively cleared from the skin when the microcirculation was encountered.

9.3.2 Vehicle Selection.

The effects of vehicle were demonstrated to some extent in this thesis. When penciclovir was topically applied in an aqueous cream-based vehicle in the studies performed using excised rat skin, there was an enhancement of the transdermal absorption of penciclovir. However, there also appeared to be reduction in the penetration enhancing effect of oleic acid. This suggested that there may have been a decrease in the partitioning of oleic acid into the stratum corneum from the aqueous cream-based formulation.

The vehicle used for a transdermal delivery system may require a finely balanced composition to achieve the desired effects of maximum drug delivery, and in a multiple delivery system the partitioning of one active ingredient may have to be compromised in order to promote delivery of another compound.

9.4 Further Study for the Enhancement of Penciclovir Penetration.

Liposomes are becoming increasingly popular as carrier systems for topically applied compounds. They are microscopic spherical vesicles, composed of phospholipids, held in a suspension (Planas M.E. *et al* 1992). The vesicles are formed when phospholipids are put under mild forces in the presence of water. The hydrated phospholipids then arrange into sheets which join to form a bilayer membrane which encloses some of the water in a sphere. Several of these vesicles can form within one another, creating a multilamellar structure of concentric phospholipid spheres, separated by layers of water. The vesicles can be used to encapsulate both lipophilic compounds within the phospholipid tails of the bilayers, or hydrophilic compounds in the water layers and in the central core of water. The walls of liposomes are very similar, physiologically, to the material of biological membranes (Schmid M.H. and Korting H.C. 1995).

The use of liposomes in the topical delivery of compounds has been used since the early 1980's (Meidan V. *et al* 1998). Since that time, the transdermal absorption of several compounds has been shown to be increased with the use of liposomes, compared to application in conventional vehicles. These include corticosteroids, progesterone, methotrexate (Gesztes A. and Mezei M. 1988), tetracaine (Planas M.E. *et al* 1992 and Gesztes A. and Mezei M. 1988), hydrocortisone, fluocinolone acetonide, inulin and cyclosporin (reviewed in Meidan V. *et al* 1998).

Liposomes have been shown to interact with the lipids of the human stratum corneum *in vitro* (Hofland H.E. *et al* 1995), and it has been demonstrated that liposomes may only deliver compounds into the stratum corneum, due to their highly lipophilic nature (Touitou E. *et al* 1998). This allows a greater penetration enhancement of lipophilic compounds, but may only deliver more hydrophilic molecules to the deeper layers of the stratum corneum (Meidan V. *et al* 1998).

The disadvantages of the use of liposomes include the high cost of production and the possible difficulty in long-term stability of the vesicles (Schmid M.H. and Korting H.C. 1995).

Touitou E. *et al* (2000) identified a modified liposome which is composed of ethanol as well as the phospholipid and water. These modified liposomes were termed ethosomes. Ethosomes consist of multilamellar vesicles, which are more flexible than those of liposomes as the lipids have a lower melting temperature (Touitou E. *et al* 2000), and have been shown to deliver molecules into the deep layers of the dermis (Touitou E. *et al* 1998).

Application of penciclovir in an ethosomal preparation may promote the drugs' percutaneous penetration. The topical application of aciclovir in these vesicles has already been shown to have significant clinical benefit for the treatment of herpes labialis. Horwitz E. *et al* (1999) demonstrated, in a clinical trial using 5% aciclovir in an ethosomal preparation, that the time to crust formation of the lesion was reduced to 1.6 days from 4.3 days with a commercial aciclovir cream, and the time to loss of crust was 3.5 days with the ethosomal preparation and 6.4 days with aciclovir cream. The same study also reported a decrease in abortive lesions (lesions that did not progress beyond the papular stage) following early treatment with the ethosomal formulation, 30% compared to 10% abortive lesions in the aciclovir cream treatment group.

Previous studies using topical aciclovir preparations have proposed that their lack of clinical benefit was due to the inadequate absorption of the compound across the skin (Raborn G. *et al* 1989 and Spruance S.L. *et al* 1984). The results of the ethosomal formulation clearly demonstrated a reduction in healing time, and in the number of abortive lesions, suggesting that aciclovir was being rapidly delivered into the skin from this topical preparation. These data suggest that the use of a similar ethosomal formulation for the topical application of penciclovir could result in similar clinical benefit for the treatment of herpes labialis.

9.5 Final Conclusion.

It was concluded from this thesis that the microdialysis technique was an effective method of the study of compounds in the dermis. This includes the assessment of percutaneous penetration of topically applied compounds and of factors affecting the concentration of the compound once present in the dermis.

Topical application of penciclovir gave very low, but measurable concentrations in the microdialysis dialysates. This suggested poor penetration of intact human skin over the time period that was studied in the experiments outlined in this thesis. However, clinical benefit for the treatment of herpes labialis may be achieved using topical penciclovir with frequent reapplication of the formulation over a period of several days.

Appendix

10. Appendix.

10.1 Photographs of Microdialysis Probes *In Vivo*.

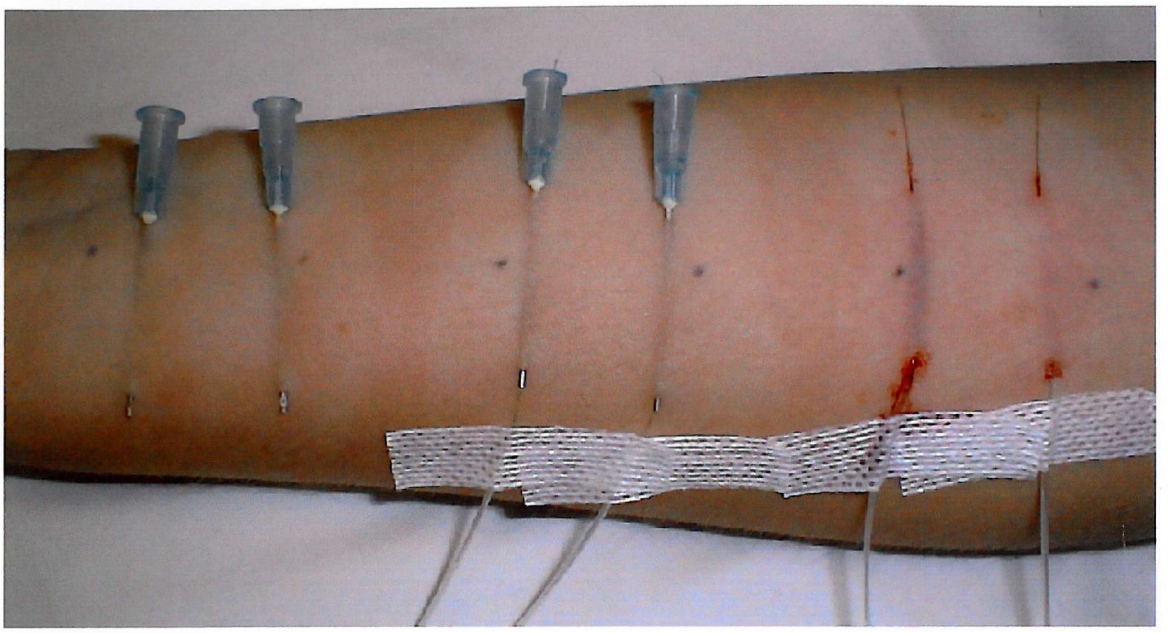


Figure 60 Insertion of the microdialysis probes *in vivo*. Left: insertion of needles under the skin surface; centre: the probes were guided through the needles and taped to the skin; right: the needles were removed leaving the probes in place.

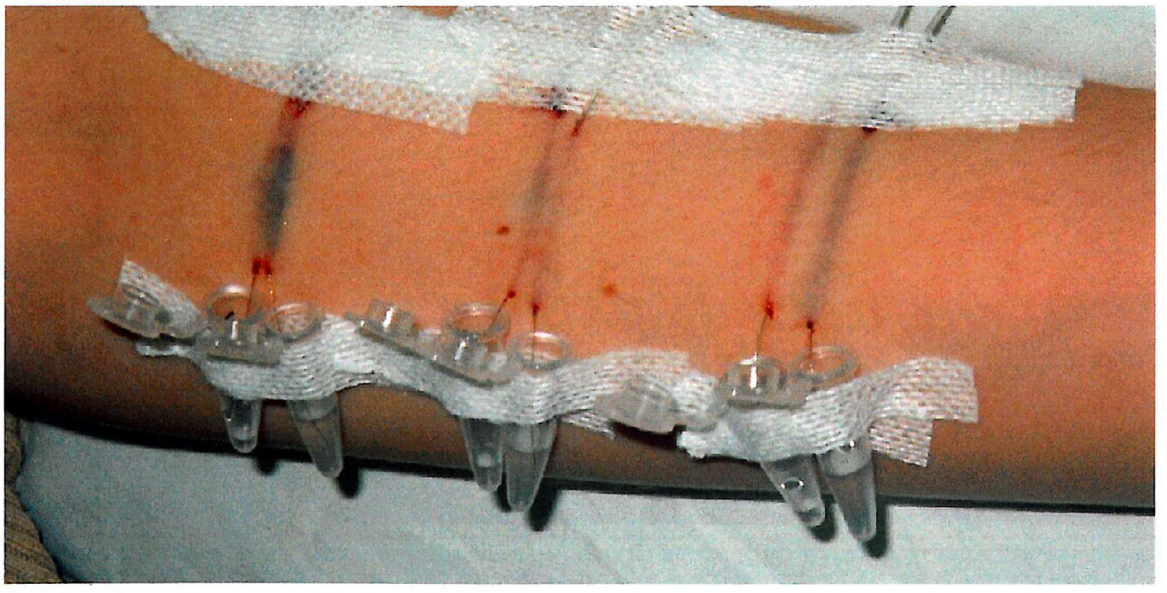


Figure 61 Positioning of the probes in the dual probe study. The photograph shows the variable distances between the pairs of microdialysis probes.

10.2 Ultrasound Scanning of Human Skin.

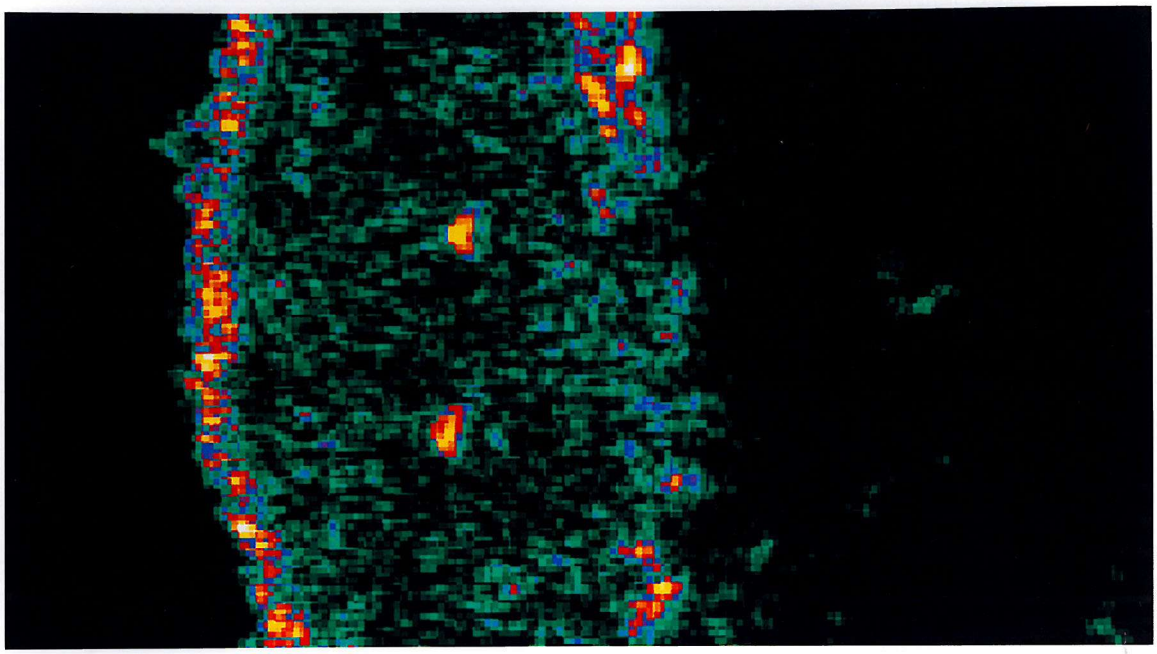


Figure 62 Cross-sectional scan of the skin and microdialysis probes. The probes can be clearly seen as two yellow and red structures in the middle of the dermis. The epidermis is seen as the brightly coloured band to the left of the image.

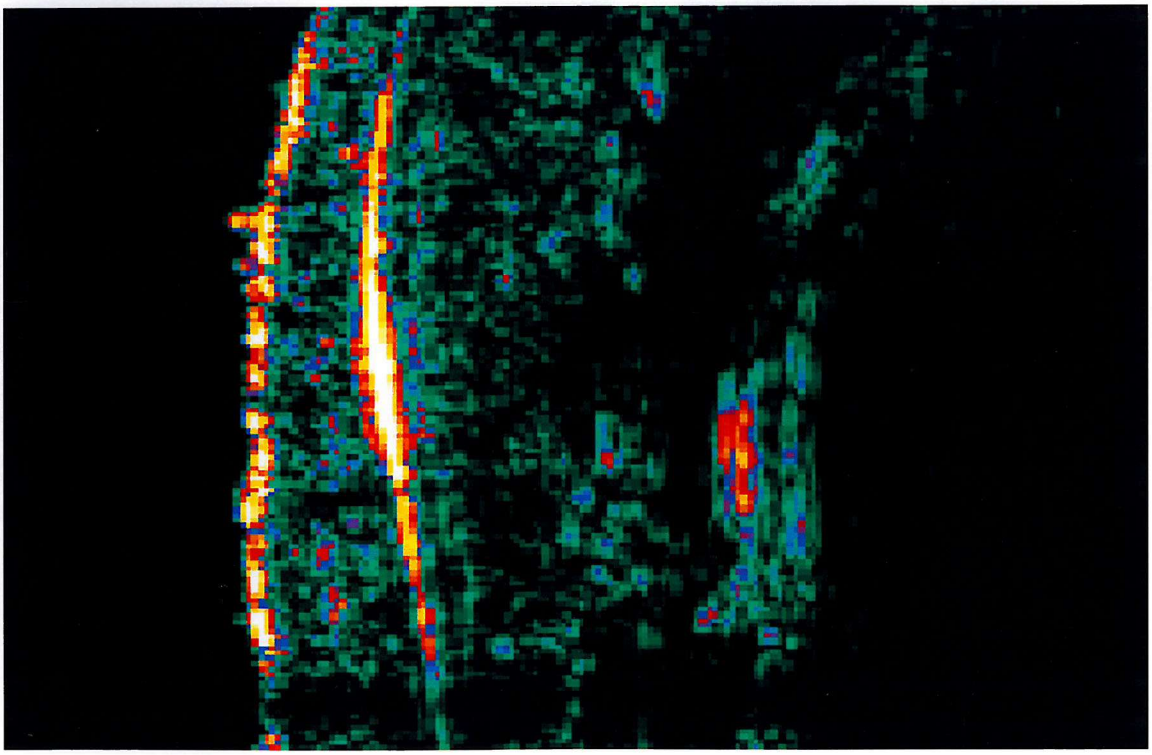


Figure 63 Cross-sectional image of the skin. This image shows a longitudinal scan of the microdialysis probe, which is seen as the white line in the dermis. The image of the probe is incomplete, as the position of the fibre moves out of the scanning plane.

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11. References

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