TRANSDERMAL PENETRATION OF ACYCLOVIR IN THE PRESENCE AND ABSENCE OF TERPENES

Mariaan Myburgh

(B.Pharm.)

Dissertation submitted in the partial fulfilment of the requirements for the degree

MAGISTER SCIENTIAE

in the

Departement of Pharmaceutics

at the

POTCHEFSTROOMSE UNIVERSITEIT VIR CHRISTELIKE HOËR ONDERWYS

Supervisor: Prof. J. du Plessis

Potchefstroom

2003

ABSTRACT

TRANSDERMAL PENETRATION OF ACYCLOVIR IN THE PRESENCE AND ABSENCE OF TERPENES

Acyclovir is an antiviral drug used in the treatment and prevention of herpes simplex and varicella-zoster viral infections. The major problem in the transdermal delivery of acyclovir is the permeation in sufficient amounts to deeper layers of the skin and into the systemic circulation. Acyclovir is a hydrophilic substance with a low partition coefficient, resulting in poor penetration through the excellent barrier of the skin, the stratum corneum.

In an attempt to enhance the transdermal permeability of acyclovir, the aim of this study was to employ terpenes as possible penetration enhancers. Terpenes are constituents of natural essential oils, with widespread medicinal use including in aromatherapy. The terpenes used in this study were 1,8-cineole, limonene, menthol, menthone, and β-myrcene.

Terpenes are not only used as penetration enhancers, but are even more often present in drugs and cosmetics. Limited studies have been done concerning the penetration of terpenes through the skin. Thus, not only the effect of the terpenes on the penetration of acyclovir, but also the penetration of the terpenes themselves were studied. The influence of acyclovir on the penetration of the terpenes was also determined.

In vitro permeation experiments were performed on human skin using Franz diffusion cells. The skin was pretreated with a 5 % solution of the terpene in ethanol and left for 30 minutes to enable ethanol evaporation and terpene incorporation into the skin. Saturated aqueous solutions of acyclovir (pH 7.4) were added in the donor compartment before and after skin pretreatment. The acyclovir concentration retrieved from the receptor compartment of the Franz cells was analyzed by HPLC. The amount of terpene that penetrated were semi-quantitatively determined by GC.

Penetration of acyclovir was significantly enhanced by two terpenes, *viz*. 1,8-cineole and menthol. The extent of enhancement was, however, not large enough to be of clinical use. The enhancement in acyclovir penetration observed upon ethanol pretreatment alone, or in the presence of limonene, menthone or β-myrcene, was not significant. Penetration enhancement of acyclovir by the terpenes was in accordance with previous studies, which postulated better enhancement of hydrophilic drugs by hydrophilic terpenes.

Large percentages of the terpenes with log P values within the optimum log P range (1 - 3) penetrated, as was found with menthone and menthol. Penetration decreased accordingly as the log P, and thus lipophilicity, increased. Stratum corneum retention is regarded as the most plausible explanation for this phenomenon. In the case of 1,8-cineole, enhancer pooling in the stratum corneum could be a possible reason for its poor penetration. Acyclovir significantly influenced the penetration profiles of some of the terpenes, but no clear explanation could be given.

Key words:
acyclovir, transdermal delivery, permeation, penetration enhancers, terpenes

OPSOMMING

TRANSDERMALE PENETRASIE VAN ASIKLOVIR IN TEENWOORDIGHEID EN AFWESIGHEID VAN TERPENE

Asiklovir is 'n antivirale geneesmiddel vir die voorkoming en behandeling van infeksies deur die herpes simplex- en varicella-zoster virus. Die grootste probleem met die transdermale aflewering van asiklovir is dat te klein hoeveelhede die dieper lae van die vel en die sistemiese sirkulasie bereik. Asiklovir is 'n hidrofiliese verbinding met 'n lae verdelingskoëffisient, wat 'n lae penetrasie deur die stratum corneum tot gevolg het.

In 'n poging om die transdermale deurgang van asiklovir te bevorder, was die doel van hierdie studie om penetrasiebevorderaars te gebruik. Terpene kom natuurlik in essensiële olies voor en word wydverspreid medisinaal gebruik, onder meer in aromaterapie. Die terpene wat in hierdie studie gebruik is, was 1,8-sineool, limoneen, mentol, mentoon en β-mirsien.

Terpene word nie net as penetrasiebevorderaars gebruik nie, maar kom dikwels in medisyne en in kosmetika voor. Tot dusver is min studies oor die penetrasie van terpene deur die vel gedoen. Dus was nie net die effek van die terpene op die deurgang van asiklovir bestudeer nie, maar ook die penetrasie van die terpene self. Die invloed van asiklovir op die penetrasie van die terpene is ook bepaal.

Eksperimente van die *in vitro*-permeasie deur menslike vel is gedoen deur Franz-diffusieselle te gebruik. Die vel is vooraf met 'n 5 % oplossing van die terpeen in etanol behandel en vir 30 minute laat staan. Sodoende is die etanol toegelaat om te verdamp en die terpene is as 'n dun film op die vel neergelaat. Versadigde waterige oplossings van asiklovir (pH 7.4) is daarna in die donorkompartement aangebring. Die konsentrasie van die asiklovir wat deur die vel tot in die reseptorkompartement gediffundeer het, is met behulp van hoëdrukvloeistofchromatografie bepaal. Die hoeveelheid terpeen wat gepenetreer het, is semi-kwantitatief met behulp van gaschromatografie bepaal.

Die penetrasie van asiklovir is betekenisvol deur twee terpene, naamlik 1,8-sineool en mentol, bevorder. Die mate van bevordering was nie groot genoeg om van kliniese belang te wees nie. Die effek van etanol, asook van limoneen, mentoon en β-mirsien op die penetrasie van asiklovir is nie betekenisvol nie. Die bevordering van die penetrasie van asiklovir deur terpene is in ooreenstemming met vorige studies, wat gepostuleer het dat daar beter bevordering is van die penetrasie van hidrofiliese geneesmiddels deur hidrofiliese terpene.

'n Groot persentasie van die terpene met log P-waardes onder 3 penetreer die vel, soos gevind met mentoon en mentol. Penetrasie het afgeneem soos wat die log P en lipofiliteit toeneem. Retensie deur die stratum corneum word beskou as die grootste rede vir hierdie verskynsel. In die geval van 1,8-sineool, word die lae penetrasie toegeskryf aan ophoping van die terpeen in die stratum corneum. Asiklovir het die penetrasieprofiele van sommige terpene betekenisvol beïnvloed, maar geen duidelike rede kon hiervoor gegee word nie.

Sleutelwoorde:
asiklovir, transdermale aflewering, permeasie, penetrasie bevorderaars, terpene

ACKNOWLEDGEMENTS

All honour to God, without His mercy, love, and guidance I would not have been able to complete this study.

I would like to express my sincerest appreciation and thanks to the following people. Without their support, assistance and supervision this study would not have been possible:

- **My parents**, to whom I dedicate this dessertation. Thank you for your constant encouragement and love this past two years.

- **Prof. Jeanetta du Plessis**, my supervisor, for your encouragement, supervision, and valuable insights. Thank you for opportunity of studying under your mentorship.

- **Prof. Jonathan Hadgraft**, thank you for your valuable advice and guidance during this study.

- **Dr. Jan du Preez**, for your help and suggestions on the validation of my analytical method and with the HPLC.

- **Mrs. Reanda Ganz**, from the Research Institute for Industrial Pharmacy, for her friendliness and help with the GC analysis.

- **Prof. Jaco Breytenbach**, for the valuable work you have done in proofreading my dissertation and your willingness to help wherever possible.

- **Dr. Suria Ellis** for her assistance with the statistical analysis of the data.

- **Wilma Steenekamp** and **Mariska Pretorius**, for your encouragement, support and friendship.

- **Rufus Terblanche**, for your advice and willingness to help.

- **Riaan**, for being a very understanding and wonderful friend. Your support, assistance, encouragement and patience during this study carried me through despondent times and really meant a lot to me.

- **My friends**, for your encouragement, patience and unchanging friendship during my studies. Thank you for the unforgettable times we shared.

TABLE OF CONTENTS

TABLE OF FIGURES

TABLE OF TABLES

CHAPTER 1:

INTRODUCTION AND STATEMENT OF THE PROBLEM

The skin is the outer covering of the body and encapsulates the organs from the environment. It serves as a multifunctional membrane, not only protecting the body from physical, chemical and microbial attacks, but also functions as a homeostatic barrier against outward loss of water. Thus, the skin has evolved to limit molecular transport into and out of the body (Suhonen *et al.*, 1999).

Because of the skin's accessibility and large surface, it poses as the most suitable alternative for oral drug administration, both for topical and systemic therapy. Avoidance of first-pass metabolism by the liver, reduced side effects, improved patient compliance, longer duration of action and more uniform plasma levels are some of the main advantages of transdermal drug delivery (Pfister, 1997).

The success of transdermal delivery depends on the permeation of sufficient amount of active agents through the skin to obtain optimum plasma levels for therapeutic effects. The fact that only eight FDA-approved drugs are currently on the transdermal market, emphasizes the limitations of the technology (Naik *et al.*, 2000).

The major limitation to transdermal drug delivery is the skin itself, and specifically the outermost layer of the skin, the stratum corneum or horny layer. This layer is the major barrier to penetration of matter through the skin, due to its integral and compact structure comprising of protein-rich cells embedded in a multilamellar lipid domain (Gao & Singh, 1998). The excellent barrier properties of this horny layer often preclude the skin as a route for systemic drug delivery of many drugs and that is why mechanisms governing transdermal drug delivery have to be understood.

Acyclovir is active in both the treatment and prevention of the herpes simplex and varicella zoster viral infections. Several trials have shown that inhibition of herpes virus replication by acyclovir is associated with improved survival (unrelated to opportunistic

herpesvirus disease) in individuals with AIDS (Dollery, 1999). Transdermal delivery is an attractive route to administer acyclovir. The advantages include more reliable uptake compared to gastrointestinal variable absorption and first-pass metabolism, continuous drug input which permits the use of drugs with short elimination half-lives, rapid termination of drug delivery, and potentially improved patient compliance.

Acyclovir transdermal therapy has a low efficiency, with lack of permeation of a sufficient enough amount of drug to target site, due the barrier characteristics of the stratum corneum to the absorption of hydrophilic acyclovir. Data obtained by Jiang *et al.* (1998) provided direct evidence that after removal of the stratum corneum, the amount of acyclovir in the viable skin layer (1.21 % *vs* 0.20 %) and the perfusion medium (2.65 % *vs* 0.25 %) was significantly and substantially increased. Consequently, it is advantageous to facilitate the transdermal transport of acyclovir by the use of permeation enhancers.

Most often permeation enhancers like terpenes are used which alter the barrier function of the stratum corneum to employ and promote permeation of poorly absorbed drugs. Terpenes are constituents of essential oils, and have widespread medicinal uses, including aromatherapy. They were reported to have good toxicological profiles, high percutaneous enhancement abilities, and low cutaneous irritancy at low concentrations (1 - 5 %). Moreover, a variety of terpenes have been shown to increase the percutaneous absorption of both hydrophilic and lipophilic drugs (El-Kattan *et al.*, 2000a). This study explores the influence of six terpenes found in eucalyptus, orange and peppermint oil on the *in vitro* skin permeation of acyclovir.

Terpenes are not only used as penetration enhancers, but even more often are present in drugs and cosmetics as components of essential oils added for some other reasons: for inhalation or for topical administration, as rubefacients, analgesics or antiseptics. Permeation enhancers, with significant biological activity and the possibility of causing side effects, should not or only in restricted quantities penetrate through the skin. Therefore, not only the promotion of penetration of other drugs but also their own penetration should be better examined. The extent of enhancer uptake into the stratum corneum during enhancer treatment is not often measured. There are very limited published studies concerning the penetration of terpenes through the skin. Penetration

studies, which have been performed on other lipophilic enhancers, often reveal surprisingly high enhancer loadings in the stratum corneum (Cornwell *et al.*, 1996).

The main objectives of this study were to determine:

- The *in vitro* effect of selected terpenes on the transdermal penetration from a saturated solution of acyclovir through human epidermis by pretreatment with a solution of ethanol and terpene, using 1,8-cineole (eucalyptol), limonene, menthol, menthone and β-myrcene.
- The *in vitro* transdermal diffusion of these terpenes with and without the presence of acyclovir.

CHAPTER 2:

TRANSDERMAL DRUG PERMEATION

2.1 THE ANATOMY AND BARRIER FUNCTION OF THE SKIN

To predict skin transport of a drug molecule, it is important to understand the barrier function of human skin and the physicochemical properties of drugs governing mass transport across the skin. The quantitative prediction of the rate and extent of percutaneous penetration and absorption of topically applied drugs and chemicals are complicated by the biological variability inherent to the skin. In order to gain perspective on this phenomenon, one should appreciate the mammalian skin as a dynamic organ with a myriad of biological functions. The most obvious is its barrier property which is of primary relevance to percutaneous absorption (Riviere, 1993).

The skin, the largest organ of the body, is a flat and thin organ with an average thickness of about 2 mm (this shows regional variations between 0.3 and 4 mm). Its weight, for a body surface of 1.5 - 2 m^2 is 3 - 4 kg, excluding subcutanous fat (Stuttgen, 1982). An average square centimeter of skin contains 10 hair follicles, 15 sebaceous glands, 12 nerves, 100 sweat glands, 360 cm of nerves, and three blood vessels (Asbill & Michniak, 2000).

The skin is composed of several layers: the stratum corneum (uppermost layer), the viable epidermis, the dermis, and the lower layers of adipose tissue. Each of these layers has different properties which poses resistances to transdermal delivery of drugs. The rate of diffusion of a drug through the skin is a result of the sum of all the resistances. The rate-limiting step is determined by the least permeable layer, almost always the stratum corneum.

2.1.1 Epidermis

The epidermis is about 0.12 mm thick (regional variations between 0.02 and 1.24 mm). There are no blood or lymph vessels in this compact cell tissue and its purpose is to synthesize the barrier, i.e. the horny layer or stratum corneum (Stuttgen, 1982). To accomplish this, keratinocytes undergo a programmed process of differentiation in which proliferative, undifferentiated cells are converted to highly differentiated, non-dividing cells. The epidermis can be divided into several layers based on the state of keratinocyte differentiation (Eckert, 1992).

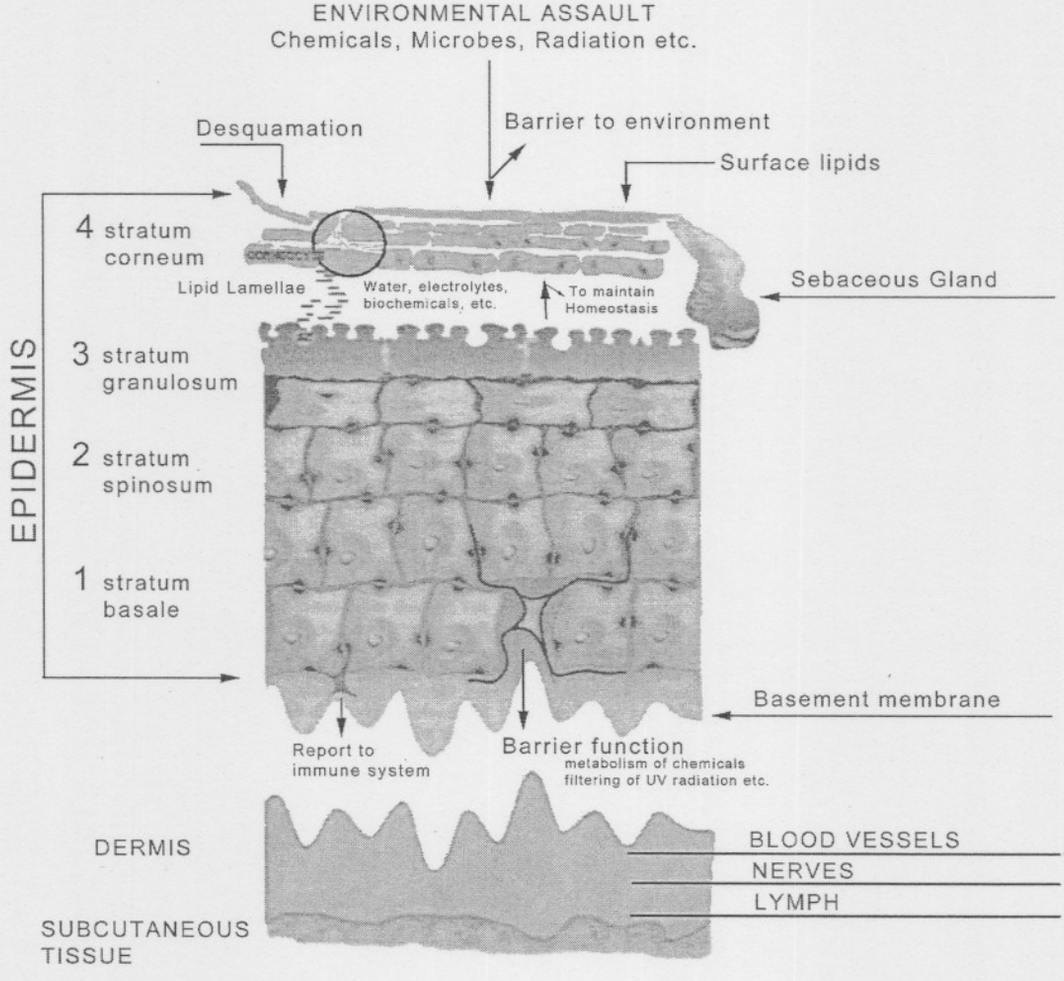


FIGURE 2-1: Skin components and functions performed (Morganti *et al.*, 2001).

The **stratum basale** is a single layer of collumnar basal cells, which remain attached to the basement membrane *via* hemidesmosomes. The basal layer contains actively dividing cells, which migrate upwards to successively form the spinous, granular and clear layers. As part of this process, the cells gradually lose their nuclei and undergo changes in composition (Foldvari, 2000; Menon, 2002).

The **stratum spinosum** is situated directly above the basal layer. Once cells leave the basal layer they lose the capacity to divide, increase in size, and flatten, and their water content diminishes (Morganti *et al.*, 2001). As the cells differentiate during their migration to the surface, the phospholipid content decreases and the sphingolipid (glucosylceramide and ceramide) and cholesterol content simultaneously increases (Foldvari, 2000).

The **stratum granulosum** contains electron-dense keratohyalin granules that contain profilaggrin. Granular layer cells are living, as evidenced by intact cellular metabolic function (Eckert, 1992).

Between the granular and cornified layer is a 'transition zone' between living and dead epidermis, the **stratum lucidum**. This is a zone of extensive cellular remodeling. Most of the existing cellular organelles, DNA and RNA, are destroyed by the activity of proteases and nucleases. In addition, the lipid contents of the membrane coating granules are released into the extracellular matrix, the keratin filaments are restructured to a more stable form, and the cornified envelope is formed (Elias, 1992).

The **stratum corneum**, or horny layer, consists of flat, roughly hexagonally shaped, partly overlapping cells, called corneocytes. The cells are composed mainly of insoluble bundled keratins, surrounded by a cell envelope stabilized through covalently bound lipid and cross-linked proteins (Asbill & Michniak, 2000). The corneocytes are embedded in a matrix of extracellular lipid bilayers (Figure 2-2). The multiple intercellular lamellae of the stratum corneum are believed to constitute the barrier to percutaneous penetration. These extracellular lipid bilayers are devoid of phospholipids and are made up of ceramides (50 %), cholesterol (25 %), free fatty acids (15 %), cholesteryl sulphate (5 %) and several minor constituents (Abraham & Downing, 1990).

The composite of the corneocytes (terminally differentiated keratinocytes), and the secreted contents of the lamellar bodies (elaborated by the keratinocytes), give it a brick-

and-mortar organization. The protein-enriched corneocytes (bricks) impart a high degree of tortuosity to the path of water or any other molecule that traverses the stratum corneum, while the hydrophobic lipids, organized into tight lamellar structures (mortar), provides a water-tight barrier property to the already tortuous route of permeation in the interfollicular domains (Menon, 2002).

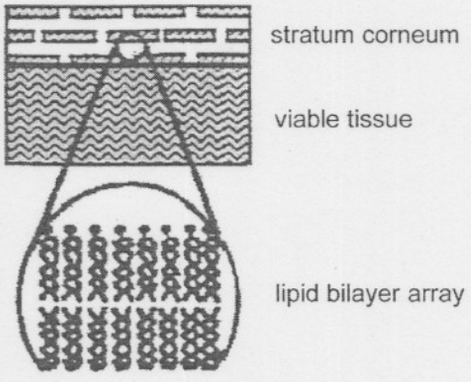


FIGURE 2-2: A schematic representation of the stratum corneum lipid bilayers (Hadgraft & Wolff, 1993).

Because of its highly organized structure, the stratum corneum is the major permeability barrier to external materials, and is regarded as the rate-limiting factor in the penetration of therapeutic agents through the skin.

While the stratum corneum is perhaps the most important phase boundary, it should be pointed out that once the penetrant has crossed the stratum corneum, it must partition into the underlying layers of epidermis, dermis, and circulatory system. These tissues are typically more hydrophilic than is the stratum corneum and can present a barrier to transport of extremely hydrophobic penetrants (Smith, 1990).

2.1.2 Dermis

Underneath the epidermis is the dermis, with fibroblasts, endothelial cells and mast cells. The cells are embedded in connective tissue composed of mainly collagenous fibers and elastic connective tissue (Asbill & Michniak, 2000)

The dermis comprises the largest fraction of the skin and is responsible for providing its structural strength. The dermis is largely acellular, but is rich in blood vessels, lymphatic

vessels and nerve endings. An extensive network of dermal capillaries connects to the systemic circulation (Foldvari, 2000).

The dermis is believed to offer no barrier to the passage of molecules that reach it, except for molecules that may be substantive to specific dermal components (Rieger, 1993). The dermal papillary layer is so rich in capillaries that most penetrants clear within minutes. Usually, deeper dermal regions do not significantly influence absorption, although they may bind a penetrant, inhibiting its systemic removal as is the case with testosterone (Barry, 2001).

Hair follicles, sebaceous glands and sweat glands are found in the dermis and subcutis, and might serve as additional specific, albeit fairly limited, pathways for drug absorption. In some cases, for example, hair follicles might act as target sites for drug delivery (Foldvari, 2000).

2.1.3 Hypodermis

This is the innermost layer of skin, consisting of adipose cells, and it functions to provide a cushion between the external skin layers and the internal structures such as bone and muscle. It also provides an energy reserve, allows for skin mobility, moulds body contours, and insulates the body (Eckert, 1992).

2.1.4 Skin appendages

The continuity of the stratum corneum is interrupted by the ducts of eccrine and apocrine glands and by hair follicles with their sebaceous glands. These sites, which probably account for no more than about 1 % of skin surface area, nevertheless serve as entry ports for externally applied substances. These so-called shunts possess a cylindrical structure, and the wall of the cylinder represents a sizable area of epithelially derived tissue that may be extensively hydrated or unprotected by terminally differentiated stratum corneum. Shunt diffusion bypasses the stratum corneum, is rapid, and may allow passage of molecules normally excluded or slowed down by the intact stratum corneum (Rieger, 1993).

2.2 PROCESS AND ROUTES OF TRANSDERMAL DELIVERY

2.2.1 The process of transdermal penetration

The release of a therapeutic agent from a formulation applied to the skin surface and its transport to the systemic circulation is a multistep process, which involves:

- Dissolution within and release from the formulation,
- partitioning into the skin's outermost layer, the stratum corneum,
- diffusion through the stratum corneum, principally *via* a lipid intercellular pathway, (i.e. the rate-limiting step for most components),
- partitioning from the stratum corneum into the aqueous viable epidermis,
- diffusion through the viable epidermis and into the upper dermis, and
- uptake into the local capillary network and eventually the systemic circulation (Kalia & Guy, 2001).

The rate determining step in the percutaneous absorption of most drugs is their permeation across the stratum corneum providing the major portion of resistance (Suhonen *et al.*, 1999).

2.2.2 Routes of penetration

2.2.2.1 Transepidermal route

The transepidermal route includes the following:

- The transcellular path, indicating that the molecules transfer sequentially and repeatedly through the "bricks" and "mortar".
- The paracellular (or intercellular) path (Mukhtar, 1992).

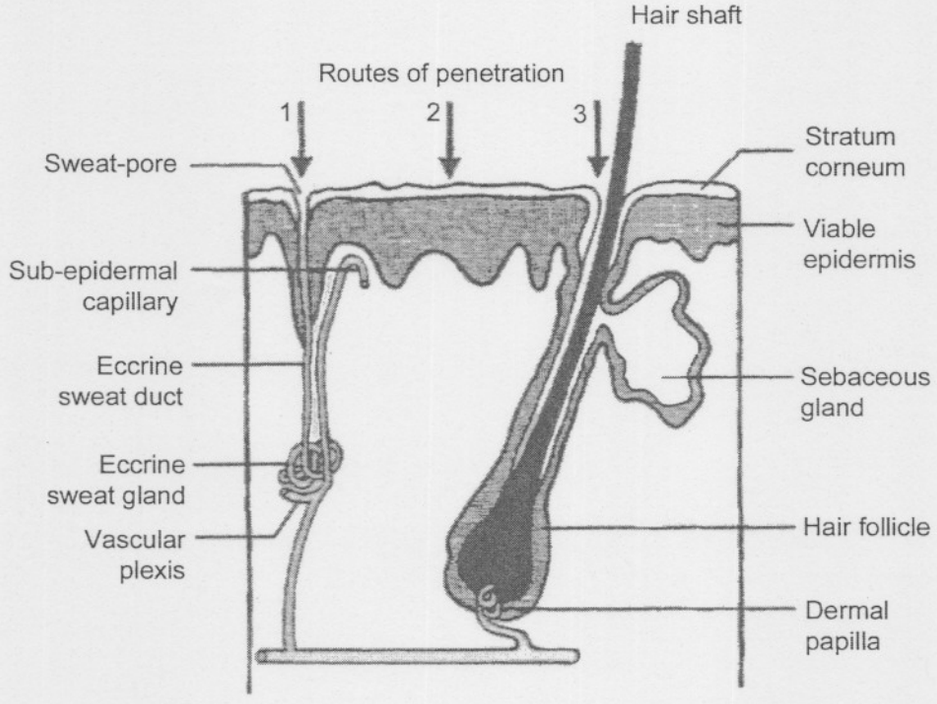


FIGURE 2-3: Simplified diagram of skin structure and macroroutes of drug penetration: (1) *via* the sweat ducts; (2) across the continuous stratum corneum or (3) through the hair follicles with their associated sebaceous glands (Barry, 2001).

The route through which permeation occurs is largely dependent on the penetrant's physicochemical characteristics, the most important being the relative ability to partition into each skin phase (Walters, 1989). The relative surface area of the intercellular route is perhaps 1 % of the total stratum corneum; furthermore the cellular arrangement would require that diffusing substances taking this route follow a highly tortuous path through the membrane. This could help to account for the very low values of apparent diffusion coefficient exhibited by most substances (Zatz, 1993). Most molecules penetrate through skin *via* this intercellular route (Barry, 2001).

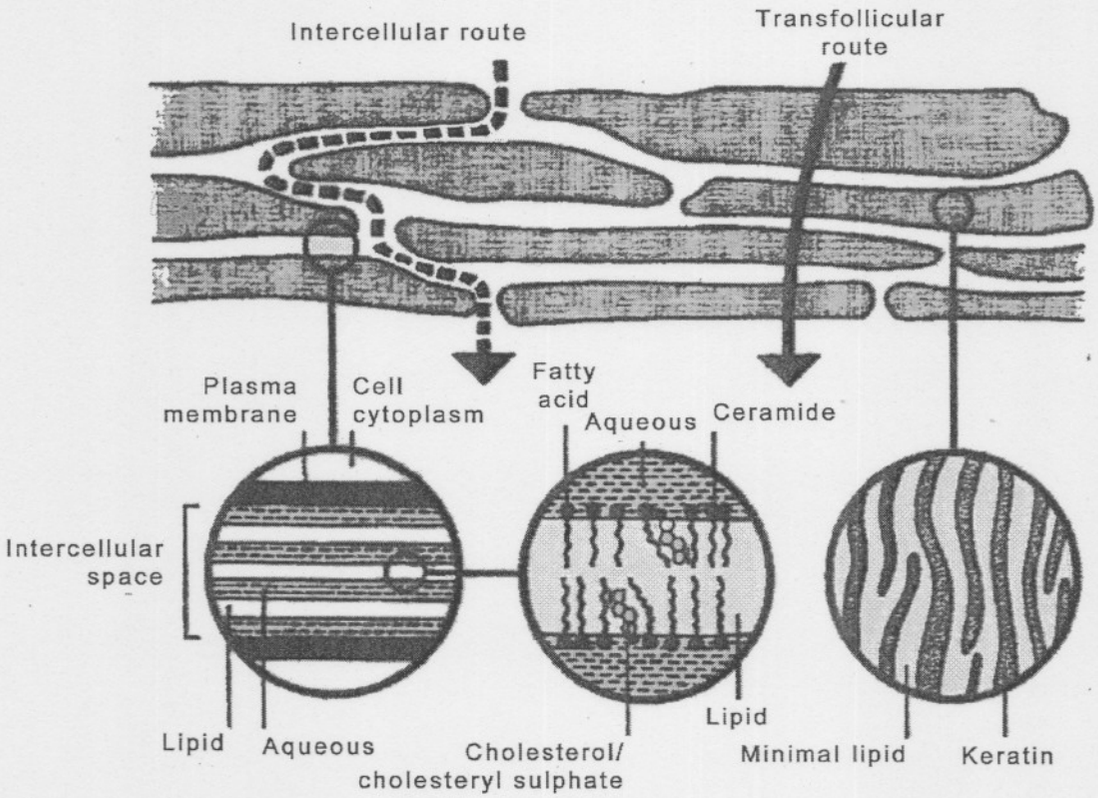


FIGURE 2-4: A scheme representing suggested routes for drug permeation through the stratum corneum i.e., (i) across the corneocytes and the intercellular lipid matrix (the transcellular pathway) and (ii) *via* the lipid matrix between the corneocytes (the intercellular pathway) (Suhonen *et al.*, 1999).

2.2.2.2 Transappendageal route

The pilosebaceous unit (hair follicle, hair shaft and sebaceous gland) provides a route that bypasses intact stratum corneum; it also represents a drug delivery target. The sebaceous gland cells are more permeable than corneocytes and thus drugs can reach the dermis by entering the follicle (bypassing the invaginated stratum corneum), passing through the sebaceous gland or penetrating the epithelium of the follicular sheath. The rich blood supply aids absorption, even though the shunt route's cross-sectional area is small. This pathway may be important for ions and for large polar molecules like polymers and colloidal particles that struggle to cross intact stratum corneum can target the follicle (Barry, 2001).

These skin appendages occupy only 0.1 % of the total human skin surface, and, therefore, it is now widely believed that the transepidermal pathway of passive diffusion is the principal pathway associated with the permeation of drugs through the skin (Suhonen *et al.*, 1999).

2.3 PHYSICOCHEMICAL PROPERTIES OF PENETRATING DRUGS

2.3.1 Solubility and partitioning

The stratum corneum barrier is lipophilic, with the intercellular lipid lamellae forming a conduit through which drugs must diffuse. For this reason, lipophilic molecules are better accepted by the stratum corneum. After diffusing through the entire thickness of the stratum corneum layer, the penetrant must repartition into the more aqueous viable epidermis beneath. Ideally, a drug must possess both lipoidal and aqueous solubilities: if it is too hydrophilic, the molecule will be unable to transfer into the stratum corneum, if it is too lipophilic, the drug will tend to remain in the stratum corneum layers (Naik *et al.*, 2000).

Partition coefficients ($P_{sc/veh}$) are directly related to the free energy of transfer of a substance between two immiscible phases. The partition coefficient ($P_{sc/veh}$) is defined as:

$$P_{sc/veh} = \frac{C_{sc}}{C_{veh}} \qquad \text{(Equation 2-1)}$$

where c_{veh} is the permeant concentration in the vehicle and c_{sc} that in the stratum corneum (Rieger, 1993).

The most common expression of lipophilicity is the logarithm of the n-octanol/water partition coefficient (log P_{oct}). The n-octanol/water two-phase system is a popular model for assessing partitioning at lipid membranes because of the similarities between the n-octanol (long hydrophobic chain and polar hydroxyl group) and membrane lipids (Guy, 1996).

The lipid/water partition coefficient of a drug is the basic determinant of drug permeability through the stratum corneum. A drug with a log P_{oct} value of approximately 2 is considered to be a potential candidate for transdermal delivery. According to Guy (1996), compounds with a log P_{oct} value between 1 and 3, with relative low molecular

weight and relatively low melting points, for example nicotine and nitroglycerin, are likely to display optimum passive skin permeation.

2.3.2 State of ionization

If the penetrant is ionizable, both charged and uncharged species are present in quantities dependent on the pH. Generally, transport of ionized species occurs much less rapidly than transport of the base or unionized species. For example, the transdermal flux of scopolamine was shown to be substantially higher at pH values above the pKa of the weak base. While it is possible that facilitated transport of ion pairs *via* a carrier vehicle may result in enhanced transdermal flux, in general higher fluxes will be obtained by maintaining the pH such that the penetrant is unionized (Kemppainene & Reifenrath, 1990).

It has recently been found that there is surprisingly significant permeation of the ionized drugs through the lipophilic pathway of the stratum corneum. It has been suggested that this could possibly be a result of ion pairing (Hadgraft & Valenta, 2000).

2.3.3 Molecular weight and size

The principal physical properties that affect absorption or transport are molecular mass and size. These factors directly influence the diffusivity, on which the rate of transport depends. Diffusivity is a kinetic term, and is a rough measure of the ease with which a molecule can move about within a medium (in this case, the skin). The larger the molecule, the more difficult it is to move about, and the lower the diffusivity (Kemppainen & Reifenrath, 1990).

Potts and Guy (1992) proposed a two-parameter model to describe permeability coefficients (k_p) of organic compounds *in vitro*. This model was based upon a measure of hydrophilicity (the octanol/water partition coefficient) and molecular size (molecular weight). For compounds ranging in molecular weight from 18 to > 750 and a log P_{oct} from – 3 to + 6, permeability through human skin can be predicted by Equation 2-2:

$$\log k_p = -6{,}3 + 0{,}71.\log P_{oct} - 0{,}0061\ MW \qquad \text{(Equation 2-2)}$$

where log k_p is the permeability coefficient, and MW is the molecular weight.

Pugh *et al.* (2000) confirmed the direct relationship between log k_p and log P_{oct} (a measure of lipophilicity), but found that the relationship between log k_p and MW is also direct (not inverse as in the Potts and Guy regression).

2.3.4 Hydrogen bonding-capacity

When a molecule penetrates the stratum corneum, it must pass through the lipid's polar head groups. There is thus the potential for drug interaction with the polar head groups (Suhonen *et al.*, 1999).

Calculations proved that the the stratum corneum was predominantly a hydrogen bonding donor (α), rather than an acceptor (β), with α_{sc}:β_{sc} = 0.6:0.4. Roberts *et al.* (1996) found that the diffusion of a penetrant is related to the number of hydrogen bonding groups on the solute. One to three hydrogen bonding groups bring pronounced reductions in diffusivity, and further groups have no effect on the minimal value. The hydrogen-bonding properties of a penetrant was found to have a dominant effect on the diffusion across the stratum corneum, but a smaller influence on the partitioning, where lipophilicity might be an important factor.

2.3.5 Melting point

According to the ideal solubility theory, the lower the melting point of a substance the greater its solubility in a given solvent, including skin lipids. A study done by Mackay *et al.* (2001), investigated the effect of melting point of chiral penetration enhancers on their stratum corneum uptake. The pure enantiomers of a chiral compound often possess different melting points, and therefore dissimilar solubilities, to the racemate because of variations in their crystal structure. In agreement with theoretical predictions, depression in melting point of menthol and neomenthol, by selection of the appropriate optical form, increased the amount of terpene delivered to the stratum corneum.

A predictive rule of thumb is that the maximum flux of a drug through the skin should decrease by a factor of 10 for an increase of 100 °C in melting point. Generally, a melting point of < 200 °C is recommended when the suitability of a drug for transdermal drug delivery is considered (Finnin & Morgan, 1999).

2.4 MATHEMATICAL MODEL - FICK'S FIRST LAW

Fick's laws are generally viewed as the mathematical description of diffusion process through membranes. Fick postulated that diffusive flow, which is the flux (J_{ss}) through a membrane should be proportional to the concentration difference between the two sides of the membrane and inversely proportional to the thickness of the membrane. The proportionality constant is defined as the permeability coefficient and this relationship is known as Fick's First Law (Rieger, 1993), as shown in the following equations:

$$J_{ss} = k_p.c_{veh} \qquad \text{(Equation 2-3)}$$

$$k_p = \frac{D.P_{sc/veh}}{h} \qquad \text{(Equation 2-4)}$$

$$J_{ss} = \frac{D.P_{sc/veh}}{h}.c_{veh} \qquad \text{(Equation 2-5)}$$

where J_{ss} is the steady-state flux (mg cm^{-2} hr^{-1}) across a membrane of thickness h cm; $P_{sc/veh}$ is the drug's stratum corneum/vehicle partition coefficient; D is the drug diffusion coefficient (cm^2 h^{-1}) in the stratum corneum; c_{veh} is the drug concentration (mg cm^{-3}) across the vehicle, and k_p is the formulation-dependent permeability coefficient of the drug. The permeability coefficient, k_p, is the product of partitioning into ($P_{sc/veh}$) and diffusion across (D/h) the stratum corneum.

From Equation 2-5, we deduce that the ideal properties of a molecule to penetrate stratum corneum well, are the following:

- Low molecular mass, preferably less than 600 Da, above which D tends to be high.
- Adequate solubility in oil and water – so that the membrane concentration gradient (the driving force for diffusion) may be high (c_{veh} is large). Saturated solutions (or suspensions having the same maximum thermodynamic activity) promote maximum flux in equilibrium systems.
- High but balanced (optimal) $P_{sc/veh}$ (too large may inhibit clearance by viable tissues).

- Low melting point, correlating with good solubility as predicted by ideal solubility theory (Barry, 2001).

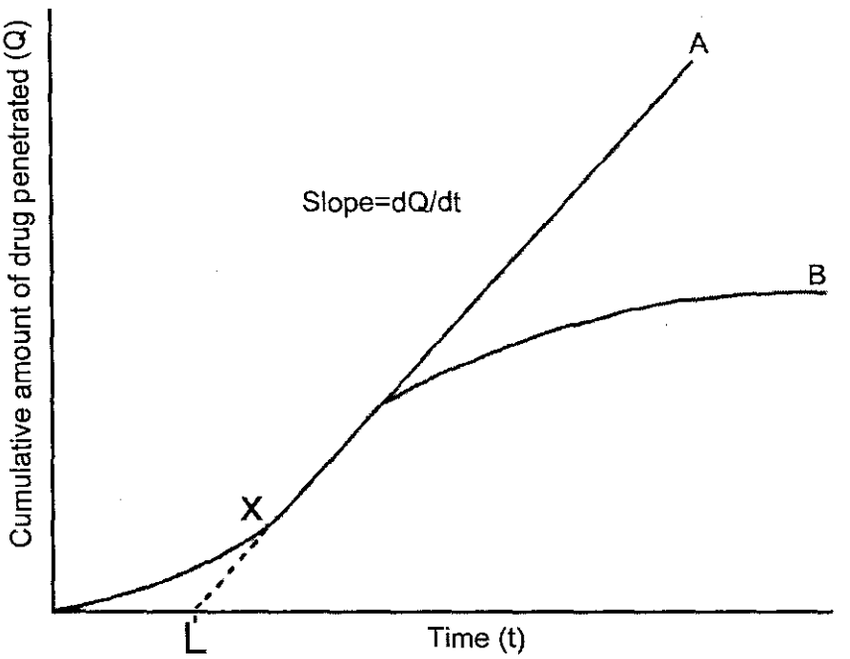


FIGURE 2-5: Plot of cumulative amount of drug (Q) in receptor fluid *vs* time for studies with diffusion cells. (A) Infinite dose with steady state conditions. (B) Depleted or finite dose (Ravis, 1990).

When plotting cumulative amount of drug in receptor fluid *vs* time for studies with diffusion cells according to Figure 2-5, steady state or equilibrium levels have been reached at point X, (Rieger, 1993). The lag time (presented as L in Figure 2-5) is dependent almost entirely on the diffusivity of the penetrant in the stratum corneum. It is also highly dependent on skin thickness, but for a given type of skin, the thickness can be considered as constant. Steady state fluxes are generally achieved within about three lag times (Smith, 1990).

The diffusion coefficient (D) of a drug is a measure of the resistance of the skin to its movement through it, and is influenced by the molecular volume of the drug and the viscosity of the surrounding medium. The diffusion coefficient is defined as the number of moles of a drug that diffuses across a membrane or within the various membrane strata of a given unit area per unit time.

$$D = \frac{h^2}{6t_L} \qquad \text{(Equation 2-6)}$$

In the barrier stratum corneum, D, may be calculated from the lag time, t_L, according to Equation 2-6, if the thickness of the stratum corneum, h, is known (Bach & Lippold, 1998).

2.5 TRANSDERMAL PENETRATION ENHANCEMENT

2.5.1 Chemical potential adjustment

In supersaturated formulations, the thermodynamic activity of the drug in the vehicle is increased above unity, thus enhancing the driving force for drug delivery and increasing skin permeation (Moser *et al.*, 2001). There appears to be an almost linear increase in drug flux with degree of supersaturation (Hadgraft, 1999).

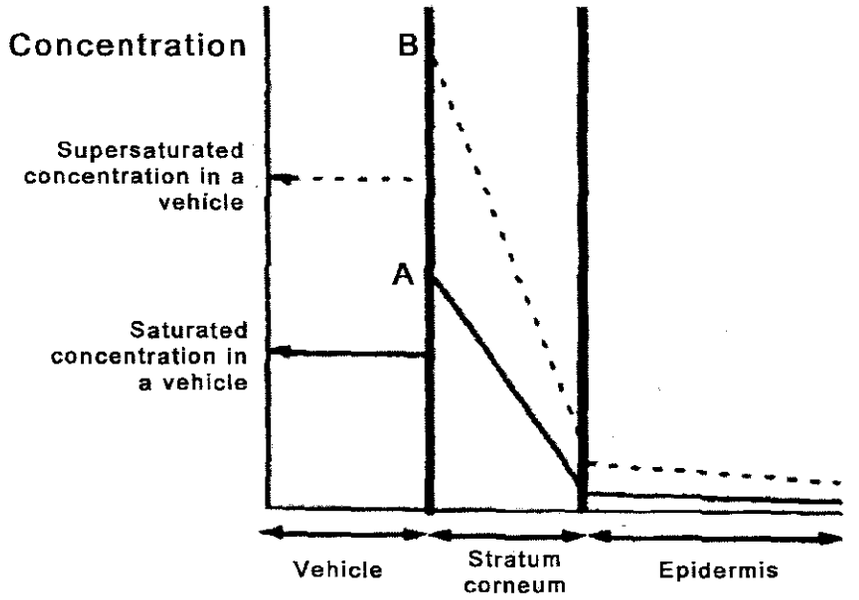


FIGURE 2-6: An adaptation of Higuchi's model of diffusion for saturated and supersaturated solutions (A and B: saturated and supersaturated concentrations in the outer layer of the stratum corneum after application of a saturated and supersaturated solution, respectively) (Pellet, 1997).

An alternative form of Equation 2-5 uses thermodynamic activities established by Higuchi, when

$$J_{ss} = \frac{aD}{yh} \qquad \text{(Equation 2-7)}$$

where a is the thermodynamic activity of drug in its vehicle and y is the effective activity coefficient in the skin barrier. For maximum penetration rate, the drug should be at its highest thermodynamic activity. Dissolved molecules in a saturated solution are in equilibrium with pure solid (which by definition is at maximum activity for an equilibrated system). The solute molecules are thus also at maximum activity (Barry, 2001).

2.5.2 Drug modification

With respect to the drug molecule itself, its lipophilicity can be modified by derivatization. This derivatization strategy is the basis of the prodrug approach in which the therapeutic entity is chemically modified to facilitate, for example, its permeation, although being designed to later yield the parent (active) drug after enzymatic or chemical release (Naik *et al.*, 2000).

2.5.3 Chemical enhancers

Penetration enhancers are chemical compounds which are themselves pharmacologically inactive, but can partition into and interact with the stratum corneum constituents (Suhonen *et al.*, 1999). These chemicals can reversibly compromise the skin's barrier function and consequently allow the entry of otherwise poorly penetrating molecules into the membrane and through to the systemic circulation. Substances reported to render the stratum corneum more permeable include alcohols, polyalcohols, pyrrolidones, amines, amides, fatty acids, sulphoxides, esters, terpenes, alkanes, surfactants and phospholipids (Naik *et al.*, 2000).

2.5.3.1 Mechanisms of enhancement

The three main enhancer mechanisms, as classified by the lipid-protein-partitioning concept, are the following:

1.5.3.1.1 Lipid action:

The actions of enhancers on intercellular lipid bilayers are related to the disordering of the packing of the lipids. This can be achieved through interactions with the polar lipid head groups, such as in the case of water, DMSO and ethanol, or with the hydrophobic lipid tails, such as in the case of oleic acid, azone and terpenes (Foldvari, 2000).

The enhancer disrupts stratum corneum lipid organization, making it more permeable. The essential action increases the drug's diffusion coefficient. The accelerant molecules jump into the bilayer, rotating, vibrating and increasing the free volume available for drug diffusion (Barry, 2001).

The molecular characteristic that typifies an enhancer which disrupts the skin lipids is a polar head group with a long alkyl chain (C_{10} to C_{14} appear to be optimal). One of the problems of such structural features is that this type of molecule also tends to have irritant properties (Hadgraft, 1999).

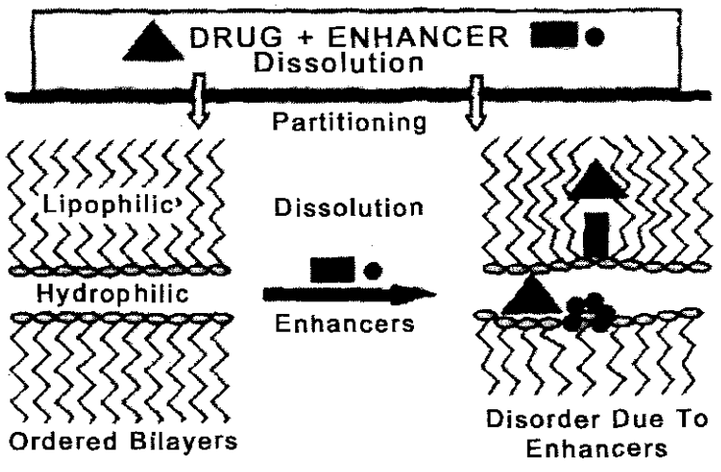


FIGURE 2-7: Schematic representation of the facilitated drug diffusion channels formed by chemical enhancer disruption of ordered intercellular lipid bilayers (Walker & Smith, 1996).

1.5.3.1.2 Protein modification:

To influence the transcellular route (a polar pathway), in which penetration is enhanced by swelling of the intracellular protein matrix, alteration of protein structure within the corneocytes is necessary (Foldvari, 2000).

Ionic surfactants, decylmethylsulphoxide and DMSO interact well with keratin in corneocytes, opening up the dense protein structure, making it more permeable, and thus increasing D (Equation 2-4). However, the intracellular route is not usually important in drug permeation, although drastic reductions to this route's resistance could open up an alternative pathway. Such molecules may also modify peptide/protein material in the bilayer domain, a feature usually neglected in the literature (Barry, 2001).

1.5.3.1.3 Partitioning promotion:

Another way in which skin permeability can be modified is to shift the solubility parameter of the skin in the direction of that of the permeant. Many solvents enter the stratum corneum, change its solution properties by altering the chemical environment, and thus increase partitioning of a second molecule into the horny layer (i.e. raise $P_{sc/veh}$ in Equation 2-5) (Barry, 2001). The solubility of the permeant in the outer layers of the skin will be increased and this, in turn, improves the flux.

2.5.3.2 Terpenes

Terpenes are naturally occurring compounds consisting of isoprene (C_5H_8) units. Terpenes are constituents of essential oils, which are the volatile and fragrant substances found mainly in flavourings, perfumes, and medicines (Gao & Singh, 1998). They were reported to have good toxicological profiles, high percutaneous enhancement abilities, and low cutaneous irritancy at low concentrations (1 - 5 %) (Okabe *et al.*, 1990).

It appears that for hydrophilic drugs, such as 5-fluorouracil, the primary effect of terpene enhancer treatment is to increase drug diffusivity in the horny layer, i.e. to reduce the barrier properties of the skin. For more lipophilic drugs, such as oestradiol, terpenes, in most instances, increase drug diffusivity but also raise drug partitioning into the stratum corneum (Cornwell *et al.*, 1996).

For terpenes, the enhancer-protein interaction might play a relatively small role because they are lipophilic compounds. The terpenes act, at least in part, by modifying intercellular lipids and disrupting its highly ordered structure to increase drug diffusivity (Gao & Singh, 1998).

The effect of enhancers on the permeation of a drug usually depends upon the physicochemical characteristics of both permeant as well as the enhancer molecule. It has been recognized that hydrophilic or oxygen-containing terpenes capable of hydrogen bonding are more active towards promoting the permeation of hydrophilic drugs, whereas, hydrocarbon terpenes (such as limonene) provide higher enhancing activity for lipophilic drugs (Moghimi, 1997; Katayama *et al.*, 1992).

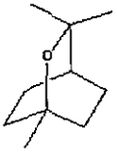
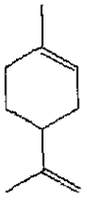
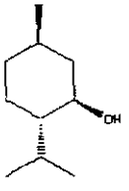
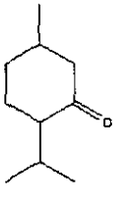
A study done by Kitagawa *et al.* (1998) was in agreement with the above statement when it was found that the permeability coefficients of the relative hydrophilic derivatives (log $P_{oct} < 2$) of benzoic acid with 1 % l-menthol (a relative hydrophilic terpene) on guinea pig dorsal skin increased more than 10-fold. No significant increase of permeability coefficients was observed for the lipophilic derivatives. Koyama *et al.* (1994) concluded that the pretreatment of the skin with lipophilic limonene resulted in a large penetration enhancement for the lipophilic butylparaben and amphiphilic 6-mercaptopurine, but had little effect on the hydrophilic mannitol.

Obata *et al.* (1993), however, found higher enhancing activity of the permeation of diclofenac by limonene than l-menthol, and attributed this phenomenon to differences in the thermodynamic activity of the enhancers in the vehicle. Limonene had a higher thermodynamic activity in the 40 % ethanol buffer solution at the evaluated concentration relative to l-menthol. Similar results were reported by El-Kattan *et al.* (2001) who found that limonene provided high enhancing activity for permeation of nicaridipine hydrochloride (log P_{oct} = -0.99) as well as hydrocortisone (log P_{oct} = 1.43). Higher enhancement activity of limonene was due to its higher thermodynamic activity in the gel at 2 % concentration.

A solution of 5 % 1,8-cineole in 50 % ethanol has been found to enhance the permeability coefficient of model hydrophilic 5-fluorouracil through porcine skin with enhancement ratio of 153.75 (Gao & Singh, 1997). In contrast, Arellano *et al.* (1996), investigated the percutaneous absorption of diclofenac sodium from carbopol gels

containing propylene glycol with 1 % terpene and found that 1,8-cineole was a poor accelerant, and that it could be related to lower thermodynamic activity of the ketone terpene in the gels.

TABLE 2-1: Physicochemical properties of the selected terpenes.

Terpene	Molecular structure	Molecular weight	Melting point (C)	log P_{oct}*
1,8-cineole		154.3	1.5	2.82
d-limonene		136.2	-95	4.58
Menthol		156.3	43	3.40
Menthone		154.3	-6	2.63
β-Myrcene		136.2	-10	4.58

*Log P_{oct} values of the terpenes were determined using the ACD software program (Advanced Chemistry Incorporated, Ontario, Canada).

2.5.4 Vehicles as chemical enhancers

2.5.4.1 Water

Water occlusion hydrates the keratin in corneocytes and increases the water content between adjacent intercellular lipid lamellae. In this way, an active compound diffusing through the intercellular lipid domains will distribute between the hydrophobic bilayer interiors and the aqueous regions separating the head groups of adjacent bilayers. Stratum corneum hydration magnifies the latter environment and increases the 'hydrophobic' character of the stratum corneum somewhat. It follows that this leads, in turn, to a reduction in the stratum corneum/viable epidermis partition coefficient to the penetrant diffusant compound (Morganti *et al.*, 2001).

2.5.4.2 Ethanol

The effect of ethanol on skin penetration is dependent on its concentration in topical vehicles as well as on the lipophilicity of the drugs used (Manabe *et al.*, 1996). Ethanol enhances skin permeability not only of lipophilic compounds at low concentration but also that of hydrophilic compounds at high concentration (Hatanaka *et al.*, 1995).

Hatanaka *et al.* (1995) investigated the time dependence of the skin permeation enhancing effect of ethanol, and concluded that it could be described by an increase in the fraction of the pore pathway and diffusivity in the lipid pathway with the replacement of lipids with ethanol in the stratum corneum.

Manabe *et al.* (1996) evaluated ethanol-water (20 - 100 %) mixed systems as vehicles that enhance skin permeation of drugs, based on the hydrodynamic pore theory. It was found that a low concentration of ethanol (20 - 60 %) extracts lipids from the skin and promotes the permeation of a drug through the pore pathway independent of the drug polarity. A high concentration of ethanol caused protein denaturation, so that it behaved like a porous membrane which is unable to distinguish drug polarity.

2.6 ACYCLOVIR

2.6.1 Identification

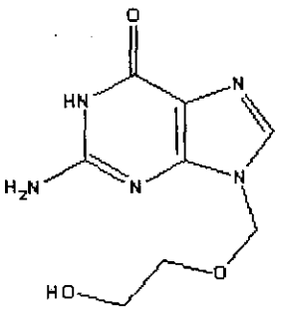


FIGURE 2-8: Molecular structure of acyclovir or 9-((2-hydroxyethoxy)methyl)guanine (Dollery, 1999).

Molecular formula: $C_8H_{11}N_5O_3$

Molecular weight: 225.2

A white powder consisting of elongated rectangular crystals (Dollery, 1999).

2.6.2 Physicochemical characteristics

2.6.2.1 Solubility

Acyclovir is slightly soluble in water, with maximum solubility of 2.5 g/l at physiological pH (Dollery, 1999). Furthermore, acyclovir is insoluble in ethanol, practically insoluble in most organic solvents and soluble in dilute aqueous solutions of alkali hydroxides and mineral acids (Lund, 1993).

2.6.2.2 Acidity and pKa

At the physiological pH of 7.4 acyclovir exists in the unionized form. The pK_{a1} and pK_{a2} values are 2.27 and 9.25, respectively (Dollery, 1999).

2.6.2.3 Partition coefficient

Calculated values for apparent partition coefficients of acyclovir were determined between water and n-octanol over a range of pH values at room temperature. Values for the 'real partition coefficient' (P) calculated, using two sets of results, by two linear regression methods were 0.024 and 0.022 (Kristl & Pečar, 1997).

2.6.2.4 Melting point

Acyclovir melts at about 230 °C, with decomposision (BP, 1993).

2.6.3 Stability

Negligible decomposition of acyclovir aqueous solutions was detected when stored for 37 days at various temperatures. Acyclovir exhibited greater stability in an alkaline solution than in an acidic solution. When acyclovir was boiled for 10 minutes in 1 N sulphuric acid and in 1 N sodium hydroxide, loss of 'potency' was about 12 % and 5 %, respectively (Lund, 1993).

2.6.4 Pharmacology

2.6.4.1 Activity

Following intracellular conversion to a pharmacologically active triphosphate metabolite, acyclovir is active *in vitro* against various Herpesviridae including herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), varicella-zoster virus, Epstein-Barr virus, herpesvirus simiae (B virus), and cytomegalovirus (McEvoy, 2002).

2.6.4.2 Therapeutic use

1. Treatment of herpes simplex keratitis.
2. Treatment and prophylaxis (suppression) of herpes simplex infections of skin and mucous membranes in immunocompetent individuals.
3. Treatment of severe and/or generalized herpes simplex infections in immunocompromised and immunocompetent individuals.

4. Treatment of varicella zoster infections in immunocompromised and immunocompetent individuals.

5. Prophylaxis of herpes simplex, varicella-zoster, and cytomegalovirus infections in the immunocompromised.

6. Improvement of survival of patients with AIDS (Dollery, 1999).

2.6.4.3 Adverse effects

Oral acyclovir is well tolerated; headache, nausea, vomiting, diarrhoea and light-headedness may occur. Intravenous administration may be nephrotoxic, resulting in reversible abnormalities of renal function. Phlebitis at injection sites is common. Abnormality of liver function may occur, and encephalopathic changes with lethargy, tremors, confusion and convulsions have been reported (Gibbon, 2000).

2.6.4.4 Pharmacokinetics

Acyclovir is slowly and poorly absorbed from the gastrointestinal tract, between 13 and 21 %, and the time to reach peak concentrations is 1.5 - 2 h. The mean plasma half-life of intravenous acyclovir is 2.93 (± 0.9) h with a range of 1.5 - 6.3 h. About 80 % of the administered dose is recovered unchanged from the urine, and a further 8.5 – 14 % is the only significant metabolite, the oxidized, 9-carboxymethoxymethyl guanine. Another minor metabolite, 8-hydroxy-9-(2-hydroxyethoxymethyl) guanine can be detected in urine at a concentration of less than 0.15 %. Neither of these metabolites is known to be pharmacologically active (Dollery, 1999).

2.6.5 Previous studies of acyclovir percutaneous penetration

Acyclovir has been shown to be effective against cutaneous HSV-1 infection in mice when administered either orally, intraperitoneally or topically. The failure of topical acyclovir therapy has been found to be due to the inadequate drug delivery to the target site, that is, basal epidermis. Adequate amounts of drug delivery to skin basal epidermis are necessary for the treatment of HSV skin infections because major virus-induced epidermal pathology occurs in the basal epidermis. It is reasonable to conclude that the limited efficiency of topical acyclovir therapy is due, at least in part, to the inability of

acyclovir to penetrate through the stratum corneum barrier layer of the skin and a lack of its reach at the target site, the basal epidermis (Bolger *et al.*, 1997).

The levels of acyclovir found in the skin were, however, considerably higher after topical administration when compared to oral administration. Consistent with its therapeutic efficacy following topical application, acyclovir was found to readily penetrate hairless mouse skin, the extent of which depended on the formulation employed (Bolger *et al.*, 1997).

A comparison of the ratio of the maximum plasma levels of acyclovir per finite dose of acyclovir (mg/kg) on topical (dose of 15 mg/kg) and oral (dose of 100 mg/kg) administration of the hairless mouse, suggests that both routes will deliver comparable amounts of acyclovir into the blood. This observation strongly supports the suggestion that systemic antiviral concentrations of acyclovir could be achieved following topical administration. In substantiation of the hypothesis by other investigators concerning the 'systemic' mode of action for topically applied acyclovir, it was clearly shown that acyclovir can achieve systemic antiviral concentrations following topical application (Bolger *et al.*, 1997).

A series of investigations have been done on the transdermal delivery efficacy and enhancement of acyclovir:

- The effects of vehicle and percutaneous penetration enhancer on the penetration of acyclovir through excised hairless mouse and rat skin was investigated. Four solvents, propylene glycol (PG), ethanol (EtOH), isopropanol (IPA), and isopropyl myristate (IPM), were employed as vehicles. Acyclovir penetration was faster from the vehicle with higher lipophilicity (IPM > IPA > EtOH > PG). When applied in combination with four enhancers, 1- farnesylazacycloheptan-2-one (7FU), 1-geranylazacyclo-heptan-2-one (7GU), 1-geranylazacyclopentan-2-one (5GU), and 1-dodecyl-azacycloheptan-2-one (Azone), the combination of hydrophilic vehicle (PG) and hydrophobic enhancer (Azone) resulted in a large enhancing effect (Okamoto *et al.*, 1990).

- *In vitro* and *in vivo* human skin model systems were used to quantify acyclovir disposition and absorption in skin and blood following oral and topical administration and to investigate whether bioavailability differences were the result of insufficient

drug delivery. A mathematical model of the acyclovir concentration gradient through the epidermis revealed that the drug concentration in the target site of HSV-1 infections, the basal epidermis, is 2 - 3 times less after topical administration than after oral administration. Thus, the observed lack of clinical efficacy with topical acyclovir therapy in the recurring HSV-1 infection likely reflected the insufficient delivery of the drug to the basal epidermis (Parry *et al.*, 1992).

- Lee *et al.* (1992) studied a novel animal model for the topical treatment of cutaneous herpes virus infections, with a focus upon the relationship between the dermal flux of the antiviral agent and the effectiveness of the topical therapy. When the acyclovir flux was 100 $\mu g/cm^2$ per day or greater, a maximum of 100 % topical efficacy was obtained. Systemic efficacy required higher fluxes: approximately 10-fold greater acyclovir fluxes were necessary to provide systemic efficacy equal to the topical efficacy.

- Imanidis *et al.* (1994) quantified the topical and systemic antiviral efficacy of acyclovir transdermal patches as a function of the drug delivery rate of the patches. Drug delivery rates required to attain systemic efficacy were found to be higher than the rates required to attain the same magnitude of topical efficacy. Equal topical and systemic efficacies were found to correspond to equal drug concentrations at the site of antiviral activity.

- Iontophoresis was employed for enhancing the transdermal delivery of acyclovir through nude mouse skin *in vitro*. Anodal iontophoresis shows potential applicability for enhancing acyclovir transport to the skin, considering that both electric transport and electroosmosis can be used by appropriately setting the pH of the donor (Volpato *et al.*, 1995).

- Bando *et al.* (1996a,b) investigated the penetration of seven acyclovir prodrugs through rat skin with or without the enhancer, 1-geranylazacyclo-heptan-2-one (GACH). Under the condition without GACH treatment, more lipophilic prodrugs gave higher partition parameters in the nonpolar route. Concerning the effect of GACH, the estimated partition parameters of prodrugs in the nonpolar route increased. In addition, GACH significantly decreased the enzymatic hydrolysis rate constant of all prodrugs in the skin.

- Patel *et al.* (1996) extensively examined the C* concept for prediction for prediction of the topical antiviral efficacies of acyclovir formulations in hairless mice for the treatment of cutaneous HSV-1 infections. This method was based on estimation of the free drug concentration at the target site (C*), which is presumed to be the basal cell layer of the epidermis. The validity of the C* concept was supported for various acyclovir formulations and the potential of future practical situations was suggested.

- Bolger *et al.* (1997) addressed the issue that topically applied acyclovir may mediate some of its antiviral actions by a systemic mode of action. When topically applied in a formulation consisting of polyvinyl alcohol (25 % w/v): DMSO: cremophor EL: linoleic acid (63:16:16:5, v/v/v/v), acyclovir penetrated hairless mouse skin in a concentration-dependent manner and dose-dependently reduced cutaneous herpes simplex virus 1 KOS infection.

- Data obtained from Jiang *et al.* (1998) show that after removal of stratum corneum the amount of acyclovir is significantly and substantially increased in viable skin layer (1.21 % *vs* 0.20 %) and in the perfusion medium (2.65 % *vs* 0.25 %), thus providing a direct evidence of barrier characteristics of stratum corneum to the absorption of acyclovir.

- Volpato *et al.* (1998) studied the *in vitro* distribution of acyclovir in human skin layers after iontophoresis, applied in order to increase the amount of drug in the basal epidermis, the site of HSV infections. It was proved that acyclovir can be accumulated at the target site more quickly and maintained at higher level through application of a iontophoretic pulse and by keeping drug reservoir on the skin.

- The goal of the study done by De Jalón *et al.* (2001) was to increase the amount of acyclovir in the basal epidermis, using microparticles as carriers. Poly(D,L-lacctic-co-glycolic acid) microparticles loaded with acyclovir were prepared using a solvent evaporation technique. Acyclovir distribution into porcine skin was determined after topical application of microparticles for 6, 24 and 88 hours. Only after 88 h, was the acyclovir reservoir in the basal epidermis higher with microparticles compared to acyclovir suspension control, but the amount in the receptor chamber was much lower. This type of carrier can improve acyclovir topical therapy since it increases

drug retention in the basal epidermis and consequently increases the time intervals between doses.

- Afouna *et al.* (2003) examined the influence of Azone upon the skin target free drug concentration (C*) and its correlation with the *in vivo* antiviral efficacies of cidofovir and acyclovir against HSV-1 infections. Although the estimated C* values for cidofovir formulations with and without Azone were comparable, formulation with Azone was much more effective than that without Azone in all treatment protocols. For acyclovir formulations, in contrast, addition of Azone has failed to show any effect on the preventive *in vivo* antiviral efficacy and the enhancement of acyclovir *in vivo* antiviral efficacy was merely the skin permeation enhancement effect of Azone.

2.7 SUMMARY

Criteria that merit consideration of the transdermal delivery route are the nature of the barrier, the balance between the physicochemical properties of the membrane and the drug, and the technologies available to the pharmaceutical scientist to facilitate transdermal transport (Naik *et al.,* 2000).

The stratum corneum has been identified as the principal barrier of the skin and is therefore of relevance for percutaneous penetration of drugs. It was indicated that the stratum corneum forms a continuous sheath of protein-enriched corneocytes embedded in an intercellular matrix, enriched in non-polar lipids organized as lamellar lipid bilayers, giving it a 'brick and mortar' structure.

Essentially, the stratum corneum barrier is lipophilic. For this reason, lipophilic molecules are better accepted by the stratum corneum. Ideally, a drug must possess both lipoidal and aqueous solubilities to partition through the various components and layers of the skin.

Despite these limiting factors, several techniques opt to promote transdermal delivery of drugs, including supersaturation, drug modification and chemical enhancement. Most molecules penetrate through the skin *via* the intercellular route and therefore many enhancing techniques aim to disrupt or bypass its elegant molecular structure and thus to enhance drug delivery.

CHAPTER 3:

IN VITRO TRANSDERMAL PERMEATION OF ACYCLOVIR AND TERPENES

3.1 INTRODUCTION

Topical treatment of herpes simplex with acyclovir has been reported to reduce the duration of viral shedding and accelerate the healing of the infections. Unfortunately, the clinical course of recurrent herpes simplex is not altered and has been attributed to inadequate acyclovir penetration through the stratum corneum. Improvements in the topical delivery of acyclovir should provide a therapeutic benefit, and vehicles which have been shown to enhance skin penetration of other agents might increase the penetration of acyclovir as well (Cooper *et al.*, 1985).

In an attempt to overcome the problems arising from skin impermeability and biological variability and to raise the number of drug candidates for transdermal drug delivery, various approaches have been investigated. Among these approaches is the use of penetration enhancers (Suhonen *et al.*, 1999). In this study, terpene penetration enhancers have been employed to increase acyclovir transdermal flux, which include 1,8-cineole, limonene, menthol, menthone and β-myrcene.

Terpene compounds combine good penetration enhancing abilities with low skin irritancy and low systemic toxicity. Ideally, penetration enhancers would promote transdermal absorption of drugs, and not penetrate simultaneously. In this study, the amount of terpenes that penetrated the skin were also measured, with and without the presence of acyclovir, to determine the amount of terpene penetrated and establish possible correlations between enhancement and penetration of the terpenes.

3.2 REAGENTS AND MATERIALS

Acyclovir, menthone, eucalyptol (1,8-cineole), menthol, β-myrcene and R-(+)-limonene were supplied by Sigma-Aldrich Co. (Johannesburg, SA). Anhydrous di-potassium hydrogen orthophosphate (K_2HPO_4), analytical grade methanol and phosphoric acid, as well as sodium chloride (NaCl), disodium hydrogen orthophosphate dihydrate ($Na_2HPO_4.2H_2O$) and sodium dihydrogen ortophosphate dihydrate ($NaH_2PO_4.2H_2O$) were supplied by Merck Laboratory Supplies (Midrand, South Africa). Absolute ethanol (99.9 %) was supplied by Saarchem (South Africa). The water used throughout this study was HPLC grade, double deionized with a Milli Q 50 water purification system (Millipore, Milford, USA).

3.3 ANALYTICAL METHODS

3.3.1 High performance liquid chromatography (HPLC)

In order to determine the amount of acyclovir that penetrated the skin, samples were taken from the receptor solution that needed to be quantified. Quantification of compounds by high performance liquid chromatography (HPLC) is the process of determining the unknown concentration of a compound in a known solution. In this study, very low concentrations of acyclovir were to be determined in the receptor fluid that contained phosphate buffered saline (PBS). Previously reported methods for estimation of acyclovir were studied (Patel *et al.*, 1996; Cooper *et al.*, 1985), and were modified and offered enhanced sensitivity as well as reduced run times.

3.3.1.1 The HPLC system

The HPLC analysis of acyclovir was performed using an Agilent 1100 Series HPLC, equipped with an Agilent 1100 pump, autosampler, UV detector and Chemstation Rev. A06.02 data aquisition and analysis software. A Luna, 5μ, 250 x 4,60 mm, C_{18} Phenomenex column with a Phenomenex Security Guard precolumn was used. Analysis was performed with a mobile phase comprising of 10 % methanol and 90 % water containing 6.96 g of anhydrous dipotassium hydrogen orthophosphate per liter. The pH was adjusted to 7.4 with a 10 % phosphoric acid solution. The mobile phase was filtered through a 0.45 μm Millipore filter. The samples were eluted at a flow rate of

1.0 ml/min and monitored at 254 nm for acyclovir. Analysis was performed at room temperature.

3.3.1.2 Preparation of acyclovir standard solutions

Ten milligram of acyclovir was dissolved in 100 ml HPLC grade water to obtain a 100 μg/ml solution. Standard solutions with concentration of 0.05, 0.3, 0.5, 2 and 5 μg/ml were prepared from the 100 μg/ml stock solution. All the acyclovir standard solutions were prepared daily.

3.3.1.3 Validation of the HPLC method

3.3.1.3.1 Calibration curve

A calibration curve for acyclovir ranging from 0.05 μg/ml – 5 μg/ml was compiled using standard solutions with concentrations of 0.05, 0.1, 0.2, 0.3, 0.5, 2 and 5 μg/ml.

3.3.1.3.2 Linearity

The linearity for acyclovir was determined by performing linear regression analysis on the plot of the peak-area ratios against concentrations in the range 0.025 – 5 μg/ml.

Table 3-1 contains the regression values obtained for the standard curves of acyclovir.

TABLE 3-1: Regression values obtained for acyclovir

Concentration (μg/ml)	R-squared	Slope	Y-intercept
0.05 – 5 μg/ml	0.9999	345.32	-1.199

3.3.1.3.3 Selectivity

Blank samples of phosphate buffered saline (PBS) used as the receptor phase in diffusion studies, as well as samples containing known concentrations of ethanol, terpenes and acyclovir were injected into the HPLC.

No interfering peaks were encountered at the retention time of acyclovir (5.8 min).

3.3.1.3.4 Precision

The precision of the method was investigated in terms of inter-day (reproducibility) and intra-day (repeatability) variations.

- **Reproducibility:**

The inter-day variability was determined performing HPLC analysis of three known concentrations (0.5, 2 and 5 μg/ml) on three different days. Results in terms of percentage recovered are shown in Table 3-2.

TABLE 3-2: The mean percentage recovered, standard deviation and percentage relative standard deviation (% RSD) by analysis of three samples on three different days (n = 6).

Concentration (μg/ml)	Mean % recovered	Standard deviation	% RSD
0.5	96.60	4.99	5.17
2	97.59	5.98	6.12
5	99.66	7.74	7.76

- **Repeatability:**

The intra-day variability was determined performing HPLC analysis of three known concentrations (0.5, 2 and 5 μg/ml) on three times during a single day. Results in terms of percentage recovered are shown in Table 3-3.

TABLE 3-3: The mean percentage recovered, standard deviation and percentage relative standard deviation (% RSD) by analysis of three samples on a single day (n = 6).

Concentration (μg/ml)	Mean % recovered	Standard deviation	% RSD
0.5	100.01	1.23	1.23
2	94.49	3.25	3.44
5	95.86	3.29	3.44

3.3.1.3.5 Sensitivity

The limit of detection of acyclovir was expressed as a concentration at a signal-to-noise ratio of three-to-one, and was determined as 0.5 ng/ml.

On a signal-to-noise ratio of ten-to-one, the limit of quantification for acyclovir was determined as 3.5 ng/ml.

3.3.1.3.6 System repeatability

A sample of a known concentration (0.2 μg/ml) of acyclovir was injected six times on the same day and under the same conditions, to evaluate repeatability of the system. The variation in response (% RSD) of the repeatability of the peak area was found to be 0.22 % and that of the retention times was 0.085 %.

3.3.2 Gas chromatography

3.3.2.1 The GC system

An Agilent 6890 series headspace gas chromatograph (Hewlett Packard) was used in the semi-quantitative analysis of the permeated terpenes, with the following conditions:

Column:	WCOT fused silica capillary column (Cronpak), with length of 30 m, inside diameter 0.25 mm, outside diameter 0.39 mm and film thickness 0.25 mm.
Stationary phase:	Cp-wax-52-CB
Oven temperature:	Oven temperature was maintained at 70 °C for 5 minutes, after which it was heated at 40 °C per minute up to a temperature of 180 °C where it was maintained for 6 minutes.
Front injector temperature:	250 °C
Front detector temperature:	260 °C

3.3.2.2 Preparation of standard solutions of terpenes

Standard solutions of menthol were prepared by diluting 50 mg of each in a 100 ml volumetric flask with 50 % ethanol and water to create a 500 μg/ml mother solution. From this solution, five standard solutions with concentrations ranging between 0.976 and 62.5 μg/ml were prepared. Mother solutions of 2 μg/ml of 1,8-cineole, limonene, menthone and β-myrcene were prepared by diluting 0.02 ml of each with aqueous ethanol in a 10 ml volumetric flask. Dilutions from these mother solutions were made to produce concentrations ranging between 0.008 μg/ml and 0.8 μg/ml.

3.3.2.3 Calibration curves

The amount of the terpenes in the receptor compartment samples was determined by comparison to a calibration curve established for each terpene from five standard solutions, as mentioned above.

3.3.2.4 Retention times

The retention times for the terpenes were the following:

1,8-Cineole = 7.4 min

Limonene = 7 min

Menthol = 11.9 min

Menthone = 10.4 min

β-Myrcene = 6.3 min

The samples contained ethanol which had a retention time of 3.6 minutes, and therefore did not interfere with any of the above peaks.

3.4 EXPERIMENTAL METHODS

3.4.1 Preparation of saturated solutions of acyclovir

A saturated solution of acyclovir was prepared in order to provide maximum thermodynamic activity of the drug. At a pH of 7.4, acyclovir exists in the unionized form. The acyclovir solutions was therefore prepared by suspension of acyclovir in phosphate buffered saline (PBS), which have the same consistancy as the PBS used as receptor solution, i.e. 4.4 g sodium chloride (NaCl), 9.2 g disodium orthophosphate dihydrate ($Na_2HPO_4.2H_2O$) and 2.1 g sodium dihydrogen ortophosphate dihydrate ($NaH_2PO_4.2H_2O$) in water to 1000 ml. For 24 hours prior to each experiment, excess acyclovir was suspended in the PBS at 37 °C. An amount of undissolved drug is maintained to keep the drug concentration at a constant level (saturation) during the whole permeation experiment. The saturated solubility of acyclovir was determine by filtering the solution through a 0.45 μm filter (Glassfibre Prefilter, Sartorius AG) and assayed by HPLC.

3.4.2 Preparation of terpene pretreatment solutions

A pretreatment solution was prepared for each terpene. An amount of 5 g of menthol, or a volume of 5 ml of 1,8-cineole, limonene, menthone or β-myrcene were diluted with absolute ethanol to create a 5 % solution.

3.4.3 Preparation of skin

To minimize the variability in the skin permeability properties between different anatomical sites (which have varying thicknesses of stratum corneum and follicle densities), only female abdominal skin was employed, obtained from cosmetic surgery. The full-thickness skin was frozen at -20 °C not longer than 24 h after removal. Excess fatty and connective tissues were removed after which the skin was immersed in water at 60 °C for 1 minute. The epidermis was carefully removed, placed on Whatman® filter paper and left to dry. The prepared skin samples were wrapped in aluminium foil and sealed in plastic bags. The prepared skin was frozen at -20 °C and stored for a maximum of two months before use. The frozen skin pieces were examined for defects before mounting them within the diffusion apparatus.

3.4.4 Diffusion studies

Vertically mounted Franz diffusion cells (see Figure 3-1) were used, with the epidermis (cut into circles with a diameter of ± 2 cm) mounted between the two compartments (stratum corneum facing the donor compartment side). The maximum capacities of both the donor and receiver compartments are 1 ml and 2 ml respectively. The effective diffusion area is 1.0751 cm^2.

Prior to experimentation, the upper side of the skin was pretreated by the application of 25 µl of a 5 % solution of terpene in ethanol, followed by a waiting period of 30 minutes to ensure ethanol evaporation. A thin film of terpene was therefore present on the skin by the time the saturated acyclovir solution was added.

The donor compartment contained 1 ml of saturated solution of acyclovir dissolved in buffered solution at a pH of 7.4. This compartment was covered with parafilm in order to prevent evaporation after the saturated solution was added.

The content of the receptor compartment was continuously stirred with a small magnetic bar at 500 rpm and the temperature maintained at 37 °C by means of a water bath. This will keep the skin surface at approximately 32 °C, which simulates the temperature of the human skin. The system was allowed to equilibrate for 1 hour before the addition of the acyclovir solution.

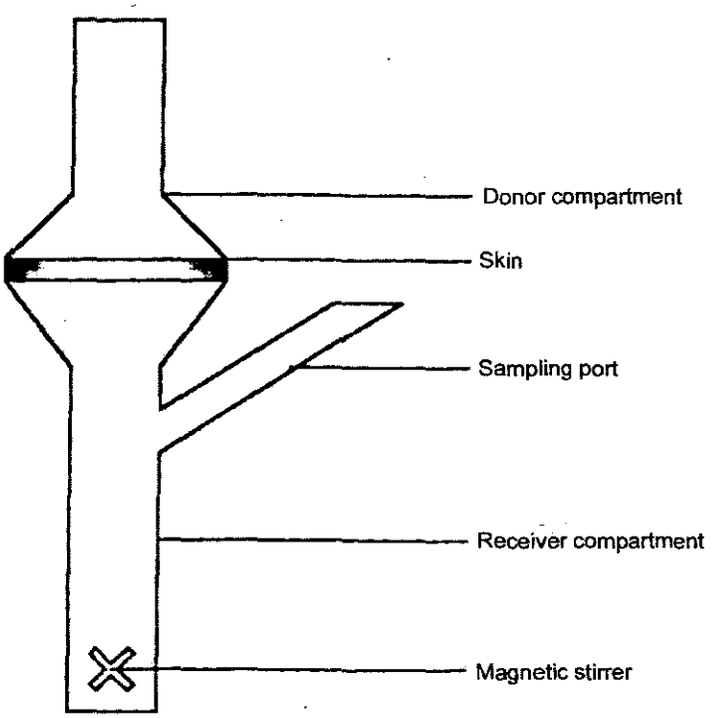


FIGURE 3-1: Schematic representation of the standard original diffusion cell developed by Franz (Bronaugh & Collier, 1993).

In the acyclovir permeation studies, the receptor compartment contained phosphate buffered saline (PBS) at physiological pH, consisting of 4.4 g sodium chloride (NaCl), 9.2 g disodium orthophosphate dihydrate ($Na_2PO_4.2H_2O$) and 2.1 g sodium dihydrogen ortophosphate dihydrate ($NaH_2PO_4.2H_2O$) in water to 1000 ml.

Considering the limited solubility of terpenes in phosphate buffered saline, the receptor compartment contained 50 % aqueous ethanol in the terpene permeation studies, which have successfully predicted *in vivo* percutaneous absorption for several chemicals (Dick *et al.*, 1996).

3.4.5 Sample collection

In the acyclovir permeation studies, the total contents of the receptor compartment from each diffusion cell was withdrawn at 2, 4, 6, 8, 10, and 12 hours, and immediately replaced with equivalent amount of fresh 37 °C PBS in order to maintain sink conditions. The amount of acyclovir that penetrated the skin was recovered in the receptor compartment and analyzed by HPLC.

In terpene penetration studies, samples were taken at 2, 4, 6, 12 and 24 hours, and replaced with equivalent amount of fresh 50 % aqueous ethanol. The amount of terpene that has penetrated the skin was analyzed by GC.

3.4.6 Calibration curves

The concentration of acyclovir retrieved from the receptor compartment was determined by comparison to a calibration curve established from standard solutions. Calibration curves for acyclovir ranging from 0.5 μg/ml – 5 μg/ml were drawn up prior to each experiment.

The amount of the terpenes in the receptor compartment samples was determined by comparison to a calibration curve established from standard solutions, as described in § 3.3.2.2 and § 3.3.2.3.

3.4.7 Data analysis

3.4.7.1 Acyclovir permeation

The cumulative amount of acyclovir that penetrated through the epidermis (n = 6) per unit area ($\mu g/cm^2$) was calculated by multiplying the concentration ($\mu g/ml$) in the receptor phase with the amount of receptor phase used (1.9 – 2.4 ml) and dividing it by the area of epidermis (1.075 cm^2).

The cumulative corrected amount of drug, *Q*, was then plotted as a function of time *t* (h). The flux (J_{ss}, $\mu g\ cm^{-2}\ h^{-1}$) was obtained from the slope of the linear part of the curve (steady state), and was calculated by the following equation:

$$Jss = \frac{\Delta Q}{\Delta t} \qquad \text{(Equation 3-1)}$$

The permeability coefficient (k_p) was calculated by curve fitting of the permeation data. The curve-fitting of data on Easyplot for Windows provided an α and β value, where:

α = Ph and

$\beta = D/h^2$

with P the partition coefficient, h the membrane thickness and D the diffusion coefficient. The product $\alpha\beta$ is equal to the permeability coefficient (k_p). The flux of acyclovir in each of the experiments is equal to the product of the permeability coefficient and the saturated solubility of acyclovir.

The lag time (t_L) was estimated by extrapolating the linear region of the cumulative amount of drug permeated *versus* time plot. The diffusion coefficient was calculated using the lag time, by the following equation:

$$D = \frac{h^2}{6t_L} \qquad \text{(Equation 3-2)}$$

The membrane thickness h, in the case of the epidermis, is assumed to be 40 μm (Volpato *et al.*, 1998).

The permeation enhancing activities were expressed as enhancement ratios of flux (E.R.) according to Equation 3-3 (Williams & Barry, 1991):

$$E.R. = \frac{flux\ of\ acyclovir\ with\ terpene}{flux\ of\ acyclovir\ without\ terpene (control)} \qquad \text{(Equation 3-3)}$$

3.4.7.2 Terpene permeation

The cumulative amount of terpenes (n = 6) that penetrated through the epidermis per unit area (μg/cm^2) were calculated by multiplying the concentration (μg/ml) in the receptor phase with the amount of receptor phase used (1.9 – 2.4 ml) and dividing it by the area of epidermis (1.075 cm^2). The percentage of the applied dose of terpene penetrated was calculated and plotted on a graph *versus* time (h).

3.5 RESULTS

3.5.1 Transdermal permeation of acyclovir

Three sets of experiments were done to determine possible acyclovir enhancement:

1. Saturated solutions of acyclovir in the donor compartment without any pretreatment of the skin.

2. Pretreatment of the skin with 20 μl of a 5 % solution of terpene in absolute ethanol, and left uncovered for 30 minutes until the ethanol has evaporated and the terpene left as a thin film on the skin. Hereafter aqueous saturated solutions of acyclovir were placed in the donor compartment.

3. Pretreatment of the skin with 20 μl of ethanol, and measuring the acyclovir penetration enhancement effects of ethanol alone.

3.5.1.1 Results

The saturated solubility of acyclovir in the buffer solution was determined as 1.458 mg/ml. Pretreatment of the skin with ethanol was used as control to study the effect of ethanol on the *in vitro* percutaneous absorption of acyclovir (control 2). The effect of ethanol pretreatment was also investigated on the permeability of acyclovir with respect to no treatment (control 1). The *in vitro* percutaneous absorption profiles (flux, J_{ss}; enhancement ratios of acyclovir, E.R.; lag time, t_L; and logarithm of permeability coefficient, log k_p) are given in Table 3-4.

TABLE 3-4: The effect of terpenes on the percutaneous parameters of acyclovir.

Pretreatment	Flux ($\mu g/cm^2/h$)	E.R.[a]	E.R.[b]	lag time (min)	log k_p
Control 1	0.15 ± 0.06	-	-	10.818	-3.966
Control 2	0.21 ± 0.06	1.34	-	0.042	-3.821
1,8-Cineole / EtOH	0.65 ± 0.26	4.205	3.130	11.34	-3.350
Limonene / EtOH	0.29 ± 0.12	1.870	1.392	0.018	-3.635
Menthol / EtOH	0.55 ± 0.24	3.536	2.632	0.024	-3.365
Menthone / EtOH	0.20 ± 0.04	1.294	0.963	25.512	-3.878
β-Myrcene / EtOH	0.30 ± 0.11	1.937	1.442	0.018	-3.599

[a]Flux of acyclovir after pretreatment with 5 % terpene in EtOH/ flux without pretreatment)
[b]Flux of acyclovir after pretreatment with 5 % terpene in EtOH/ flux with EtOH pretreatment)

The steady state flux (J_{ss} ± standard deviation) was determined from the slope of the linear portion of the cumulative amount *versus* time plot. Considering that ethanol was present in all pretreatment solutions, it was assumed to have a constant influence on the flux of acyclovir across the skin in the presence of 5 % of the terpenes. The enhancement ratios of acyclovir with terpene pretreatment are therefore expressed as two parameters: E.R.[a] and E.R.[b], calculated from the flux of control 1 and control 2, respectively. E.R.[a] is the calculated value obtained by the division of the flux of acyclovir

after terpene pretreatment of the skin by the acyclovir flux obtained when no pretreatment was employed. E.R.^{b} is the result of the division of the flux of acyclovir after terpene pretreatment of the skin by the acyclovir flux after ethanol pretreatment. The lag time (t_L) was determined by extrapolating the linear portion of the curve to its intersection with the x-axis. The permeability coefficient, k_p, was determined as described in § 3.4.7.1.

The mean flux and standard deviations of acyclovir with the various pretreatments of the skin as in Table 3-4 are presented in Figure 3-2. The transdermal flux of acyclovir with no pretreatment of the skin (control 1) was the lowest, followed by the flux of acyclovir after skin pretreatment with ethanol (control 2). The most prominent flux values of acyclovir was achieved after 1,8-cineole or menthol pretreatment. When the skin was pretreated with limonene or β-myrcene, a moderate increase in acyclovir flux in comparison with control 1 was observed, but only a mild increase in comparison with control 2. A slightly higher flux value was detected after menthone pretreatment in comparison with control 1. No difference in flux value was found between the acyclovir fluxes of control 2 and menthone pretreatment.

Figure 3-3 represents the enhancement ratios of acyclovir in the presence of the different pretreatment protocols in relation to control 1 (E.R.^{a}) and control 2 (E.R.^{b}). E.R.^{a} is a value of 0.34 higher than E.R.^{b} in all the experiments, representing the difference in the flux of acyclovir control 1 and control 2, with ethanol having an enhancement ratio of 1.34. From this graphical representation (Figure 3-3) the extent of the enhancing effects of 1,8-cineole and menthol are evident, with enhancement ratios of 4.205 and 3.130 for 1,8-cineole and 3.536 and 2.632 for menthol. Low enhancement was observed after limonene (1.87 and 1.392) and β-myrcene (1.937 and 1.442) pretreatment. E.R.^{b} of ~ 1 which was found in the case of menthone pretreatment of the skin, provided evidence that there were no difference in the enhancing of acyclovir penetration with these terpenes in comparison to control 2.

From Table 3-4, a lag time of 10.818 minutes obtained from control 1 was observed. A significant decrease in lag time was observed when the skin was pretreated with ethanol and limonene, menthol or β-myrcene. The lag time increased after menthone pretreatment, and a slight increase after 1,8-cineole pretreatment.

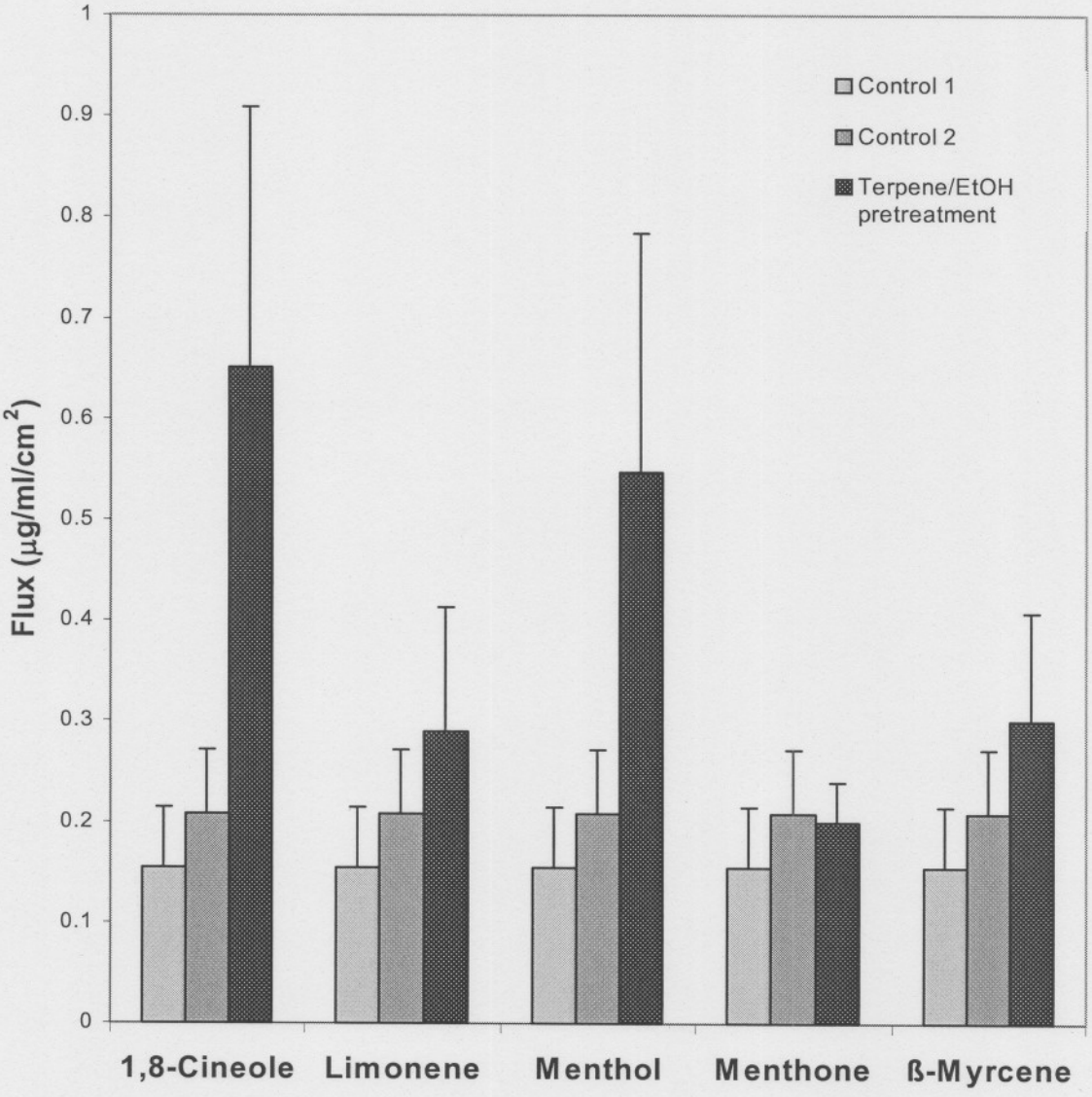


FIGURE 3-2: The mean steady state transdermal flux ± SD of acyclovir without pretreatment (control 1), with ethanol pretreatment (control 2) and with 5 % terpene in ethanol.

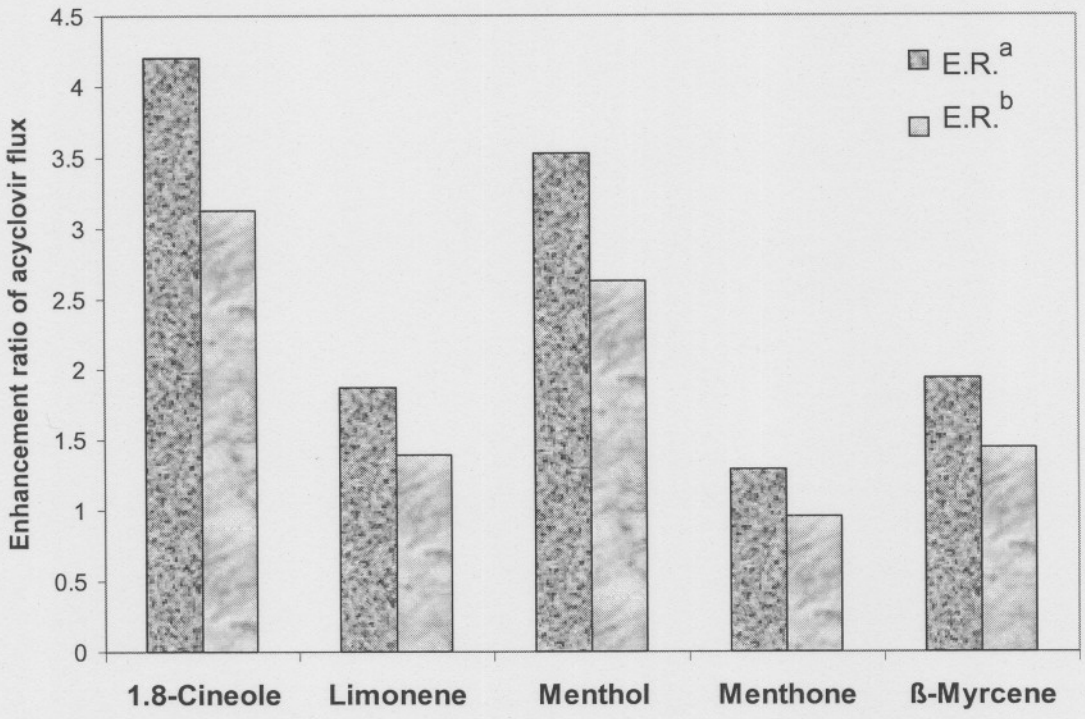


FIGURE 3-3: The enhancement ratios of acyclovir flux after terpene pretreatment with respect to no pretreatment (E.R.^{a}) and ethanol pretreatment (E.R.^{b}).

3.5.1.2 Statistical analysis

An analysis of variance (ANOVA) was done to determine if there were statistically significant differences between the mean of the flux values ($\mu g/cm^2/h$) of acyclovir. The Dunnett's t Tests was used to determine the differences in flux values when the skin was pretreated with the terpenes, compared to control 1 and control 2. Statistically significant differences, with the level of probability taken as $p < 0.05$ as the level of confidence by Student's t test, were found between the flux values of 1,8-cineole and menthol pretreatment, in comparison to control 1 and 2. No statistically significant differences could be shown between control 1 and 2. The difference between limonene, menthone and β-myrcene pretreatments and the two controls was also not significant.

3.5.1.3 Discussion

At a concentration of 1.458 mg acyclovir/ml in the donor compartment, acyclovir was capable of passing the human epidermis at a rate of 0.16 $\mu g/cm^2/h$, taken as control flux rate (control 1). A lag time of about 10 minutes was observed. The stratum corneum barrier properties (i.e. porosity and tortuosity) are associated with the polar regions of the stratum corneum intercellular lipids for the transport of polar permeants. The pore pathway may be viewed as the transport along the polar/aqueous regions between bilayers of the intercellular lipids. This route is highly tortuous and the physicochemical properties of acyclovir permits it to a resulted slower diffusion through the stratum corneum compared to a more lipophilic compound, and a higher flux and longer lag time are expected under control conditions.

As terpene were dissolved in ethanol, skin was also pretreated with this substance, in order to elucidate if ethanol itself acted as a percutaneous enhancer for acyclovir permeation. When the skin was pretreated with ethanol (control 2), the maximum flux of acyclovir increased by an enhancement factor of 1.34. No statistically significant differences could be shown between the fluxes of control 1 and 2. This means that ethanol had no or only a slight influence.

In contrast to this finding, ethanol is regarded as a permeation enhancer. Ethanol enters the skin and removes measurable quantities of the barrier material (Bommannan *et al.*, 1991). The transdermal enhancing effects are regarded to be dependent on concentration and time.

The concentration-dependent enhancing effect is well known (Hatanaka *et al.*, 1995): ethanol enhances skin permeability not only of lipophilic compounds at low concentration but also of hydrophilic compounds at high concentration. Using the parallel permeation pathway model, Hatanaka *et al.* (1995) have suggested that ethanol can create new pores in the stratum corneum and then increase the fraction of the aqueous pathway (ϵ). The increase in ϵ may be time-dependent because of slow progress of delipidization. There is also a possibility that ethanol acts on the stratum corneum *via* a lipid-disordering mechanism. Such an action would cause an increase in diffusivity of permeants in the lipid pathway (D_L) with time, with the replacement of lipids by ethanol in

the stratum corneum. In conclusion, the study suggested that both ϵ and D_L increase as pretreatment with ethanol is prolonged.

Furthermore, leaching of lipids from the skin into ethanol results from replacement of lipids by ethanol in the stratum corneum, so that the amount of lipids leached served as an index of ethanol concentration in the stratum corneum. Hatanaka and co-workers (1995) attempted to describe the time-dependent effect of ethanol on the skin permeation in relation to lipid leaching. The cumulative amount of sterols leached (Q_s) could be described mathematically by Equation 3-4:

$$Q_s = 0.357t + (40.2t/0.979+t) \qquad \text{(Equation 3-4)}$$

where t represents the pretreatment time (h).

In the current study, the pretreatment time with ethanol was 30 minutes. Using equation 4-4, the amount of sterol leached in this time span is calculated as 13.608 $\mu g/cm^2$, much lower than ~ 50 $\mu g/cm^2$ as in the case of 32 hour pretreatment. Therefore, a short pretreatment time could be a possible reason for the low enhancing effect of ethanol.

A study done by Manabe *et al.* (1996) analyzing the skin penetration enhancing effect of drugs by mixed ethanol-water systems with the hydrodynamic pore theory, found decreased skin permeability when absolute ethanol was used. No clear reason was stated. It was suggested that the low permeation produced by pure ethanol were related to the amount and structure of protein in the skin. A possible suggestion made by the same investigators, was that the fraction of lipid pathway was lowered and that the pore pathway (solvent filled domain) was increased by delipidization at a higher concentration of ethanol. The pore paths can be assumed as a domain filled with the mixed solvent. If this assumption is correct, the solubility of acyclovir in the skin under these conditions is dependent on its solubility in this mixed solvent, or ethanol, as was used in this study.

It has been generally accepted that solvents such as ethanol or propylene glycol used with enhancers accumulate in the tissue and increase the partitioning of drugs due to large affinities of drugs for the solvents (Gao & Singh, 1998). Ethanol can shift the solubility parameter in the direction of the permeant, increasing the solubility of the permeant in the outer layers of the skin and this improves flux (Hadgraft, 1999). Acyclovir, however, has a lower ethanol solubility (0.2 mg/ml) than water solubility

(2.5 mg/ml) (McEvoy, 2002). Ethanol treatment is suggested to remove stratum corneum intercellular lipids and water. The loss of transepidermal water caused by ethanol could in fact lower acyclovir permeation through the polar pathway. On the other hand, acyclovir has a higher affinity for ethanol than the stratum corneum lipids, which present as the main barrier. The combination of these two factors could be a possible explanation for the effect of ethanol found on the transepidermal permeation of acyclovir.

The relatively low enhancement factor of ethanol compared to those found in other studies could also be explained by the fact that in the present study the skin was pretreated with an amount of 25 µl ethanol. A study done by Pendlington *et al.* (2001) describes the fate of ethanol topically applied to skin. The rate of evaporation of ethanol from skin was measured *in vitro*. [^{14}C]ethanol was diluted with absolute ethanol, and 20 µl was dosed onto pieces of whole pig skin (32 °C). The skin and remaining [^{14}C]ethanol were measured at intervals of 10 seconds. The half-life ($t_{1/2}$) of evaporation of ethanol from the resulting hyperbolic curve of % ethanol still in or on the skin *versus* time was determined as 11.7 seconds. Skin penetration experiments by the same investigators, found that the total recovery of ethanol for non-occluded cells was less than 3 %, indicating that most of the ethanol had evaporated before being able to penetrate the skin. Since pretreatment in the present study occurred without occlusion, it can be assumed that evaporation of ethanol occurred rapidly, and that only a little amount of ethanol partitioned into the stratum corneum.

Ethanol not only affected the penetration of acyclovir in the control study, but also the activity of the terpenes. The composition of the pretreatment formula changes as ethanol evaporates. The terpene becomes more concentrated, which result in higher thermodynamic activity and its partitioning into the skin is elevated (Flynn & Weiner, 1993).

As shown in Table 3-4, the highest acyclovir flux values obtained in this study was after skin pretreatment with 1,8-cineole (0.65 ± 0.26 µg/cm^2/h) and with menthol (0.55 ± 0.24 µg/cm^2/h). Expressed as an enhancement ratio for acyclovir flux in the presence of 1,8-cineole and menthol compared to the control flux of acyclovir, the values were 4.205 and 3.536, respectively, and compared to the value of control 2 were 3.130 and 2.632. In the current study, only these two terpenes demonstrated a significant enhancing effect on acyclovir permeation ($p > 0.05$). The degree of enhancement by both 1,8-cineole and

menthol was much greater than that by ethanol itself. 1,8-Cineole and menthol are relatively hydrophilic terpenes. 1,8-Cineole is an oxygen containing terpene with a log P value of 2.82. Menthol is an alcoholic terpene, with a log P of 3.20. It has been reported that the effect of an enhancer on the permeation of a drug usually depends upon the physicochemical characteristics of both the permeant as well as the enhancer molecule. Williams & Barry (1991) found that hydrocarbon terpenes show minimal activity as compared to the oxygen containing terpenes in enhancing 5-fluorouracil (hydrophilic permeant) penetration. Apparently, hydrocarbon terpenes are effective for lipophilic drugs and oxygen-containing terpenes are effective for hydrophilic drugs. This finding has also been demonstrated by Hori *et al.* The effects of terpenes on the permeation of propranolol hydrochloride (hydrophilic drug) and diazepam (lipophilic drug) were evaluated. The purely hydrocarbon terpenes promoted both propranolol and diazepam permeation, whereas the terpenes with hydrogen-bonding ability only enhanced the flux of propranolol (Hori *et al.*, 1991).

In contrast, menthone pretreatment of the skin had no significant enhancing influence on acyclovir transdermal flux. The flux observed in the presence of menthone was 0.20 ± 0.04 $\mu g/cm^2/h$, with an enhancement ratio of 1.294 compared to control 1 and 0.963 compared to control 2. This finding was somewhat surprising, since menthone is a hydrophilic ketone terpene with a log P of 2.63. Furthermore, a lag time of 25.512 minutes was observed after menthone pretreatment, which is the longest lag time observed in this study. The late onset and low flux suggest that menthone could in fact retard acyclovir flux across the stratum corneum. The reason for this is not clear. The effect of menthone could possibly be masked by vehicle effects or may be ascribed to the physicochemical properties of the permeant, possible interactions or skin compositional factors.

Limonene is a hydrocarbon terpene with a log P of 4.58. β-Myrcene is the only non-cyclic terpene employed and has the same log P value as limonene. Their enhancing effect on transdermal permeation of acyclovir was mild, but not statistically significant. At a flux of 0.29 ± 0.12 $\mu g/cm^2/h$, the enhancement ratios of acyclovir in the presence of limonene was 1.870 and 1.392 compared to control 1 and control 2, respectively. The flux initiated by β-myrcene was 0.30 ± 0.1 $\mu g/cm^2/h$ with enhancement ratios of 1.937 and 1.442 compared to control 1 and control 2, respectively. It is interesting to note the

important role of the partition coefficient as is evident in the case of these two terpenes. Limonene and β-myrcene both have the same log P, molecular weight and enhanced acyclovir to the exact same extent, even though they differ in structure and melting point.

Except for the effect obtained from menthone pretreatment, these results are in good agreement with the findings of Williams & Barry (1991). Of all the cyclic terpenes chosen from the chemical classes of hydrocarbons, alcohols, ketones and ethers, 1,8-cineole showed the greatest activity in enhancing 5-fluorouracil (enhancement factor of 95) out. The alcohols were shown to be generally less effective than ethers (1,8-cineole). Menthol was not included in that study, but having a lower enhancement factor in the present results, the trends in the enhancement of the permeability of acyclovir are similar. The hydrocarbons, *α*-pinene and limonene, showed the lowest enhancement factors, less than three fold increase in the case of 5-fluorouracil. Menthone, however, was the most effective ketone with an enhancement factor of approximately 40 (Williams & Barry, 1991).

Synergistic permeability enhancement by ethanol with some terpenes has been demonstrated by Obata *et al.* (1993). Morimoto *et al.* (1993) also found that the combination of L-menthol and ethanol provided higher skin permeation of morphine hydrochloride compared to either alone. In the present study, no significant synergy could be stated. It is possible that the synergy may not arise for some drug molecules from the combined effect of ethanol and terpenes on skin structure. For other molecules, part of the effect may be an increased partitioning of the drug into the stratum corneum.

The lag times of the various experimental pretreatment protocols are summarized in Table 4-1. A lag time of acyclovir transdermal permeation was determined to be ~ 10 minutes when no pretreatment was employed. When the skin was pretreated with ethanol, a dramatic reduction in lag time was observed (0.042 min). When terpenes were added to the pretreatment solution, the lag time decreased further in the case of limonene (0.018 min), menthol (0.024 min) and β-myrcene (0.018 min). Increases in lag time were observed with 1,8-cineole (11.34 min) and menthone (25.512 min).

According to Equation 3-3, the lag time increases as the diffusion pathlength increases. During lag time periods, the stratum corneum would be conditioned for higher

permeability and permeation reaches steady state after lag time. Therefore, the increase in lag time could also be due to slow stratum corneum conditioning. A shortening of the diffusion pathlength could be the result of one of more of the following: fluidization of stratum corneum lipids, extraction of lipids, increasing the solubility of drug in the stratum corneum or enhancer interaction with keratin (Vaddi *et al.*, 2002).

It is important to note that the lag time of acyclovir was significantly decreased after ethanol pretreatment of the skin. The increase in the permeability of the stratum corneum and the decrease in its transport lag time after ethanol pretreatment have also been obtained with urea by Li *et al.* (1998). It was, however, found that high tortuosity after ethanol treatment remained and it was suggested that urea may continue to travel primarily around the corneocytes, the highly resistive barriers around the corneocytes (Li *et al.*, 1998). After limonene, menthol and β-myrcene pretreatment, further decrease in lag time was observed. This situation may thus correspond to greatly reduced tortuosity.

Similar delayed onset of action observed with 1,8-cineole has been previously observed. Yamane *et al.* (1995) studied permeation of model hydrophilic 5-fluorouracil through human epidermal membranes treated with neat d-limonene, 1,8-cineole and menthone for times ranging from 1 to 12 hours. An initial reduction in the partitioning of the drug was observed after 1 h pretreatment with 1,8-cineole or d-limonene. The pooling of enhancers inside stratum corneum has been proposed as an explanation for the uptake of large quantities of 1,8-cineole and d-limonene into the stratum corneum which were reported to be 26.3 and 8.90 % w/dry weight of stratum corneum (Cornwell *et al.*, 1994). If the enhancers replace water in the stratum corneum, it was expected that the observed initial reduction in drug partitioning into the stratum corneum treated with 1,8-cineole or d-limonene, since 5-flourouracil is relatively less soluble in 1,8-cineole and d-limonene than water. However, nerolidol (larger solubility of 5-fluorouracil in nerolidol than in the other two terpenes) did not cause the initial reduction and hence the decrease in drug partitioning is unlikely to be due to a simple solvent effect (Yamane *et al.*, 1995). In the present study, the pooling of 1,8-cineole may be the reason for the slow onset of acyclovir permeation. Limonene, however, did not show this initial slow onset of acyclovir flux represented by a longer lag time. A possibility is that enhancer pooling of limonene either did not have an effect on acyclovir permeation onset, or it could have been the cause for the low flux value obtained after limonene permeation.

3.5.2 Transdermal permeation of terpenes

In this study, the transdermal permeation of five terpenes (1,8-cineole, limonene, menthol, menthone and β-myrcene) were studied. Two sets of experiments were done on each. Firstly, terpene penetration was determined by pretreatment of the skin for 30 minutes with the pretreatment solution, after which PBS at a pH of 7.4 was added to the donor compartment of the Franz cell. Secondly, the influence of acyclovir on the permeation of the terpenes was determined which was added to the PBS at saturated concentration. The amount of terpene recovered in the receptor solution, was regarded as having penetrated. The terpene penetration were semi-quantitatively determined by gas chromatography and expressed as percentage of applied dose.

3.5.2.1 Results

The cumulative percentage of applied dose of terpenes that permeated the skin after 24 hours in the presence and absence of acyclovir are given in Table 3-5. The skin was pretreated with 25 µl of 5 % solutions of terpenes. The amount of applied dose as 1.25 µl of liquid 1,8-cineole, limonene, menthone and β-myrcene; and 1.24 mg of menthol.

TABLE 3-5: The cumulative amount of terpenes permeated after 24 hours in the presence and absence of acyclovir expressed as mean percentage of applied dose ± standard deviation.

	% TERPENE PERMEATED IN ACYCLOVIR ABSENCE	% TERPENE PERMEATED IN ACYCLOVIR PRESENCE
1,8-Cineole	21.436 ± 4.322	26.296 ± 3.432
Limonene	2.922 ± 1.043	19.133 ± 1.581
Menthol	70.208 ± 7.481	70.054 ± 5.004
Menthone	96.509 ± 21.963	84.673 ± 12.738
β-Myrcene	6.902 ± 0.917	10.939 ± 1.652

The mean percentage and standard deviations of permeation observed of the various terpenes over a 24 hour period with and without acyclovir in Table 3-5 are presented in Figure 3-4. Figure 3-5 is the permeation profiles of each of the terpenes with and without acyclovir influence taken at times 6, 12 and 24 hours.

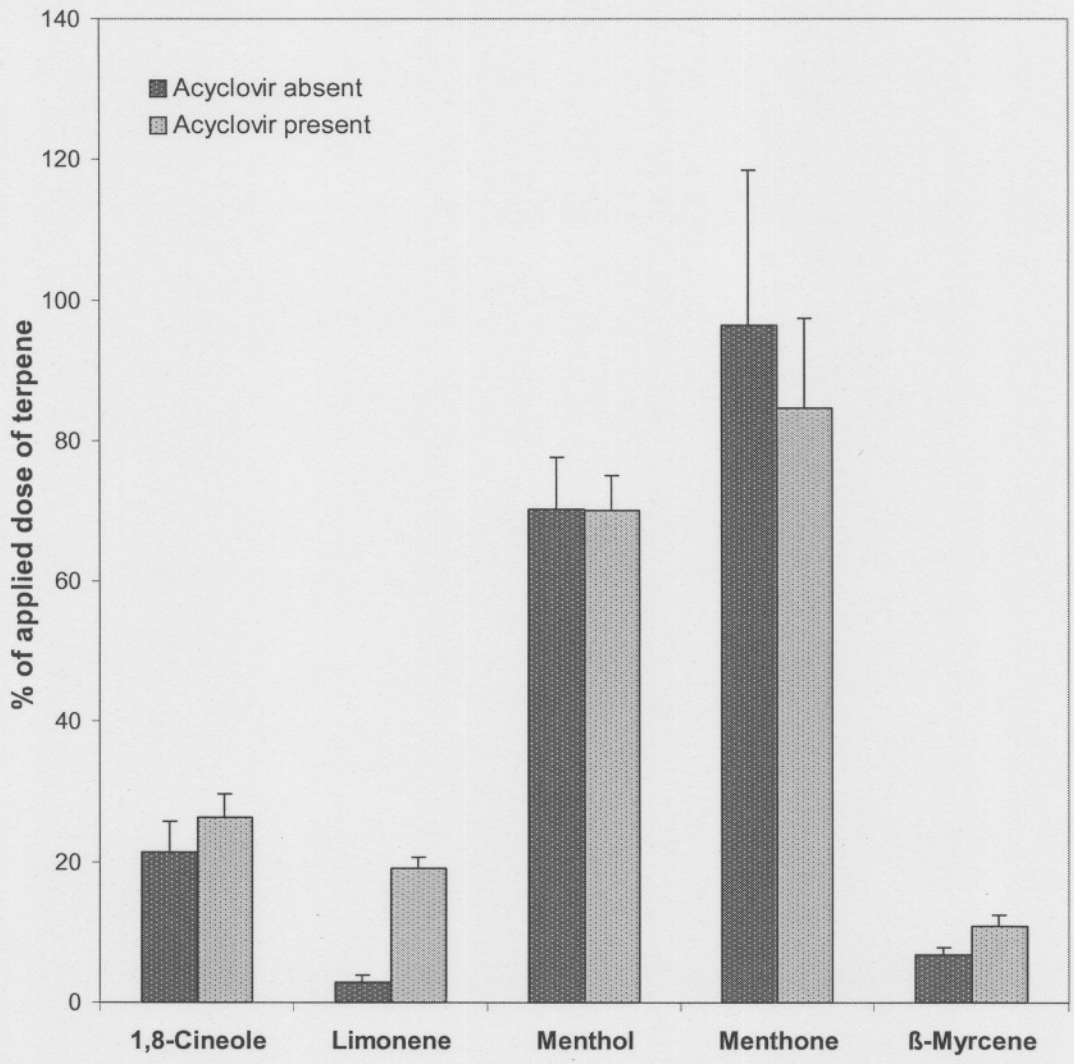


FIGURE 3-4: The mean percentage and standard deviations of permeation observed of the various terpenes over a 24 hour period with and without acyclovir influence.

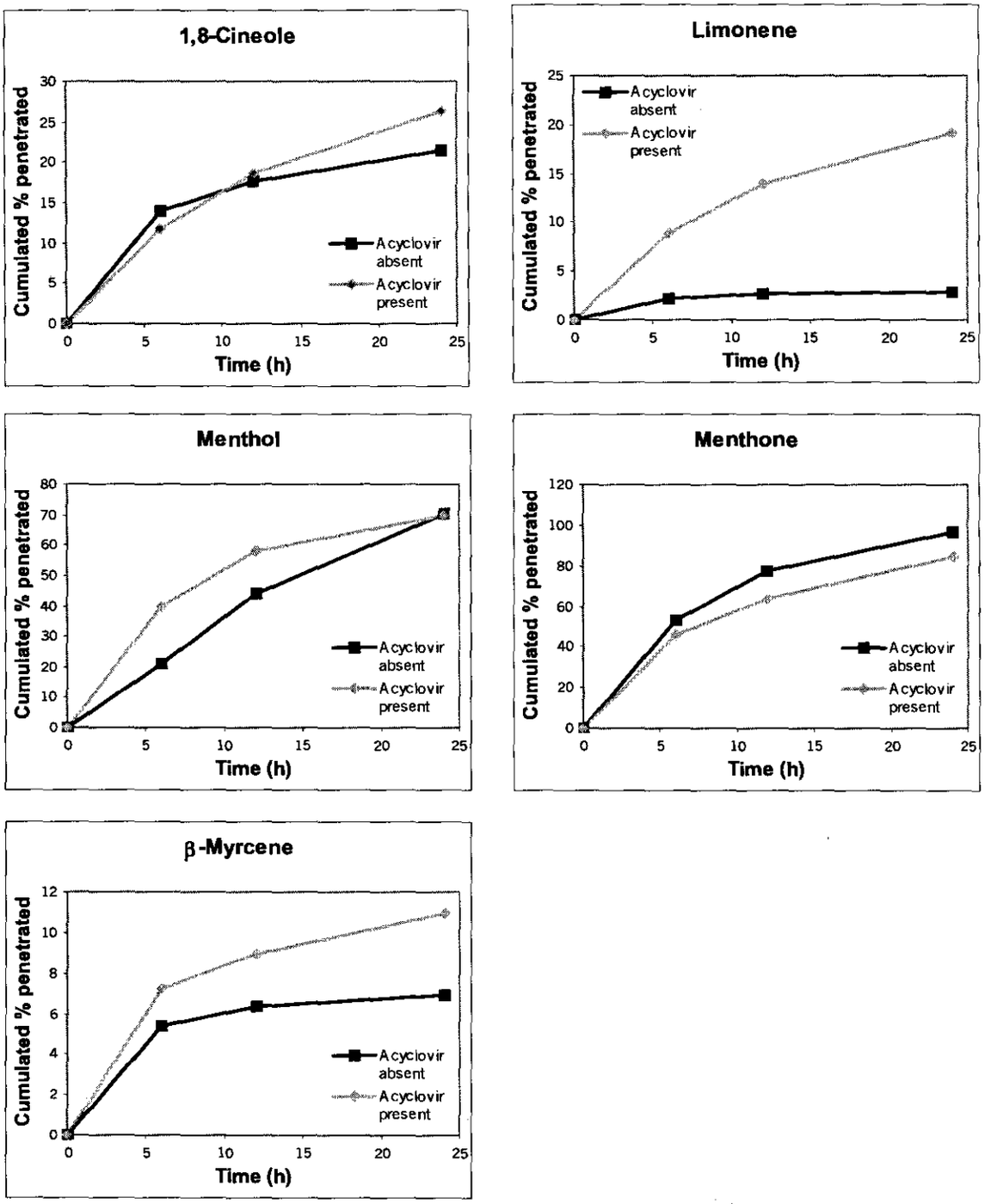


FIGURE 3-5: The permeation profiles of the cumulative percentage of applied amount of the different terpenes that penetrated in the presence and absence of acyclovir.

3.5.2.2 Statistical analysis

The statistical analysis of differences was performed using analysis of variance (ANOVA), and applied to the mean cumulative percentage of applied dose. In each of the permeation studies of the terpenes, the % permeated in the presence of acyclovir was compared to the % permeated in the absence of acyclovir. Statistical comparisons were made of the cumulative % of terpene penetrated at the three time points 6, 12 and 24 hours as well as between the time intervals of 6 – 24 h and 12 – 24 h. Statistically significant differences, with the level of probability taken as $p < 0.05$ as the level of confidence by Student's t-test. Statistically significant differences were found at the three time points of 1,8-cineole as well as the time interval 6 – 24 h, but no difference in interval 12 – 24 h ($p = 0.1193$). Menthone showed difference in none of the time points or time intervals; where limonene showed significant difference in all time points and intervals. In the case of menthol, all time points and time intervals showed significant difference, except at the time level of 24 h ($p = 0.9674$). β-Myrcene showed significant differences in all time points and intervals, except at the time point of 6 h ($p = 0.0937$).

3.5.2.3 Discussion

The terpene with the highest % permeation through the skin was menthone, followed by menthol, isomenthol, 1,8-cineole, limonene and β-myrcene. When their partition coefficients are taken into account, it is clear that permeation was highest with the terpenes with the lowest log P, and permeation decreased accordingly as the log P increased. One exception, however, is the permeation of 1,8-cineole, as will be explained later.

Optimal log P values for transdermal delivery is in the range 1 – 3 (Finnin & Morgan, 1999). Too high log P values may cause retention of these compounds within the stratum corneum, inhibiting clearance from the stratum corneum. During percutaneous absorption, the skin can act as a reservoir. In particular, lipophilic chemicals may remain in the stratum corneum for an extended period of time. Once in the skin, it is indicated that substances are likely to eventually be systemically absorbed (Poet & McDougal, 2002).

The more lipophilic terpenes employed in this study, limonene and β-myrcene (log P = 4.58 each), showed the lowest % permeation, less than 20 % after 24 h. Most likely,

these two terpenes have partitioned into the stratum corneum, but partitioning out of the stratum corneum into the receptor solution is probably the rate-limiting step in the permeation process.

Menthone, which showed the highest % penetration after 24 h (> 80 %), has a log P value of 2.63 which is within the optimum range suggested by research. Relatively easy partitioning into the stratum corneum, between the different substances within the stratum corneum, and into the receptor solution is the most probable cause for the high % recovered.

The intermediate log P value of 3.20 presented menthol, gives cause to intermediate permeation compared to the other terpenes. The % of menthol that permeated after 24 hours (70 %) was considerably higher than the more lipophilic terpenes, limonene and β-myrcene.

1,8-Cineole penetration was not in concurrence with the expectation. A much lower amount (~ 20 – 26 %) was recovered in the receptor solution. 1,8-Cineole is a relative hydrophilic terpene with a log P value of 2.82. Partitioning into, through and out of the stratum corneum was expected to be relatively easy, since 1,8-cineole have optimal solubility in both oil and water phases. The question arises as to where this terpene could have been retained.

It is most probable that enhancer pooling could be the reason. Since the terpenes under study are lipophilic, it is likely that they would distribute into the intercellular spaces of the stratum corneum. Enhancer uptake studies have shown that terpenes are incorporated into the stratum corneum in large quantities when applied as undiluted oils (Cornwell *et al.*, 1996). Wide-angle X-ray diffraction investigated the effect of limonene and 1,8-cineole on human stratum corneum *in vitro*. These studies showed that, following enhancer treatment, liquid terpenes formed a separate phase within the stratum corneum from undisrupted lipid bilayers. In addition to this finding, it has been noted that when a molten mixture of octadecanol (selected as a simple model lipid) and 1,8-cineole is cooled to room temperature, the mixture solidifies and most of the terpene separates as droplets (Cornwell *et al.*, 1994).

The reported values of enhancer uptake, as a percentage of dry tissue weight, obtained following treatment with neat (+)-limonene and 1,8-cineole were 8.90 % and 26.2 %

respectively. The ratio of 1,8-cineole to lipid molecules was calculated as 10:1 (Cornwell *et al.*, 1994). In these studies, the membranes were soaked in the enhancer solution. In the present study, enhancer pretreatment solution was only placed on the upper side of the skin. In addition, a finite amount of 5 % terpene was employed in ethanol vehicle. A considerable amount of 1,8-cineole applied probably pooled inside the stratum corneum lipids, and thus permeation of 1,8-cineole was to a much lesser extent.

The amount of 1,8-cineole that permeated the skin after 24 hours was 21.4 %. Acyclovir had a significant effect on the permeation of 1,8-cineole. At the time of 6 h, more 1,8-cineole^{PBS}(1,8-cineole permeation in absence of acyclovir) have permeated than 1,8-cineole^{ACV} (1,8-cineole permeation in presence of acyclovir). However, after 12 and 24 h, more 1,8-cineole^{ACV} has penetrated, with an amount of 26.3 % 1,8-cineole^{ACV} after 24 hours. The difference in amount of 1,8-cineole^{PBS} and 1,8-cineole^{ACV} that penetrated between 12 and 24 h was insignificant. This means that acyclovir had probably only an effect on 1,8-cineole permeation within the first 6 hours after which the rate of diffusion was constant.

The amount of limonene^{PBS} (limonene permeation in absence of acyclovir) that permeated the skin after 24 hours was 2.9 %. Acyclovir had a significant effect on the permeation of limonene. Limonene^{ACV} (limonene permeation in presence of acyclovir) recovered in the receptor solution at the three time points, as well as the time intervals was much higher than limonene^{PBS}.

The amount of menthol^{PBS} (menthol permeation in absence of acyclovir) that permeated the skin after 24 hours was 70.2 %. Acyclovir had a significant effect on the permeation of menthol ($p > 0.05$). At both 6 and 12 h, the % menthol^{ACV} (menthol permeation in presence of acyclovir) recovered in the receptor solution was higher than menthol^{PBS}. At the time intervals of 6 – 12 h and 12 – 24 h, the rate of menthol^{ACV} permeation occurred at a significant lower rate than that of menthol^{PBS}. At 24 h, however the amount of menthol^{PBS} permeated was the same as the amount of menthol^{ACV} (70.1 %).

The amount of menthone^{PBS} (menthone permeation in absence of acyclovir) that permeated the skin after 24 hours was 96.5 %, which was the greatest amount of all the terpenes tested. Acyclovir had no significant effect on the permeation of menthone. At 6, 12 and 24 h, menthone^{PBS} was slightly higher, but not significantly ($p > 0.05$) than

menthone^{ACV} (menthone permeation in presence of acyclovir). The amount of menthone^{ACV} recovered in the receptor solution after 24 h was 84.7 %.

The amount of β-myrcene^{PBS} (β-myrcene permeation in absence of acyclovir) that permeated the skin after 24 hours was 6.9 %. The amount of β-myrcene^{PBS} recovered in the receptor solution up to 6 h were not significantly different ($p > 0.05$) than β-myrcene^{ACV} (β-myrcene permeation in presence of acyclovir). Thereafter, at time points of 12 and 24 h and time intervals of 6 – 12 h and 12 – 24 h, β-myrcene^{ACV} were significantly higher, suggesting that acyclovir had not an initial effect on myrcene permeation, but only after about 6 h. The amount of β-myrcene^{ACV} permeated after 24 h was 10.9 %.

When acyclovir was present in the transdermal studies of the terpenes, significant differences could be noted on the permeation profiles of 1,8-cineole, limonene, menthol and β-myrcene. Partitioning of the terpenes into the stratum corneum was probably not influenced by acyclovir, since the skin was pretreated with the terpenes prior to the addition of the acyclovir solution. In the case menthone, no significant influence could be detected.

The outcome was mostly higher percentage of terpene penetrated after 24 hours in the presence of acyclovir, as found with 1,8-cineole, limonene and β-myrcene. These were the terpenes that were mostly retained by the stratum corneum. A possibility could be that acyclovir increased the aqueous environment within the stratum corneum, and in turn increased partitioning out of the stratum corneum into the receptor solution.

The effect acyclovir had on the permeation kinetics of the terpenes seems to vary with time. No direct correlation could be made between solubility parameters of the terpenes and acyclovir influence. Penetration enhancement of acyclovir by the terpenes and permeation of the terpenes itself also showed no correlation. The mechanism of acyclovir influence on terpene permeation is not clear. Further investigation is required.

3.6 CONCLUSIONS

The HPLC method for the analysis of acyclovir has been validated and proven to be simple, rapid, specific and sensitive. Under the chromatographic conditions described previously, the run time was less than 7 minutes. Good levels of reproducibility, repeatability and sensitivity were obtained. The properties mentioned render this procedure suitable for the quantitative analysis of acyclovir. *In vitro* techniques for measuring the ability of a chemical to penetrate the skin are quite diverse and they are useful screens. It was concluded that the simple diffusion cell is an appropriate model for this type of assessment, and that human skin is the membrane of choice. An experimental method has been established by which the objected aim of this study can

The transdermal permeation of acyclovir in the presence and absence of ethanol and terpenes were investigated. Ethanol was found to have no significant influence on acyclovir flux, and one or a combination of the following factors could be possible explanations:

- The pretreatment time of 30 minutes could have been too short, since ethanol effects on the stratum corneum are time-dependent.
- The use of absolute ethanol caused an unexplicable decrease in permeability as was also observed by other investigators.
- The solubility of acyclovir in the stratum corneum did not significantly increase, since its solubility in ethanol is very low.
- Extensive evaporation of ethanol occurred and thus much lower amounts than expected reached and partitioned into the stratum corneum.

Significant enhancement of transdermal flux of acyclovir was observed when the skin was pretreated with 1,8-cineole and menthol. Both these terpenes are hydrophilic, oxygen-containing terpenes. The lipophilic, hydrocarbon terpenes limonene and β-myrcene had no significant enhancing effect on acyclovir flux. These results are in accordance to results obtained by Williams & Barry (1989) with hydrophilic 5-fluorouracil. The exception was found with hydrophilic menthone, that showed no enhancement and a long lag time. No clear reason can be given as to what the cause could be.

The lag time of acyclovir decreased when the skin was pretreated with ethanol. When menthol, limonene and β-myrcene were included in the pretreatment solution, the lag time decreased even further, suggesting shortened diffusion pathlength and tortuosity of the stratum corneum. 1,8-Cineole, however, increased the lag time of acyclovir. This may be due to enhancer pooling within the stratum corneum, causing a reduction in the onset of action.

In agreement with theoretical predictions, the amount of terpene permeated through the skin was related to their log P values. Menthone, with a log P value of 2.63, showed the highest amount to penetrate the skin, expressed as percentage of applied dose. In accordance, as log P values increased, the amount permeated decreased. The reasons for this phenomenon were ascribed to binding and retention in the stratum corneum as the terpenes become more lipophilic. 1,8-Cineole's low permeation was found to be 'out of line' in accordance with its solubility parameters and pooling in the stratum corneum was regarded as the most possible cause.

Acyclovir had a significant influence on the permeation profiles of most of the terpenes. Influence that has shown significance over 24 hours were mostly promoting or enhancing. The mechanism of influence of acyclovir on terpene permeation is unknown, and found to be independent on terpene solubility and enhancing properties.

CHAPTER 4:

SUMMARY AND FINAL CONCLUSIONS

When using a biological membrane, the variability thereof it is important to consider. In the case of the stratum corneum, the precise composition, texture, appendageal distribution, water/lipid/protein contents and compaction of the stratum corneum varies amongst humans, from site to site and with race, age, and gender. This can make the permeability to membrane thickness relationship a variable factor (Kemppainen & Reifenrath, 1990). The skin preparation and separation technique as well as storage time could influence the barrier function of the skin.

In order to deliver therapeutic agents through the human skin for systemic effects, the comprehensive morphological, biophysical, and physicochemical properties of the skin should be considered (Rieger, 1993). The skin is a complex organ and is regarded as a nearly impermeable membrane that protects the body from external environment. The molecular weight, water solubility, melting point, and oil/water partition coefficients are some of the important attributes which determine the diffusivity of the permeant. Most drugs do not have all the suitable characteristics which promote transdermal delivery. To overcome this limitation, enhancement strategies have been developed, among which the use of chemical permeation enhancers are the most widely utilized approach.

Acyclovir, an antiviral agent, has previously been proven to penetrate the skin (Bolger *et al.*, 1997; Imanidis *et al.*, 1994). Clinical efficiency, however, lacked due to insufficient delivery and penetration through the stratum corneum.

In the present study, the influence of terpenes on the transdermal permeation of acyclovir was investigated. The results were in agreement with previous studies, which stated that the hydrophilic terpenes enhance transdermal permeation of hydrophilic drugs (Williams & Barry, 1989). An exception was found with menthone.

1,8-Cineole and menthol were the only terpenes that enhanced penetration of acyclovir. Enhancement by these two terpenes, however, was not to a large enough extent to be of

clinical use. A flux value of 1.25 μg/ml/h, which were reported as useful for a 50 % inhibition of the viral cytopathic effect (IDI_{50}) in herpes simplex type 1 infections, can be taken as a reference (Lee *et al.*, 1992; Imanidis *et al.*, 1994). The flux values obtained after pretreatment of 1,8-cineole and menthol, were only half of the flux value required for efficiency.

In conclusion, none of tested terpenes proved to be promising future enhancers for transdermal delivery of acyclovir. It must, however, be noted, that only one combination was used throughout the study. Five percent of terpene in ethanol was used as the pretreatment solution in an amount of 25 μl.

In further continuation of this study it is suggested that variations of the following factors might result in a more favourable enhancement effect of these terpenes on acyclovir permeation:

- The use of higher concentrations, or even neat terpenes, for skin pretreatment.
- A larger amount of pretreatment solution.
- Pretreatment of the skin by soaking the skin in the solution for a certain amount of time.
- Formulation of an acyclovir and terpene solution, where permeant and enhancer application in the donor compartment are concurrent.

Furthermore, terpene penetration was proven to be in agreement with theoretical predictions. The amount of terpene permeated through the skin was related to the partition coefficients. Lipophilic terpenes was mainly retained by the skin, and hydrophilic terpenes penetrated the skin in greater than expected amounts.

Acyclovir had a significant influence on the permeation of most of the terpenes, and was shown to be mostly promoting or enhancing. The mechanism of influence of acyclovir on terpene permeation is unknown. For future studies, the following are suggested:

- Terpene penetration studies *in vivo*.
- Penetration studies on aromatherapeutic oils to estimate biovailability of the terpenes found in these oils commonly used.

- Studies on the influence of drugs on penetration enhancers. These studies could reveal and explain possible enhancement mechanisms of the enhancers and possibly correlate enhancer permeation with permeant enhancement.

- Penetration studies on other commonly used transdermal enhancers.

REFERENCES

ABRAHAM, W. & DOWNING, D.T. 1990. Factors affecting the formation, morphology and permeability of stratum corneum lipid bilayers *in vitro*. (*In* SCOTT, R.C., GUY, R.H. & HADGRAT, J. *eds.* Prediction of percutaneous penetration: methods, measurements, modeling. London: IBC Technical Services, p. 110-122).

AFOUNA, M.I., FINCHER, T.K., ZAGHLOUL, A.A. & REDDY, I.K. 2003. Effect of Azone upon the *in vivo* antiviral efficacy of cidofovir or acyclovir topical formulations in treatment/prevention of cutaneous HSV-1 infections and its correlation with skin target site free drug concentration in hairless mice. *International journal of pharmaceutics*, 253:159-168.

ARELLANO, A., SANTOYO, S., MARTIN, C. & YGARTUA, P. 1996. Enhancing effect of terpenes on the *in vitro* percutaneous absorption of diclofenac sodium. *International journal of pharmaceutics*, 130:141-145.

ASBILL, C.S., MICHNIAK, B.B. 2000. Percutaneous penetration enhancers: local *versus* transdermal activity. *Pharmaceutical science & technology today*, 3:36-41.

BACH, M. & LIPPOLD, B.C. 1998. Percutaneous penetration enhancement and its quantification. *European journal of pharmaceutics and biopharmaceutics*, 46:1-13.

BANDO, H., SAHASHI, M., MOHRI, S., YAMASHITA, F., TAKAKURA, Y. & HASHIDA, M. 1996. *In vivo* skin penetration enhancement of acyclovir by theoretical design of prodrug-enhancer combination, *International journal of pharmaceutics*, 145:103-113.

BANDO, H., SAHASHI, M., TAKAGI, T., YAMASHITA, F., TAKAKURA, Y. & HASHIDA, M. 1996. Analysis of *in vitro* skin penetration of acyclovir prodrugs based on a diffusion model with a metabolic process. *International journal of pharmaceutics*, 135:91-102.

BARRY, B.W. 2001. Novel mechanisms and devices to enable successful transdermal drug delivery. *European journal of pharmaceutical sciences*, 14:101-114.

BOLGER, G.T., ALLEN, T., GARNEAU, M., LAPEYRE, N., LIARD, F. & JARAMILLO, J. 1997. Cutaneously applied acyclovir acts systemically in the treatment of herpetic infection in the hairless mouse. *Antiviral research*, 35:157-165.

BOMMANNAN, D., POTTS, R.O. & GUY, R.H. 1991. Examination of the effect of ethanol on human stratum corneum *in vivo* using infrared spectroscopy. *Journal of controlled release*, 16:299-304.

BRITISH PHARMACOPOEIAE. 1993. Vol. 1. London: HMSO. 714p.

BRONAUGH, R.L. & COLLIER, S.W. 1993. *In vitro* methods for measuring skin permeation. (*In* ZATZ, J.L. *ed.* Skin permeation: fundamentals and application. Illinois: Allured Publishing Corporation. p. 93-111).

CAL, K., JANICKI, S. & SZNITOWASKA, M. 2001. *In vitro* studies on penetration of terpenes from matrix-type transdermal systems through human skin. *International journal of pharmaceutics*, 224:81-88.

COOPER, E.R., MERRIT, E.W. & SMITH, R.L. 1985. Effect of fatty acids and alcohols on the penetration of acyclovir across human skin *in vitro*. *Journal of pharmaceutical sciences*, 74:688-689.

CORNWELL, P.A., BARRY, B.W., BOUWSTRA, J.A. & GOORIS, G.S. 1996. Modes of action of terpene penetration enhancers in human skin; differential scanning calorimetry, small-angle X-ray diffraction and enhancer uptake studies. *International journal of pharmaceutics*, 127:9-26.

CORNWELL, P.A., BARRY, B.W., STODDART, C.P. & BOUWSTRA, J.A. 1994. Wide-angle X-ray diffraction of human stratum corneum: effects of hydration and terpene enhancer treatment. *Journal of pharmacy and pharmacology*, 46:938-950.

DE JALóN, E.G., BLANCO-PRíETO, M.J., YGARTUA, P. & SANTOYO, S. 2001. Topical application of acyclovir-loaded microparticles: quantification of the drug in porcine skin layers. *Journal of controlled release*, 75:191-197.

DICK, I.P., BLAIN, P.G. & WILLIAMS, F.M. 1996. Improved *in vitro* skin absorption for lipophilic compounds following the addition of albumin to the receptor fluid in flow-through cells (*In* BRAIN, K.R., JAMES, V.J. & WALTERS, K.A. *eds.* Prediction of percutaneous penetration. Vol. 5. STS Publishing: Cardiff. p. 267-270).

DOLLERY, D. 1999. Therapeutic drugs, second edition, volume 1. London: Churchill Livingstone. 1473p.

EL-KATTAN, A., ASBILL, C.S. & MICHNIAK, B.B. 2000. The effect of terpene enhancer lipophilicity on the percutaneous permeation of hydrocortisone formulated in HPMC gel systems. *International journal of pharmaceutics*, 198:179-189.

EL-KATTAN, A., ASBILL, KIM, N. & MICHNIAK, B.B. 2001. The effects of terpene enhancers on the percutaneous permeation of drugs with different lipophilicities. *International journal of pharmaceutics*, 215:229-240.

ELIAS, P.M. 1992. Role of lipids in barrier function of the skin. (*In* Mukhtar, H., *ed.* Pharmacology of the skin. Florida: CRC Press. p. 30-38).

ECKERT, R.L. 1992. The structure and function of the skin. (*In* Mukhtar, H., *ed.* Pharmacology of the skin. Florida: CRC Press. p. 4-12).

FINNIN, B.C. & MORGAN, T.M. 1999. Transdermal penetration enhancers: applications, limitations, and potential. *Journal of pharmaceutical sciences*, 88:955-957.

FOLDVARI, M. 2000. Non-invasive administration of drugs through the skin: challenges in delivery system design. *Pharmaceutical science & technology today*, 4:417-423.

GAO, S. & SINGH, J. 1997. Mechanism of transdermal transport of 5-fluorouracil by terpenes: carvone, 1,8-cineole and thymol. *International journal of pharmaceutics*, 154:67-77.

GAO, S. & SINGH, J. 1998. *In vitro* percutaneous absorption enhancement of a lipophilic drug tamoxifen by terpenes. *Journal of controlled release*, 51:193-199.

GIBBON, C.J. 2000. South African medicines formulary, fifth edition. Cape town: South African medical association. 537p.

FLYNN, G.L. & WEINER, N.D. 1993. II. Topical and transdermal delivery – provinces of realism. (*In* GURNY, R. & TEUBNER, A. *eds.* Dermal and transdermal drug delivery. Stuttgart: Wissenschaftliche Verlagsgesellschaft, p. 33-64).

GUY, R.H. 1996. Current status and future prospects of transdermal drug delivery. *Pharmaceutical research*, 14:1765-1769.

HADGRAFT, J. 1999. Passive enhancement strategies in topical and transdermal drug delivery, *International journal of pharmaceutics*, 184:1-6.

HADGRAFT, J. & VALENTA, C. 2000. pH, pK_a and dermal delivery. *International journal of pharmaceutics*, 200:243-247.

HADGRAFT, J. & WOLFF, M. 1993. Physicochemical and pharmacokinetic parameters affecting percutaneous absorption. (*In* GURNY, R. & TEUBNER, A. *eds.* Dermal and transdermal drug delivery. Stuttgart: Wissenschaftliche Verlagsgesellschaft, p. 161-172).

HATANAKA, T., KATAYAMA, K., KOIZUMI, T., SUGIBAYASHI, K. & MORIMOTO, Y. 1995. Time-dependant percutaneous absorption enhancing effect of ethanol. *Journal of controlled release*, 33:423-428.

HORI, M., SATOH, S., MAIBACH, H.I. & GUY, R.H. 1991. Enhancement of propranolol hydrochloride and diazepam skin absorption in vitro: effect of enhancer lipophilicity. *Journal of pharmaceutical sciences*, 80:32-35.

IMANIDIS, G., SONG, W., LEE, P.H., SU, M., KERN, E.R. & HIGUCHI, W.I. 1994. Estimation of skin target site acyclovir concentrations following controlled (trans)dermal drug delivery in topical and systemic treatment of cutaneous HSV-1 infections in hairless mice. *Pharmaceutical research*, 11:1035-1041.

JIANG, M., QURESHI, S.A., MIDHA, K.K. & SKELLY, J.P. 1998. *In vitro* evaluation of percutaneous absorption of an acyclovir product using intact and tape-stripped human skin. *Journal of pharmacology and pharmaceutical sciences*, 3:102-107.

KALIA, Y.N. & GUY, R.H. 2001. Modeling transdermal drug release. *Advanced drug delivery reviews.* 48:159-172.

KATAYAMA, K., TAKAHASHI, O., MATSUI, R., MORIGAKI, S., AIBA, T., KAKEMI, M. & KOIZUMI, T. 1992. Effect of l-menthol on the permeation of indomethacin, mannitol and cortisone through excesed hairless mouse skin. *Chemical & pharmaceutical bulletin*, 40:3097-3099.

KEMPPAINEN, B.W. & REIFENRATH, W.G. 1990. Methods for skin absorption. Florida: CRC Press. 207p.

KITAGAWA, S., HOSOKAI, A., KASEDA, Y., YAMAMOTO, N., KANEKO, Y. & MATSUOKA, E. 1998. Permeability of benzoic acid derivatives in excised guinea pig dorsal skin and effects of L-menthol. *International journal of pharmaceutics*, 161:115-122.

KOYAMA, Y., BANDO, H., YAMASHITA, F., TAKAKURA, Y., SEZAKI, H. & HASHIDA, M. 1994. Comparative analysis of percutaneous absorption enhancement by d-limonene and oleic acid based on a skin diffusion model. *Pharmaceutical research*, 11:377-383.

KRISTL, A., SRČIČ, S., VREČER, F., ŠUŠTAR, B. & VOJNOVIC, D. 1996. Polymorphism and pseudopolymorphism: influencing the dissolution properties of the guanine derivative acyclovir. *International journal of pharmaceutics*, 139:231-235.

KRISTL, A. & PEčAR, S. 1997. Hydrolipophilic anomalies of some guanine derivatives. *European journal of medicinal chemistry*, 32:3-8.

LEE, P.H., SU, M.H., KERN, E.R. & HIGUCHI, W.I. 1992. Novel animal model for evaluating topical efficacy of antiviral agents: flux *versus* efficacy correlations in the acyclovir treatment of cutaneous herpes simplex virus type 1 (HSV-1) infections in hairless mice. *Pharmaceutical research*, 9:979-989.

LI, S.K., SUH, W., PARIKH, H.H., GHANEM, A., MEHTA, S.C., PECK, K.D. & HIGUCHI, W.I. 1998. Lag time data for characterizing the pore pathway of intact and chemically pretreated human epidermal membrane. *International journal of pharmaceutics*, 170:93-108.

MACKAY, K.M.B., WILLIAMS, A.C. & BARRY, B.W. 2001. Effect of melting point of chiral terpenes on human stratum corneum uptake. *International journal of pharmaceutics*, 228:89-97.

MANABE, E., SUGIBAJASHI, K. & MORIMOTO, Y. 1996. Analysis of skin penetration enhancing effects of drugs by ethanol-water mixed systems with hydrodynamic pore theory. *International journal of pharmaceutics*, 129:211-221.

MCEVOY, G.K. 2002. AHFS Drug information. Bethesda: American society of health-system pharmacists. 3740p.

MENON, G.K. 2002. New insights into skin structure: scratching the surface. *Advanced drug delivery reviews*, 54: S3-S17.

MOGHIMI, H.R., WILLIAMS, A.C. & BARRY, B.W. 1996. A lamellar matrix model for stratum corneum intercellular lipids. IV. Effects of terpene penetration enhancers on the permeation of 5-fluorouracil and oestradiol through the matrix. *International journal of pharmaceutics*, 145:49-59.

MOGHIMI, H.R., WILLIAMS, A.C. & BARRY, B.W. 1997. A lamellar matrix model for stratum corneum intercellular lipids. V. Effects of terpene penetration enhancers on the structure and thermal behaviour of the matrix. *International journal of pharmaceutics*, 146:41-54.

MORGANTI, P., RUOCCO, E., WOLF, R. & ROUCCO, V. 2001. Percutaneous absorption and delivery systems, *Clinics in dermatology*, 19: 489-501.

MORIMOTO, Y., SUGIBAYASHI, K., KOBAYASHI, D., SHOJI, H., YAMAZAKI, J. & KIMURA, M. 1993. A new enhancer-coenhancer system to increase skin permeation of morphine hydrochloride *in vitro*. *International journal of pharmaceutics*, 91:9-14.

MOSER, K., KRIVET, K., KALIA, Y.N. & GUY, R.H. 2001. Enhanced skin permeation of a lipophilic drug using supersaturated formulations. *Journal of controlled release,* 73: 245-253.

MUKHTAR, H. 1992. Pharmacology of the skin. Florida: CRC Press. 416p.

NAIK, A., KALIA, N.Y. & GUY, R.H., 2000. Transdermal drug delivery: overcoming the skin's barrier function. *Pharmaceutical science & technology today,* 3: 318-326.

OBATA, Y., TAKAYAMA, K., MAIBANI, Y., MACHIDA, Y. & NAGAI, T. 1993. Effect of pretreatment of skin with cyclic monoterpenes on permeation of diclofenac in hairless rat. *Biological & pharmaceutical bulletin*, 16:312-314.

OKABE, H., OBATA, Y., TAKAYAMA, K. & NAGAI, T. 1990. Percutaneous absorption enhancing effect and skin irritation of monocyclic monoterpenes. *Drug design and delivery*, 6:229-238.

OKAMOTO, H., MUTA, K., HASHIDA, M. & SEZAKI, H. 1990. Percutaneous penetration of acyclovir through excised hairless mouse and rat skin: effect of vehicle and percutaneous penetration enhancer. *Pharmaceutical research*, 7:64-67.

PARRY, G.E., DUNN, P., SHAH, V.P. & PERSHING, L.K. 1992. Acyclovir bioavailability in human skin. *The journal of investigative dermatology*, 98:856-863.

PATEL, P.J., GHANEM, A., HIGUCHI, W.I., SRINIVASAN, V. & KERN, R. 1996. Correlation of in vivo topical efficacies with *in vitro* predictions using acyclovir formulations in the treatment of cutaneous HSV-1 infections in hairless mice: an evaluation of the predictive value of the C* concept. *Antiviral research*, 29:279-286.

PELLET, M.A., ROBERTS, M.S. & HADGRAFT, J. 1997. Supersaturated solutions evaluated with an in vitro stratum corneum tape stripping technique. *International journal of pharmaceutics,* 151:91-98.

PENDLINGTON, R.U., WHITTLE, E., ROBINSON, J.A. & HOWES, D. 2001. Fate of ethanol topically applied to skin. *Food and chemical toxicology*, 39:169-174.

PFISTER, W.R. 1997. Transdermal and dermal therapeutic systems: current status. (In GOSH, T.K., PFISTER, W.R. & YUM, S.I., *eds.* Transdermal and topical drug delivery systems. Illinois: Interpharm Press, p.33-100).

PHARMACEUTICAL CODEX. 1994. 12th ed. London: Pharmaceutical press. 1117p.

POET, T.S. & McDOUGAL, J.N. 2002. Skin absorption and human assessment. *Chemical-biological interactions,* 140:19-34.

POTTS, R.O. & GUY, R.H. 1992. Predicting skin permeability. *Pharmaceutical research*, 9:663-669.

PUGH, W.J., DEGIM, I.T. & HADGRAFT, J. 2000. Epidermal permeability-penetrant structure relationships: 4. QSAR of permeant diffusion across human stratum corneum in terms of molecular weight, H-bonding and electronic charge. *International journal of pharmaceutics*, 197:203-211.

RAVIS, W.R. 1990. Data interpretation and analysis in percutaneous absorption studies. (*In* KEMPPAINEN, B.W. & REIFENRATH, W.G., *eds.* Methods for skin absorption. Florida: CRC Press, p. 147-164).

RIEGER, M.M. 1993. Factors affecting sorption of topically applied substances. (*In* Zatz, J.L. *ed.* Skin permeation: fundamentals and application. Illinois: Allured Publishing Corporation. p. 33-72).

RIVIERE, J.E. 1993. Biological factors in absorption and permeation. (*In* Zatz, J.L. *ed.* Skin permeation: fundamentals and application. Illinois: Allured Publishing Corporation. p. 113-125).

ROBERTS, M.S., PUGH, W.J. & HADGRAFT, J. 1996. Epidermal permeability: penetrant structure relationships. 2. The effect of H-bonding groups in penetrants on their diffusion through the stratum corneum. *International journal of pharmaceutics*, 132:23-32.

SMITH, K.L. 1990. Penetrant characteristics influencing skin absorption. (*In* KEMPPAINEN, B.W. & REIFENRATH, W.G., *eds.* Methods for skin absorption. Florida: CRC Press, p. 23-34).

STUTTGEN, G. 1982. Drug absorption by intact and damaged skin. (*In* BRANDAU,R. & LIPPOLD, B.H. *eds.* Dermal and transdermal absorption. Stuttgart: Wissenschaftliche Verlagsgesellshaft, p. 27-39.

SUHONEN, T.M., BOUWSTRA, J.A. & URTTI, A. 1999. Chemical enhancement of percutaneous absorption in relation to stratum corneum structural alterations. *Journal of controlled release*, 59:149-161.

VADDI, H.K., HO, P.C., CHAN, Y.W. & CHAN, S.Y. 2002. Terpenes in ethanol: haloperidol permeation and partition through human skin and stratum corneum changes. *Journal of controlled release*, 81:121-133.

VOLPATO, N.M., NICOLI, S., LAURERI, C., COLOMBO, P. & SANTI, P. 1998. *In vitro* acyclovir distribution in human skin layers after transdermal iontophoresis. *Journal of controlled release*, 50:291-296.

VOLPATO, N.M., SANTI, P. & COLOMBO, P. 1995. Iontophoresis enhances the transport of acyclovir through nude mouse skin by electrorepulsion and electroosmosis. *Pharmaceutical research*, 12:1623-1627.

WALKER, R.B. & SMITH, E.W. 1996. The role of percutaneous penetration enhancers. *Advanced drug delivery reviews*, 18:295-301.

WALTERS, K.A. 1989. Penetration enhancers and their use in transdermal therapeutic systems. (*In* HADGRAFT, J. & GUY, R.H. *eds.* Transdermal drug delivery: developmental issues and research initiatives. Vol. 35. New York: Marcel Dekker, p. 197-232).

WILLIAMS, A.C. & BARRY, B.W. 1991. Terpenes and the lipid-protein-partitioning theory of skin penetration enhancement. *Pharmaceutical research*, 8:17-24.

YAMANE, M.A., WILLIAMS, A.C. & BARRY, B.W. 1995. Effects of terpenes and oleic acid as skin penetration enhancers towards 5-fluorouracil as assessed with time; permeation, partitioning and differential scanning calorimetry. *International journal of pharmaceutics*, 116:237-251.

ZATZ, J.L. 1993. Scratching the surface: Rationale and approaches to skin permeation. (*In* Zatz, J.L. *ed.* Skin permeation: fundamentals and application. Illinois: Allured Publishing Corporation. p. 11-31).