THE TRANSDERMAL ABSORPTION OF 5-FLUOROURACIL IN THE PRESENCE AND ABSENCE OF TERPENES

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ABSTRACT

The transdermal absorption of 5-fluorouracil in the presence and absence of terpenes as penetration enhancers

The skin is an amazingly resilient and relatively impermeable barrier that provides protective, perceptive and communication functions to the body (Ramachandran & Fleisher, 2000). The stratum corneum is widely accepted as the barrier of the skin – limiting the transport of molecules into and across the skin. One of the bottlenecks in the successful development of transdermal drug delivery devices is the fact that the skin (more accurately, the stratum corneum - SC) tends to control the rate of drug transport. This makes it very difficult to influence or regulate the transdermal drug absorption kinetics from outside, i.e. by means of the vehicle. A possible, and even elegant, solution may be the use of so-called "penetration enhancers", thereby suppressing the dominant role of the stratum corneum penetration barrier (Bodde *et al.,* 1990).

For this study 5-fluorouracil (5-FU), a polar hydrophilic drug, was chosen as model drug to study its penetration through the stratum corneum. Terpenes used as possible penetration enhancers for 5-FU were menthol, isomenthol, menthone, β-myrcene, limonene and 1,8-cineole. In previous studies, terpenes with low skin irritancy and low systemic toxicity, were found to be effective penetration enhancers for a number of hydrophilic and lipophilic drugs (Cornwell & Barry, 1994; Cornwell *et al.,* 1996; Godwin & Michniak, 1999).

The objective of this study was to determine the different flux rates of 5-FU in the absence of any pretreatment of the stratum corneum and also through ethanol and selected terpene pretreated SC. The effect of each terpene on the penetration of 5-FU was determined. The penetration of the selected terpenes themselves through the human stratum corneum was also determined.

In vitro permeation studies were performed using vertical Franz diffusion cells with human skin (stratum corneum). A saturated aqueous solution of 5-fluorouracil in the absence and presence of pretreatment of the SC was used as the donor phase. Pretreatment was performed by applying a 5 % terpene solution or absolute ethanol to the SC half an hour before the saturated

solution was applied in the donor compartment. A 50/50 ethanol/water solution was used as the receptor phase. All the experiments were conducted over a 24 h period. The 37 °C temperature was held constant by means of a water bath. For the analysis of 5-FU flux rates, samples from the receptor compartment were obtained and were analysed by means of high-pressure liquid chromatography (HPLC). In order to determine the cumulative percentage of terpenes penetrated through human stratum corneum, the samples were analysed by gas chromatography (GC).

In this study, only menthol and isomenthol (both oxygen-containing terpenes) showed a statistically significant increase on the flux of 5-FU, with flux values of 1.13 ± 0.38 and 1.45 ± 0.68 $\mu g/cm^2/h$, respectively, compared to untreated skin with a flux value of 0.54 ± 0.23 $\mu g/cm^2/h$ for 5-FU. It was also proved that ethanol itself had an enhancing effect on 5-FU and showed synergistic effects on the enhancement activities of all the terpenes. It was found that all the terpenes (applied as a 5 % solution in ethanol) penetrated through the stratum corneum in the absence of 5-fluorouracil. 5-Fluorouracil had either an increasing or decreasing effect on the penetration of the terpenes.

From these findings, it could be concluded that oxygen-containing terpenes had the best penetration enhancing effect on 5-FU and that menthol and isomenthol were the most effective penetration enhancers, although the extend of penetration enhancemant is not large enough for clinical application. All the terpenes have the ability to penetrate through human stratum corneum, and 5-FU either had an increasing or decreasing effect on their penetration.

Key words:	Penetration; 5-fluorouracil; terpenes; penetration enhancers; stratum corneum

OPSOMMING

Transdermale absorpsie van 5-fluoorurasiel in die teenwoordigheid en afwesigheid van terpene as penetrasiebevorderaars

Die vel is 'n ongelooflike elastiese en relatief ondeurlaatbare skans wat die liggaam teen die omgewing beskerm en dit daarmee in kontak plaas. (Ramachandran & Fleisher, 2000). Die stratum corneum word algemeen aanvaar as die skans van die vel wat die beweging van molekules in en deur die vel beperk. Een van die struikelblokke vir die suksesvolle ontwikkeling van transdermale afleweringsisteme is die feit dat die vel (of meer akkuraat, die stratum corneum - SC) die tempo reguleer waarteen die vervoer van geneesmiddels plaasvind. Dit maak dit baie moeilik om die transdermale geneesmiddelabsorpsie van buite af, deur byvoorbeeld die draerstof, te beïnvloed of te reguleer. 'n Moontlike, en selfs elegante, oplossing mag wees om sogenaamde "penetrasiebevorderaars" te gebruik om die dominerende rol van die stratum corneum as penetrasieskans te onderdruk (Bodde *et al.,* 1990).

5-Fluoorurasiel (5-FU), 'n polêre hidrofiliese geneesmiddel, is as modelgeneesmiddel vir hierdie studie gekies waartydens die penetrasie daarvan deur die menslike stratum corneum bestudeer is. Terpene wat as moontlike penetrasiebevorderaars vir 5-FU gebruik is was mentol, isomentol, mentoon, β-mirseen, limoneen en 1,8-sineool. Uit vorige studies het dit geblyk dat terpene met lae velirritasie en 'n lae sistemiese toksisiteit effektiewe penetrasiebevorderaars vir 'n aantal hidrofiliese sowel lipofiliese geneesmiddels is (Cornwell & Barry, 1994; Cornwell *et al.,* 1996; Godwin & Michniak, 1999).

Die doel van hierdie studie was om die fluks van 5-FU voor en na voorafbehandeling van die SC met etanol en die onderskeie terpene te bepaal. Die effek van elke terpeen op 5-FU was ook bepaal. Die penetrasie van die onderskeie terpene, as sulks, deur menslike stratum corneum is ook gemeet.

Die *in vitro*-diffusie van die verbindings deur menslike vel (stratum corneum) is met behulp van vertikale Franz-diffusieselle bepaal. Versadigde waterige oplossings van 5-FU is as skenkerfase met onbehandelde en behandelde SC gebruik. Voorafbehandeling is uitgevoer

deur 'n 5 % terpeenoplossing of absolute etanol vir 'n halfuur op die SC te plaas voordat die skenkerfase toegevoeg is. 'n 50/50 etanol/water-oplossing is as reseptorfase gebruik. Alle eksperimente is oor 'n tydperk van 24 uur uitgevoer. Die temperatuur is met 'n waterbad konstant op 37 °C gehou. Vir bepaling van die fluks van 5-fluoorurasiel is monsters wat vanuit die reseptorkompartement onttrek is met behulp van hoëdrukvloeistofchromatografie (HDVC) ontleed. Gaschromatografie is gebruik om die hoeveelheid terpeen wat deur stratum corneum gedring het te bepaal.

In hierdie studie het slegs mentol en isomentol (suurstofbevattende terpene) statisties betekenisvolle verhoging in die fluks van 5-fluoorurasiel teweeg gebring met flukswaardes van 1.13 ± 0.38 en 1.45 ± 0.68 $\mu g/cm^2/h$, onderskeidelik, in vergelyking met onbehandelde vel wat 'n fluks van 0.54 ± 0.23 vir 5-FU toon. Dit is ook gevind dat etanol self 'n versnellende effek op die penetrasie van 5-FU het, asook 'n sinergistiese effek op die penetrasiebevorderende aktiwiteit van die onderskeie terpene. Dit is gevind dat al ses terpene in die teenwoordigheid en afwesigheid van 5-FU deur die stratum corneum penetreer. In die teenwoordigheid van 5-FU is 'n verhoging of verlaging in die penetrasie van die terpene waargeneem.

Uit hierdie bevindings kan afgelei word dat suurstofbevattende terpene die beste penetrasiebevorderende effek op 5-FU het en dat mentol en isomentol die mees effektiewe penetrasiebevorderaars is, hoewel die mate van bevordering nie voldoende vir kliniese toepassing is nie. Al die terpene het die vermoë om deur menslike stratum corneum te penetreer, en 5-FU het 'n verhogende of verlagende effek op hierdie penetrasie.

Sleutelwoorde:	Penetrasie; 5-fluoorurasiel; terpene; penetrasiebevorderaars; stratum corneum

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CHAPTER 1
INTRODUCTION AND PROBLEM STATEMENT

The skin, which is the largest human body organ, envelops and protects the body from the external environment while helping to maintain the integrity and appropriate functioning of the complex inner body organs. The rate determining step for transdermal delivery of most drugs is provided by the stratum corneum (SC) (Scheuplein, 1965). Its structure has been depicted in the brick and mortar model (Elias, 1981; Michaels *et al.,* 1975) in which anucleate keratinized cells are embedded in a lipid mortar. The SC lipids are arranged in multiple bilayers providing alternate hydrophobic and hydrophilic barriers (Williams & Barry, 1991a). *In vitro* skin permeability studies can provide an understanding of the nature and origin of the barrier properties of the skin in terms of physicochemical characteristics of the SC (Kurihara-Bergstrom & Good, 1994).

Only a handful of drugs are suitable for transdermal administration, because in order to achieve significant plasma concentrations, drug absorption must be substantial. For this to occur, the drug absorption must be potent, the drug preferably of low molecular weight, lipophilic and unionized at a physiological pH (Alexander-Williams & Rowbotham, 1998).

5-Fluorouracil (5-FU) is an important drug in the topical treatment of actinic keratoses and basal cell carcinomas of the face and other, more permeable areas of the skin (Goette, 1981). 5-FU is a polar hydrophilic compound with pKa values of 8 and 13 (Ritchel & Hussain, 1988) and a log P value (octanol/water partition coefficient) of -0.78 ± 0.31 (ACD software, Toronto, Canada). Due to these characteristics, 5-FU itself is not a good penetrant through skin. It was, however, found that hydrophilic drugs have great potential for enhancement of their skin penetration because their permeability coefficients are low (Flynn & Stewart, 1988; Williams & Barry, 1991b).

One of the approaches in improving transdermal absorption is to include a penetration enhancer in the formulation of the drug.

Traditionally, penetration enhancers were designed to deliver high drug concentrations through the skin into the systemic circulation. The use of many of these agents, however, resulted in unpleasant or toxic side effects (Asbill & Michniak, 2000). The ideal penetration enhancer should be non-toxic, effective over a wide pH range and act in a reversible way. Other characteristics, like reliability with respect to site-specific drug release or controlled release, may add to the potential utility of such an ideal absorption-enhancing agent (Kotzé *et al.,* 1999).

Terpenes are hydrocarbons with the general formula $(C_5H_8)_n$ together with their oxygenated derivatives. Essential plant oils are the main source of terpenes (Cal *et al.,* 2001). Terpenes are of low cutaneous irritancy, possess good toxicological profiles, provide excellent enhancing abilities and appear to be promising candidates for pharmaceutical formulations. Terpenes are more often present in drugs and cosmetics as components of essential oils added for some or other reason: for inhalation or for topical administration, as rubefacient, analgesics or antiseptics. A variety of terpenes have been shown to increase the transdermal absorption of both hydrophilic and lipophilic drugs (Gao & Singh, 1998). The terpenes used in this study were menthol, isomenthol, menthone, β-myrcene, limonene and 1,8-cineole (eucalyptol).

The main objectives of this study were to:

- Determine the effect of the selected terpenes (with different physicochemical characteristics) on the transdermal delivery of 5-fluorouracil.
- Determine the penetration of the selected terpenes through the human stratum corneum (SC).

In order to achieve these objectives, the following aims had to be done:

- Investigate available literature and information on the issues of transdermal drug delivery, 5-FU and terpenes.
- Perform *in vitro* skin diffusion studies of 5-FU.
- Development of an analytical method on a HPLC that was reliable and sensitive enough to determine the concentration of 5-FU that penetrated through the SC.
- Development of an analytical method on a gas chromatograph that was reliable and sensitive enough to determine the cumulative percentage of terpenes penetrated through the SC.

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CHAPTER 2 TRANSDERMAL DELIVERY

2.1 INTRODUCTION

Until the 1900, the skin was believed to be an impervious barrier, designed to protect the body from foreign organisms as well as from chemicals and drugs. This view changed with a serendipitous finding that polar compounds such as dimethyl sulphoxide were absorbed into blood circulation rapidly after exposure to skin. This discovery led to active research towards development of transdermal methods for systemic administration of drugs (Chaubal, 2002).

The potential of using intact skin as the site of administration for dermatological preparations to elicit pharmacological action in the skin tissue has been recognized for several years (Barr, 1962). The permeation of chemicals, toxic agents and drugs are much slower across the skin when compared to other biological membranes in the body. The understanding of this complex phenomenon has led to the development of transdermal drug delivery systems, in which the skin serves as the site for the administration of systemically active drugs. It is only after skin permeation, that the drugs reach the systemic circulation. The drug molecules are then transported to the target site, which could be relatively remote from the site of administration, to produce their therapeutic action (Baker, 1986). In addition to the relationship between rate of drug delivery to the skin and maximum achievable drug permeation across the skin, the choice of drugs to be delivered transdermally, clinical needs and drug pharmacokinetics are some of the important considerations in the development of transdermal drug delivery systems (Chien, 1988; Ritschel & Hussain, 1988). Doctors around the world are calling transdermal delivery the "Delivery System of the Future" (Anon, 2003).

2.2 THE SKIN AS BARRIER TO TRANSDERMAL ABSORPTION

The skin of an average adult body covers a surface area of approximately 2 square meters and receives about one-third of the blood circulating through the body. It is one of the most readily accessible organs of the human body. With a thickness of only a few millimeters (2.97 ± 0.28 mm),

the skin separates the vital organs from the outside environment, serves as a protective barrier against physical, chemical or microbial attacks, acts as a thermostat in maintaining body temperature, plays a role in the regulation of the blood pressure, and protects the human body against the penetration of ultraviolet rays.

Microscopically, the skin is a multi-layered organ composed of many histological layers. It is generally described in terms of three major multi-laminate layers: the epidermis, the dermis, and the hypodermis, as shown in Figure 2-1. The epidermis is further divided into five anatomical layers with the outermost layer of stratum corneum exposed to the external environment (Chien, 1987).

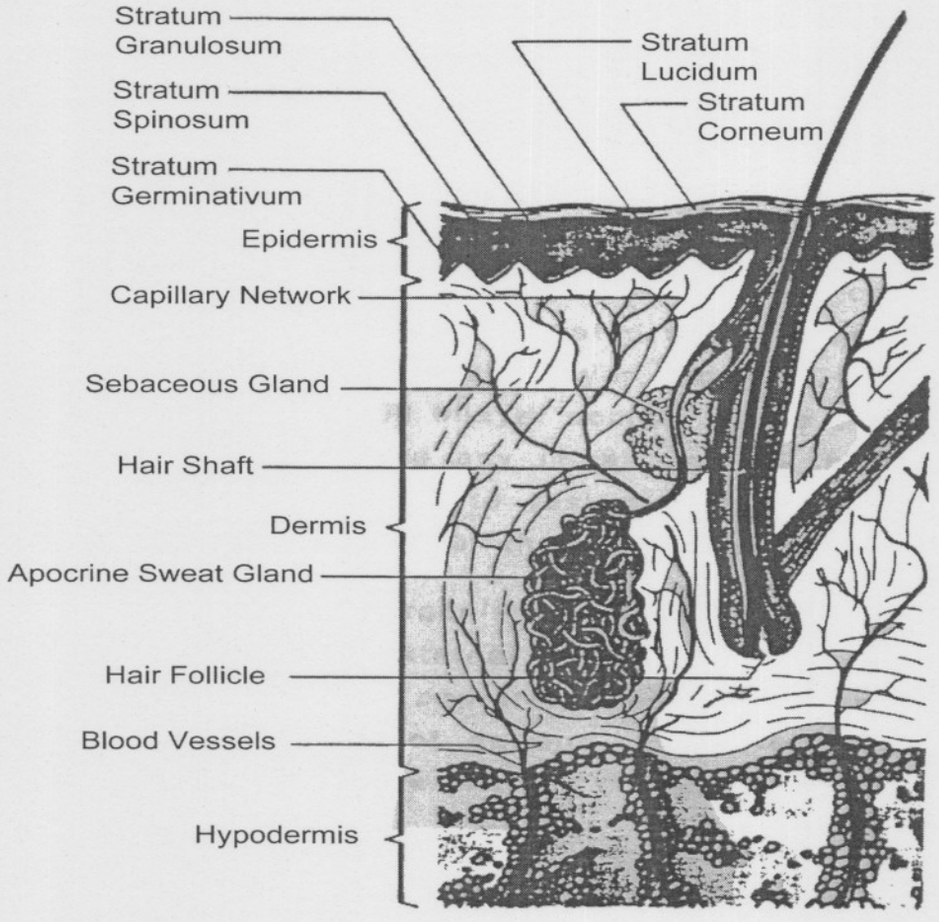

Figure 2-1: A cross sectional view of human skin, showing various skin tissue layers and appendages (Chien, 1987).

2.2.1 THE STRATUM CORNEUM (SC)

The surface layer (10-20 μm), the SC, is highly hydrophobic and contains 10-15 layers of interdigitated corneocytes, which are constantly shed and renewed. Its organization can be described by the "brick and mortar" model, in which extracellular lipid accounts for ~10 % of the dry weight of this layer, and 90 % is intracellular protein (mainly keratin). The SC lacks phospholipids, but is enriched in ceramides and neutral lipids (cholesterol, fatty acids, cholesteryl esters) that are arranged in a bilayer format and form so called 'lipid channels'. Interdigitated long-chain ω-hydroxy-ceramides provide cohesion between corneocytes by forming tight lipid envelopes around the corneocyte protein component. The barrier function of the skin is created by lamellar granules, which are synthesized in the granular layer and later become organized into the intercellular lipid bilayer domain of the SC. Barrier lipids are tightly controlled and any impairment to the skin results in active synthetic processes to restore them. The skin's barrier function appears to depend on the specific ratio of various lipids.

Because of its highly organized structure, the SC 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. The ability of various agents to interact with the intercellular lipid therefore dictates the degree to which absorption is enhanced (Foldvari, 2000).

2.2.2 VIABLE EPIDERMIS

The viable epidermis consists of multiple layers of keratinocytes at various stages of differentiation. 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. The role of the viable epidermis in skin barrier function is mainly related to the intercellular lipid channels and to several partitioning phenomena. Depending on their solubility, drugs can partition from layer to layer after diffusing through the SC.

Several other cells (eg. melanocytes, Langerhans cells, dendritic T cells, epidermotropic lymphocytes and Merkel cells) are also scattered throughout the viable epidermis, which also contains a variety of active catabolic enzymes (e.g. esterases, phosphatases, proteases, nucleotidases and lipases). Lipid catabolic enzymes (such as acid lipase, phospholipase, sphingomyelinase, steroid sulfatase), although mainly concentrated in the SC and granulosum, have been demonstrated throughout the epidermal layers. Although the basal and spinous

layers are rich in phospholipids, 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.

2.2.3 DERMIS AND HYPODERMIS

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, with considerable horizontal branching from the arterioles and venules in the papillary dermis to form plexuses and to supply capillaries to hair follicles and glands. Dermal lymphatic vessels help drain excess extracellular fluid and clear antigenic materials.

The elasticity of the dermis is attributed to a network of protein fibres, including collagen (type I and III) and elastin, which are embedded in an amorphous glycosaminoglycan ground substance. The dermis also contains scattered fibroblast, macrophages, mast cells and leucocytes. Hair follicles, sebaceous glands and sweat glands are found in the dermis and subcutis, and might serve as additional, specific pathways for drug absorption. In some cases, for example, hair follicles might act as target sites for drug delivery (Foldvari, 2000).

2.2.4 SKIN APPENDAGES

The skin has interspersed hair follicles and associated sebaceous glands, the so-called pilosebaceous glands, and in specific regions two types of sweat glands, the eccrine and apocrine glands. Collectively these are called the skin appendages (Flynn, 1990). The sebum which is produced by the sebaceous glands consists of a mixture of fatty acids, triglycerides, waxes, cholesterol and cellular debris (Montagna, 1965). Lipophilic drugs that are compatible with sebum will diffuse through the follicles, while hydrophilic drugs that are incompatible with the sebaceous lipids will not be able to utilize this pathway for passive diffusion (Ramachandran & Fleisher, 2000).

2.3 TRANSDERMAL ABSORPTION

The sequential steps in transdermal absorption are shown in Figure 2-2 and are:

1. Diffusion or transport of the penetrant to the skin surface.
2. Partitioning of the chemical into the stratum corneum.
3. Diffusion through (the intercellular lipids of) the stratum corneum.
4. Partitioning of the chemical from the lipophilic stratum corneum into the aqueous viable epidermis.
5. Diffusion through the viable epidermis and upper dermis.
6. Uptake of penetrant into a cutaneous blood vessel and systemic access (Guy & Hadgraft, 1989a).

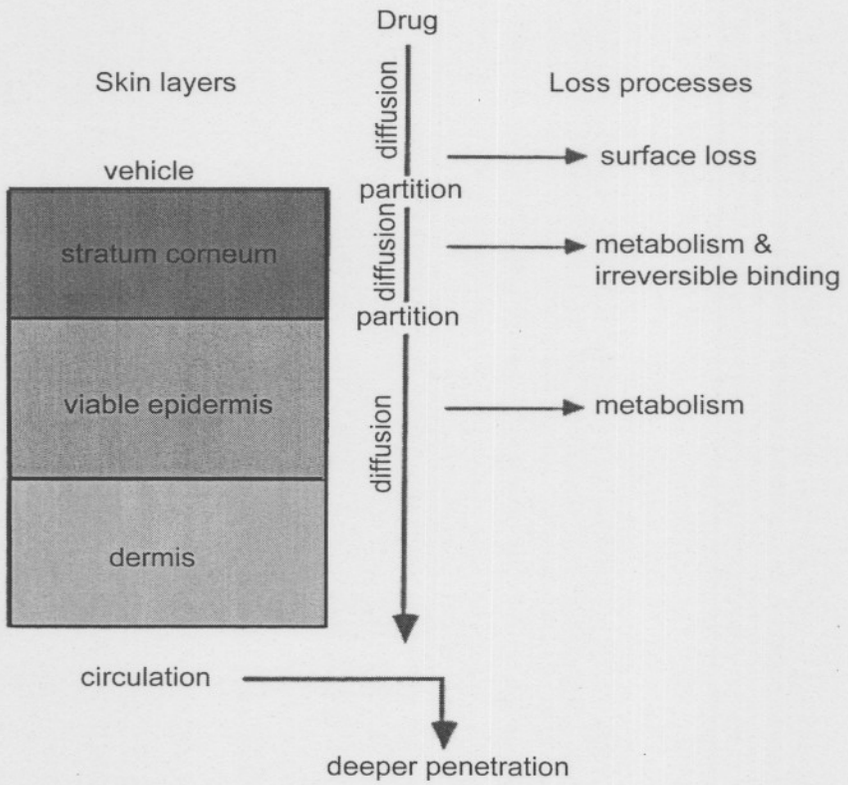

Figure 2-2: Sequential physicochemical steps involved in transdermal absorption (Guy & Hadgraft, 1989a).

Transdermal absorption is a step-wise process and can be divided into three parts:

- **Penetration** is the entry of a substance into a particular layer.
- **Permeation** is the penetration from one layer into another.
- **Absorption** is the uptake of a substance into systemic circulation (Panchagnula, 1997).

2.4 ROUTES OF PENETRATION

Figure 2-3 illustrates the possible macro routes of drug permeation across intact skin.

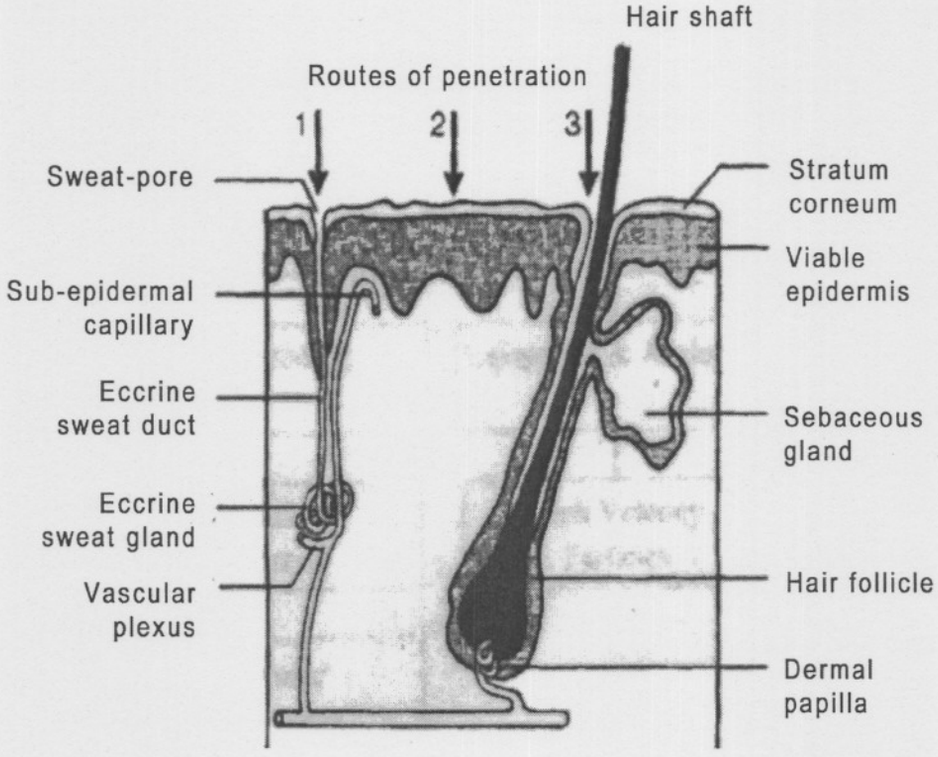

Figure 2-3: The three potential routes of penetration of a diffusant into the subepidermal tissue of the skin: (1) *via* the sweat ducts, (2) across the continuous stratum corneum or (3) through the hair follicles with their associated sebaceous glands (Barry, 1983).

The transappendageal route transports substances *via* the sweat glands and the hair follicles with their associated sebaceous glands. The transepidermal route across the continuous stratum corneum comprises transport via intracellular and intercellular spaces. Both polar and

non-polar substances diffuse *via* transcellular and intercellular routes by different mechanisms (Blank *et al.*, 1967). The polar molecules mainly diffuse through the polar pathway consisting of "bound-water" within the hydrated stratum corneum, whereas the non-polar molecules dissolve and diffuse through the non-aqueous lipid matrix of the stratum corneum. Figure 2-4 describes possible micro routes of drug permeation. The transappendageal route is considered to be of minor importance because of their relatively small area (less than 0.1 % of total surface). However, this route may be of some importance for large polar compounds (Barry, 1987).

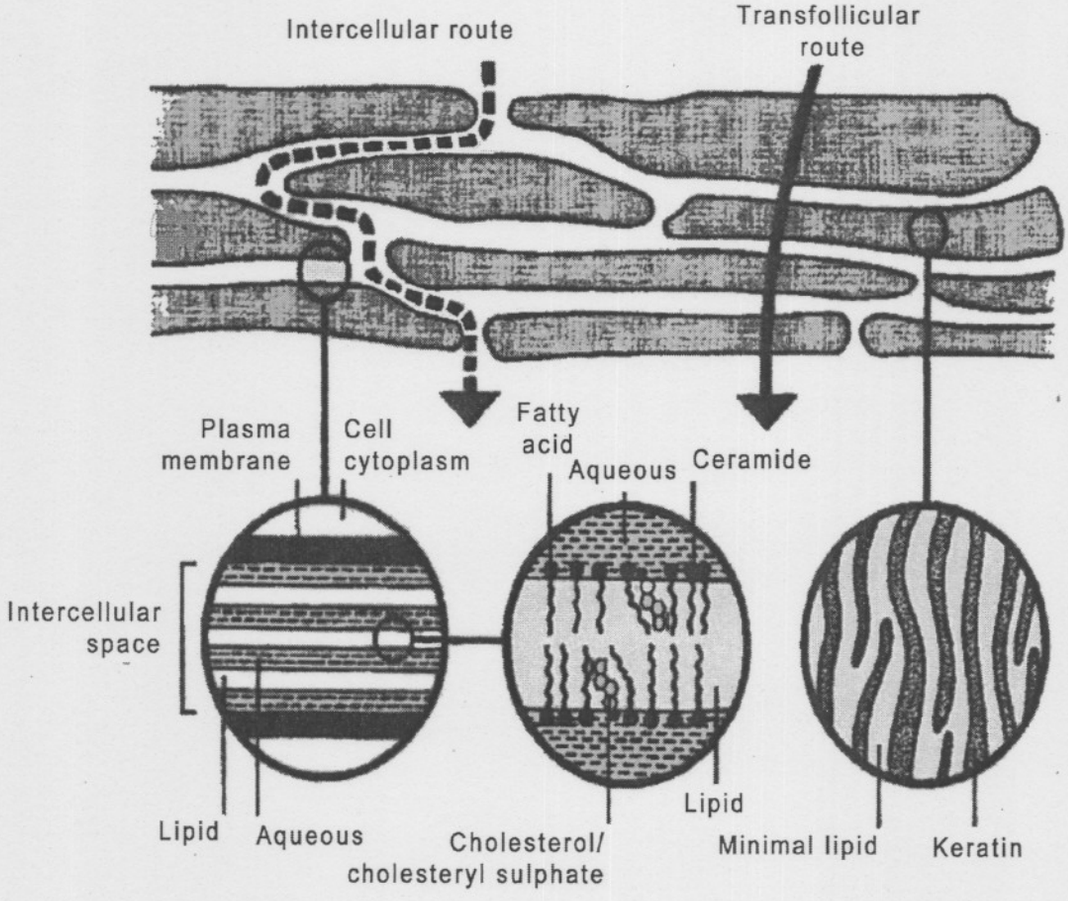

Figure 2-4: The possible micro-routes for drug entry through the stratum corneum – transcellular or intercellular (Barry, 1987).

2.5 ADVANTAGES OF TRANSDERMAL DELIVERY

Transdermal delivery differs from traditional topical drug delivery in that the latter involves drug transport to viable epidermal and/or dermal tissues of the skin for local therapeutic effect, with only a very meager fraction of drug transported into the systemic blood circulation, while with transdermal delivery the aim is to deliver the required dose systemically.

Transdermal drug delivery (TDD) has several advantages over conventional oral dosage forms:

- It avoids peaks and valleys in serum levels often seen with discrete oral dosages.
- It avoids first-pass metabolism in the stomach and liver upon oral administration of a drug, as skin metabolism extremely low.
- In many instances, zero-order delivery is maintained and can be sustained for a longer period of time, leading to less frequent dosing regimens.
- Less side effects, like nausea.
- Better patient compliance
- Relatively less intersubject variability with the transdermal system as compared to oral drug administration because of unavoidable food effects and adverse physiological conditions that might interfere with the oral absorption process (Roy, 1997).

2.6 APPROACHES TO DETERMINE SKIN ABSORPTION

A mathematical model for absorption is given by Fick's first law. The simplest way of modeling the process of skin absorption is to assume that Fick's first law of diffusion is applicable (Guy & Hadgraft, 1989b). In terms of Fick's law of diffusion, the skin can be regarded as a composite membrane, taking into account the effects of the circulation. Fick's first law states that the quantity of a diffusing substance, J, which migrates in 1 second through 1 cm^2 in the direction X from the skin surface into the stratum corneum, is equal to the diffusion coefficient D, multiplied by the concentration gradient (dc/dx):

$$J = -D\left(\frac{dc}{dx}\right) \qquad \text{(Equation 2-1)}$$

However, during the diffusion of a drug into the stratum corneum, the concentration gradient in the distribution space is reduced. Fick's second law defines this decrease of the gradient with time:

$$\left(\frac{\delta C}{\delta t}\right) = D\left(\frac{\delta^2 c}{\delta x^2}\right) \qquad \text{(Equation 2-2)}$$

Transformation shows that the quantity, which diffuses from the drug depot to a given distance, is proportional to the square root of the diffusion time; this means that the substance spreads with a decreasing velocity. Over short distances, however, diffusion is rather constant.

The assumption that the diffusion coefficient is constant is only a good approximation. Furthermore, neither the stratum corneum nor the whole skin is an unique inert membrane. Therefore the drug concentrations in the formulation are not the same as at the skin surface but are related to them by the vehicle-membrane distribution coefficient K_m. When the difference between the concentration at the upper membrane surface and its lower surface is ΔC and the thickness of the membrane is δ, then the equation can be stated as follows:

$$J = \frac{K_m \cdot D \cdot C}{\delta} = k_p \cdot \Delta C \qquad \text{(Equation 2-3)}$$

Where:

J = flux of drug

K_m = vehicle-membrane distribution coefficient

D = diffusion coefficient

ΔC = concentration difference between the upper surface and lower surface of the membrane

δ = thickness of the membrane

k_p = permeability coefficient

The parameters in Equation 2-3 can be measured or calculated (Schalla & Schaefer, 1982).

Thus, it can be concluded that the diffusion coefficient (D) and the permeability coefficient (k_p) are the determinant factors in the transdermal delivery of drugs. By increasing any of these two

parameters, the transdermal delivery can be increased. This can be accomplished by physical and chemical penetration enhancement that will be discussed in 2.8.

2.7 FACTORS INFLUENCING TRANSDERMAL DELIVERY

The skin influences transdermal delivery through its control of the transport of drug across its barrier layers, the adhesion of a transdermal system, and by providing a warning system for the body by regulating against xenobiotics entering the skin in the form of irritation and sensitization. None of the factors that contribute to these influences are mutually dependent phenomena between the drug, system composition and skin. The biological, chemical and physical properties of the drug, skin and formulation influence the ability of the transdermal system to deliver the drug on a continuing basis (Cleary, 1993).

2.7.1 BIOSYSTEMIC FACTORS AFFECTING TRANSDERMAL ABSORPTION

2.7.1.1 Composition of the stratum corneum

The stratum corneum consists of both hydrophilic (protein rich intercellular space between the corneocytes) and lipophilic (membranes around the cells) components, the water content of which may vary depending on the body location and environmental conditions. This property of the stratum corneum causes it to act selectively in the absorption of most substances and is being seen as the rate-limiting step of the absorption process (Göpferich & Lee, 1992; Squire & Lees, 1992). When the water content increases as a result of water diffusing from underlying epidermal layers or because of excessive environmental humidity, permeability of polar and nonpolar substances increases (Ansel, 1985).

2.7.1.2 Dermatological conditions

Physical injuries to the skin surface, such as cuts, abrasions and burns, destroy the natural barrier function of the stratum corneum, enhancing the absorption of almost any substance. Increased permeability of substances may be allowed by skin diseases such as atopic dermatitis, inflammatory/allergic conditions and exfoliative dermatitis, because these conditions may alter the integrity of the stratum corneum (Barry, 1988).

2.7.1.3 Reservoir effects

Drug molecules may bind to certain sites or be retained by certain organelle, for example proteins, within any of the various skin layers. These bound molecules cannot diffuse further and are thus not able to be taken up by the general circulation and are, therefore, not immediately bioavailable. A process whereby drug molecules are "held back" in the stratum corneum because of insufficient water solubility for diffusion into the aqueous tissues under the SC, was described by Guy and Hadgraft (1989c). Although this might not be considered entirely as a depot or reservoir effect, it also leads to the drug not being bioavailable immediately.

2.7.2 PHYSICOCHEMICAL FACTORS

2.7.2.1 Drug related factors that affect release, rate and permeation

Physicochemical properties of a drug substance are the most important determinants for its permeation through the skin. The molecular weight, molecular volume, water solubility, melting point, and oil/water partition coefficient are some of the important physicochemical attributes that should be taken into account for selecting potential transdermal candidates (Roy, 1997).

2.7.2.1.1 Drug solubility

The solubility characteristics of a substance greatly influence its ability to penetrate biological membranes. In the formulation of preparations for topical application, it is profitable to select or prepare compounds having the required solubility characteristics before attempting to promote their penetration by pharmaceutical manipulation. The activity becomes less as the derivatives become more lipid and less water soluble. Compounds that are more soluble in water and less soluble in lipids are similarly less active after topical application (Idson, 1975). The relationship presumably exists because a certain amphiphilicity (hydrophilic/lipophilic) balance is required.

In order to permeate through the skin, the molecules need to penetrate from the vehicle into the outermost lipophilic tissue – the SC (i.e. possess a reasonable lipophilicity). Subsequently, the molecule needs to partition out of the SC into the essentially aqueous viable epidermis (i.e. possess a reasonable hydrophilicity). For very lipophilic molecules the rate-determining step may be the partitioning of the drug from the SC to the epidermis, whereas for hydrophilic

molecules, it is penetration into the SC. Optimum skin permeation is therefore reached with molecules having "mixed" lipophilic/hydrophilic properties (Surber *et al.*, 1993).

If the drug is only partially soluble in the vehicle, that is, if it is partially in solution and partially present as an undissolved solid, release from the vehicle may be less than maximal, possibly compromising bioavailability. Poor release onto and into the subsurface strata in this case is a kinetic problem related to the diffusion of the drug through the applied vehicle film. On the other hand, if the drug is extremely soluble in the vehicle film so that it is in solution in a highly unsaturated state, then the drug will tend to remain in the film, which has a high affinity for it, rather than to partition into the skin tissue. The optimum between these two extremes is obtained by adjusting the solubility of the drug by appropriate choice of vehicle components so that essentially all the drugs are in solution and at the same time the vehicle is saturated or nearly saturated with the drug (Flynn, 1990).

The solubility constraint in the SC (σ_{SC}/μg.cm^{-2}) can be estimated using equation 2-4 or 2-5:

$$\text{Log } \sigma_{SC} = 1.911\ (10^3/\text{mp}) - 2.956 \qquad \text{(Equation 2-4)}$$

$$\text{Log } \sigma_{SC} = 1.31 \log [\text{oct}] - 0.13 \qquad \text{(Equation 2-5)}$$

Where mp is the permeant melting point (Kelvin) and [oct] is the octanol solubility of the permeant (g/l) (Hadgraft & Wolff, 1993).

The solubility parameter or cohesive energy density of a drug is synonymous with lipophilic/hydrophilic properties. In general, materials which have low melting points penetrate the skin more readily (Hadgraft & Wolff, 1993).

2.7.2.1.2 Ionic state

The non-polar nature of the horny layer suggests that charged compounds should encounter high resistance to permeation. This proposition is most easily studied by use of ionigenic compounds, for which the ratio of charged to uncharged species can be manipulated by changing the pH of the vehicle. The two species are of about equal size, so their diffusion coefficients should have about the same value (Zatz, 1993a).

Most drugs are weak acids or bases and may exist in an ionized or non-ionized form. The movement of a drug through membranes is governed to a large extent by the degree of ionization. Because of the greater lipophilicity of non-ionized forms, these forms have a higher

potential to permeate the skin than ionized forms (Abdou, 1989 and Jack *et al.*, 1991). Ionized drugs are either bound to or repelled by the membranes which are highly charged, or they may bind with water becoming larger molecules and, therefore, lose their capacity to permeate (Parry *et al.*, 1990).

Therefore, it can be stated that the importance of the ionic state of a drug as a factor in the absorption process could be disregarded provided the pH of the vehicle has been demonstrated as being optimal for the specific drug.

2.7.2.1.3 Molecular size and mass

Molecular size and mass play an important role in determining the release of the drug from a transdermal therapeutic system (TTS) as the drug molecules have to diffuse through the matrix components, the membrane of the TTS, the stratum corneum and the underlying tissues. Small molecules penetrate more rapidly than larger molecules, but within a narrow range of molecular size. An optimal drug diffusion rate is documented for molecular masses of 800 to 1000, but molecules as large as 5000 can also penetrate the skin, although slower (Parikh *et al.*, 1984). Compounds of small molecular size may penetrate through the aqueous pathway more readily than larger molecules, which penetrate more readily through the lipoidal pathway (Zatz, 1993b; Takahashi, 1993).

2.7.2.1.4 Log P (octanol/water partition coefficient)

The lipid/water partition coefficient indicates the ratio of components of a drug in two (practically) immiscible phases. This partition coefficient is the basic decisive factor for specific drug permeability through the stratum corneum of the skin (Ritschel, 1988).

For transdermal delivery a drug effectively undergoes three major partitioning movements, namely, from the vehicle into the SC, from the SC into the epidermis and from the epidermis into the dermis. Here, the drug may partition into the capillary system and thus enter the systemic circulation to become bioavailable, or it may partition into the subcutaneous fat to form a fat depot (Barry, 1988). It is therefore obvious that bioavailability and the resultant pharmacological action are to a large extent dependent on the partitioning behaviour of the drug (Al-Khamis *et al.*, 1987).

The partition coefficient is most often determined between octanol and water and is in fact a measure of the preference of the drug for a hydrophilic or lipophilic phase (York, 1988; Idson, 1983). The partition coefficient of the drug may be determined according to the following equation (Ansel, 1985):

$$Kp = \frac{\text{concentration of drug in octanol}}{\text{concentration of drug in water}} \qquad \text{(Equation 2-6)}$$

This equation indicates that a proper balance in the partition coefficient is necessary to ensure optimum drug release and permeation into the SC and through the skin layers. The drug characteristics required to ensure the optimum functioning would, therefore, favour a drug with only sufficient water solubility to dissolve in the aqueous phase but with sufficient lipid solubility to prevent entrainment in the water layer and the ideal octanol/water partition coefficient would therefore be one or slightly greater than one (Abdou, 1989). According to Guy (1996) compounds with a log P value between 1 and 3, with modest melting points and with relatively low molecular weights, are likely to have decent passive skin permeabilities. Potts and Guy (1992) determined that the optimal log P value for the range of non-steroidal anti-inflammatory agents and salicylates is ~ 2.5.

2.7.2.2 Skin hydration

Hydration of skin is a major factor affecting the rate and extent of transdermal absorption. This effect is usually more important for nonpolar than polar molecules, and is most likely secondary to an increase in diffusivity of the penetrating molecule.

However, hydration may also affect the partitioning and concentration gradient of the penetrating molecule in the stratum corneum as well as the overall thickness of the effective barrier. Changes in these parameters could alter the size of the stratum corneum reservoir for different penetrants, an event which would change the shape of the permeation profile (Riviere, 1993).

The mechanism of transport of a drug through hydrated SC may be quite different from that through normal SC. Schalla and Schaefer (1982) suggested that the mechanism of hydration was to increase the size of the pores. There is not only a physical alteration of the tissue due to hydration, but at high water activities there are also changes in both the diffusion coefficient and

activity coefficient of the penetrating agent. This leads to an increase in the flux of most substances.

Environments with high relative humidity (greater than 80 %) may also result in significant skin hydration. An assessment of the degree of hydration can be made by monitoring the permeability of the stratum corneum to water through measurement of transepidermal water loss. The effect of hydration has also been demonstrated using *in vitro* model systems in which the epidermal surface (donor reservoir) is immersed in water; in this case the relative humidity is effectively 100 %. In *in vitro* diffusion cell studies where the donor chamber is fully immersed in fluid (e.g. infinite-dose static cell studies), one is actually studying the absorption across fully-hydrated skin, a situation rarely encountered *in vivo* in humans. This limitation should be recognized when extrapolating data from such studies (Riviere, 1993).

2.7.3 PHYSICOCHEMICAL PROPERTIES OF 5-FLUOROURACIL

The transdermal route of administration cannot be utilized for a wide range of drugs, and it is therefore necessary to identify the factors that limit drug suitability, and secondly, to address the problems involved in the selection of suitable drugs (Guy & Hadgraft, 1989c). Of primary importance is the determination of the rate at which a drug penetrates the skin as well as a drug's physicochemical properties. The relevant physicochemical properties can be determined by examining the mechanisms by which a drug penetrates the skin (Hadgraft & Wolff, 1993). These properties are listed below and the chemical structure is shown in Figure 2-5.

One anti-viral (anti-neoplastic) drug was chosen as model for this study, namely 5-fluorouracil (5-FU). 5-FU, firstly introduced as a rationally synthesised anticancer agent 30 years ago, continues to be widely used in the management of several common malignancies including cancer of the colon, breast and skin. 5-Fluorouracil is an important drug in the topical treatment of actinic keratoses and basal cell carcinomas of the face and other, more permeable areas of skin (Goette, 1981). This drug is a weak acid and an analogue of the naturally occurring pyrimidine uracil, and can also be classified as a polar hydrophilic penetrant (Diasio & Harris, 1989).

2.7.3.1 Structure and properties of 5-fluorouracil

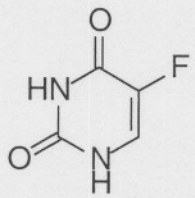

Figure 2-5: The chemical structure of 5-fluorouracil (Rudy & Senkowski, 1973).

- **Chemical name:** 5-Fluoro-2,4(1H,3H)-pyrimidinedione, 2,4-dioxo-5-fluoro pyrimidine or 2,4(1H,3H)-pyrimidinedione, 5-fluoro (Bayomi & Al-Badr, 1989).
- **Formula:** $C_4H_3FN_2O_2$ (Bayomi & Al-Badr, 1989).
- **Molecular weight:** 130.08 (Bayomi & Al-Badr, 1989).
- **Appearance, colour and odour:** White to practically white, odourless, crystalline powder (Bayomi & Al-Badr, 1989).
- **Melting point:** 282 ºC – 283 ºC (Bayomi & Al-Badr, 1989).
- **Dissociation coefficient:** pKa = 8 and 13 (Rudy & Senkowski, 1973).
- **Stability:** 5-fluorouracil is stable in solutions which are not strongly basic (pH less than 9). When subjected to strongly basic conditions, 5-fluorouracil is hydrolyzed to urea, fluoride, and an aldehyde. This hydrolysis is enhanced by increased pH and temperature (Rudy & Senkowski, 1973).
- **Solubility:** The solubility data obtained at 25 ºC for 5-FU and can be seen in Table 2-1.
- **The log octanol water partition coefficient:** - 0.78 ± 0.31 (ACD software, Toronto, Canada).
- **% Unionised pH 6:** 99,99 % with a pKa of 13 and 99,01 % with a pKa of 8 (Ritschel & Hussain, 1988).

It is clear that 5-fluorouracil (5-FU) is a polar hydrophilic compound. Together with its other physicochemical properties, like its low log octanol/water partition coefficient and pKa values, 5-FU would not be a good candidate for transdermal delivery. Hydrophilic drugs have great potential for enhancement because of their low permeability coefficients (Flynn & Stewart, 1988; Williams & Barry, 1991a). The ideal octanol/water partition coefficient for a compound for optimum permeation into the SC and through the skin, is believed to be ~ 2.5, and because of 5-FU octanol/water partition coefficient of -0.78 ± 0.31, penetration enhancers can be of great benefit (Potts & Guy, 1992).

Table 2-1: 5-Fluorouracil solubility (Rudy & Senkowski, 1973).

Solvent	Solubility (mg/ml)
Benzene	< 0.1
Chloroform	< 0.1
95 % ethanol	5.54
Ethyl ether	< 0.1
Isopropyl alcohol	2.15
Methanol	9.37
Petroleum ether (30 °C – 60 °C)	< 0.1
Water	12.2

According to Moghimi and co-workers (1996) it is predicted that 5-FU would follow the transcellular route for penetration through enhancer treated skin. For a hydrophilic drug, partitioning into the corneocytes should not be a rate limiting step, and if such a drug do not permeate the SC through the transcellular route, the rate limiting step should be a diffusional barrier (Moghimi *et al.*, 1996).

A popular technique to enhance the permeability of 5-FU is the use of penetration enhancers which reduce reversibly the permeability barrier of the SC (Barry, 1983). The lipophilicity of the permeant as well as the enhancer molecule is thought to play an important role in determining

the enhancers' promoting activity on the permeation of the drug across the skin (El-Kattan *et al.*, 2001). Terpenes were reported to be effective penetration enhancers for both hydrophilic and lipophilic drugs, with low skin irritancy and low systemic toxicity (Cornwell & Barry, 1994), and therefore terpenes were chosen as penetration enhancers in this study.

The use of terpenes as penetration enhancers will be discussed in § 2.8.2.2.3.

2.8 PENETRATION ENHANCERS

Currently, the most widely utilized approach to drug permeation enhancement involves the use of chemical permeation enhancers. Permeation enhancers, also referred to as penetration enhancers, accelerants, or absorption promoters, assist the transfer of the drug through the skin. Chemical penetration enhancers generally partition into the skin, and interact with different skin constituents to elicit temporary and, ideally, reversible reduction of barrier properties (Büyüktimkin *et al.*, 1997). Firstly, physical enhancers will be discussed and then chemical enhancers.

2.8.1 PHYSICAL ENHANCERS

Although many different physical approaches to enhancing percutaneous absorption have been attempted, the most notable approaches are iontophoresis, ultrasound (sonophoresis) and electroporation. None of these enhancement methods are passive since they require the input of energy to achieve their effects. To date, these methods show the most promising effect for transdermal drug delivery systems that incorporate a large drug reservoir on the surface of the skin and that need to deliver very large molecular weight compounds in the kilodalton range (Finnin & Morgan, 1999).

2.8.1.1 Iontophoresis

Most drugs cannot permeate through human skin in therapeutic quantities by passive diffusion alone, and almost no peptide or protein drugs can penetrate into the skin at all because of their large molecular size and hydrophilicity. The need for penetration enhancement techniques brought iontophoresis research to the front line (Sun, 1997).

Iontophoresis is a process which causes an increased penetration of solute molecules into tissues by the use of an applied current through the tissue. Iontophoresis may provide a safe, economical and convenient way to administer charged or uncharged drugs transdermally in a controlled manner (Burnette, 1989).

The potential advantages of iontophoresis include:

- An increased capability of delivering larger amounts of therapeutic agents compared to passive delivery systems.
- Its ability to deliver significantly higher amounts of relatively large molecular weight compounds.
- Better control of the delivery profile, including nonzero-order profiles.

Potential disadvantages of iontophoresis include:

- Complexity of the delivery system. Even though prototype iontophoresis patches which are identical in appearance to transdermal patches are available, they are significantly more complex in nature.
- Chemical stability of the therapeutic agent.
- Relatively unknown toxicology of prolonged exposure to current. Historically, iontophoresis has been used for short-term treatments.
- Cost. It is obvious that the costs of development and manufacture for the iontophoretic patch would be significantly higher than the passive transdermal patch.

Some of the critical factors that significantly affect iontophoretic transport are

- electrical considerations,
- pH,
- efficiency,
- current and
- chemical enhancement (Sarpotdar, 1991).

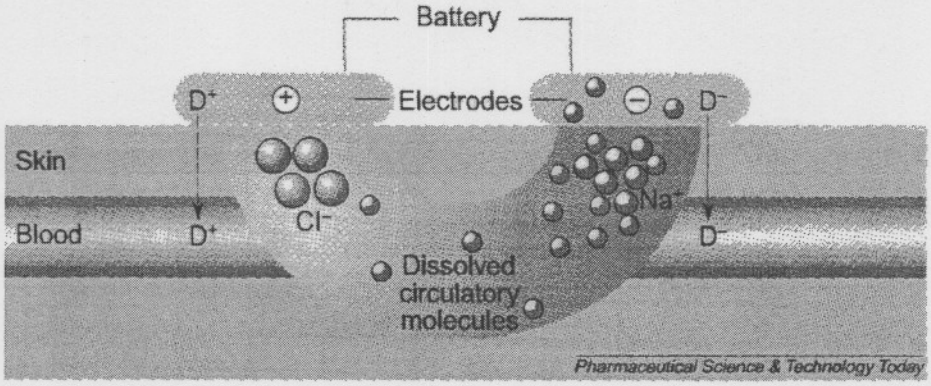

Figure 2-6: Schematic presentation of an iontophoretic device. An iontophoretic assembly principally consists of a pair of (formulation containing) electrode chambers, which are placed in contact with the skin surface. When the device is powered, the passage of a small electrical current drives positively charged drugs into the skin from the anode and, likewise, negatively charged drugs from the cathode. At the same time circulating anions (e.g. Cl^-) and cations (e.g. Na^+) make their way towards the anode and cathode, respectively. In addition, the permselective nature of the skin favours the transport of cations resulting in a net convective flow of solvent in the anode to cathode direction. Consequently, dissolved analytes (e.g. glucose) are also transported towards the cathode where they can be extracted and monitored (Naik *et al.,* 2000).

2.8.2 CHEMICAL PENETRATION ENHANCERS

The most extensively investigated enhancement strategy involves the use of chemicals that can reversibly compromise the skin's barrier function and consequently allow the entry of otherwise poorly penetrating molecules into the membranes and through to the circulation (Naik *et al.,* 2000). Currently, the most widely used approach to drug penetration enhancement across the stratum corneum barrier is the use of chemical penetration enhancers (sorption promoters and accelerants) and prodrugs (Asbill & Michniak, 2000).

2.8.2.1 Prodrugs

Because the physicochemical properties of the drugs themselves are usually not optimal for their delivery into or through topical membranes, the topical administration of drugs is not always effective (Sloan, 1992).

One approach to deal with the problem is to make a transient derivative – a prodrug of the drug – which imparts to the drug the desired transient change in its physicochemical properties. This approach has many advantages. Firstly, the changes in the physicochemical properties and the pharmacological profile of the drug are transient, so that once the prodrug has delivered the drug, one is left with a well-characterised and well-understood molecule with which to work. Secondly, the breadth of the possible transient changes is limited only by the imagination and resourcefulness of those responsible for designing the prodrug (Sloan, 1992). The aim of the prodrug approach is changing the pharmaceutical and/or pharmacokinetic character of the parent drug and thereby enhancing its skin permeation, efficacy and therapeutic value (Rautio *et al.,* 2000).

Earlier efforts concentrated on the chemical modification of drug molecules in order to increase drug flux through the production of derivatives with optimum lipid solubilities. This concept is also applicable in the prodrug approach, in which inactive but highly absorbable prodrug molecules are subsequently activated within the skin (Foldvari, 2000).

Chemical modification of the drug structure in order to improve its therapeutic index provides the most versatile approach. The classical way is to design the molecule to best fit the target receptor. Since the structure of the receptor site is generally not known in detail, this is an iterative process: we get to know the structure of the receptor from the various substrates. However, this approach cannot generally lead to the elusive "magic bullet", not only because of the limitations of the receptor specificity, but also owing to the distribution of the receptors. These facts require additional chemical modifications to be considered. It was suggested that prodrugs, the inactive chemical precursors of the active drugs, could improve drug specificity. Prodrugs are essentially designed in such a way that their major or preferably single metabolic pathway is the one leading to the active drug (Bodor, 1987).

This approach, however, is rarely possible with proteins and DNA. The remaining and most feasible option might therefore involve manipulation of the skin barrier (Foldvari, 2000)

2.8.2.2 Penetration enhancers

The most popular solution for overcoming the intrinsic resistance of the stratum corneum and its biological variability is to incorporate penetration enhancers (accelerants or absorption promoters) into the skin products or transdermal devices. A penetration enhancer is a chemical that displays the only characteristic that it reversibly reduces the barrier nature of the stratum corneum without the accelerant damaging any viably cells.

We can list the desirable attributes of the ideal penetration enhancer as follows (Barry, 1991 & Shah, 1994):

- The material should be pharmacologically inert and should possess no action of itself at receptor sites in the skin or in the body in general.
- The material should not be toxic, irritating or allergenic.
- On application, the onset of penetration-enhancing action should be immediate; the duration of the effect should be predictable and suitable.
- When the material is removed from the skin, the tissue should immediately and fully recover its normal barrier property.
- The barrier function of the skin should reduce in one direction only, so as to promote penetration into the skin. Body fluids, electrolytes or other endogenous materials should not be lost to the atmosphere.
- The enhancer should be chemically and physically compatible with a wide range of drugs and pharmaceutical adjuvants.
- The substance should be an excellent solvent for drugs.
- The material should spread well over the skin and it should have a suitable skin "feel".
- The chemical should have the ability to be formulated into lotions, suspensions, ointments, creams, gels, aerosols, transdermal devices and skin adhesives.
- It should be inexpensive, odourless, tasteless and colourless so as to be cosmetically acceptable.
- The enhancer must increase the diffusivity of the drug in the skin.

- It should cause stratum corneum lipid fluidisation, which leads to decreased barrier function (a reversible action).
- It should increase and optimize the thermodynamic activity of the drug in the vehicle and in the skin.
- It should result in a reservoir of drug within the skin.
- It should affect the partition coefficient of the drug, increasing its release from the formulation into the upper layers of the skin.

Promoters may, of course, be used in conjunction with a thermodynamic control approach, iontophoresis or ultrasound (Barry, 1991).

2.8.2.2.1 Lipid-Protein-Partitioning (LPP) Theory

An overall concept, which explains how penetration enhancers work, is known as the Lipid-Protein-Partitioning (LPP) Theory. It proposes that enhancers usually work by one or more of three main mechanisms. Accelerants

- can alter the *lipid* domain of the stratum corneum,
- may interact with the *protein* elements of the tissue and
- may increase the *partitioning* of a drug, a co-enhancer or water or and combination of these three, into the stratum corneum.

A range of factors controls the relative importance of each route, including the physicochemical properties of the penetrant, diffusional time scale, follicle and gland densities, properties of the stratum corneum, vehicle effects, metabolism, and hydration (Barry, 1991).

The theory assumes the following:

1) At steady state, most molecules permeate human skin across the intact stratum corneum and shunt route penetration is negligible. (The concepts would also apply to that component of penetrant mass using the follicular route and passing through the stratum corneum of the follicle).

2) The rate-limiting step in the percutaneous absorption process lies in permeation across the stratum corneum. Thus, effects within the vehicle (dissolution of crystals, diffusion, evaporation, dilution by transdermal water, etc.) are not rate determining, the stratum corneum is essentially intact and clearance into the viable tissues and blood is fast and unaffected by the enhancer.

3) Enhancer effects are essentially reversible. Thus, the theory does not consider, for example, the corrosive effects of strong acids and alkalies, osmotic shock produced by asymmetric concentrations of materials like dimethyl sulfoxide, and lipid extraction by solvents such as chloroform/methanol.

4) Increases in permeation by maximizing drug chemical potential in the vehicle, by producing supersaturation in the vehicle, and by iontophoresis, ultrasound or heat are all excluded.

Barry (1991) stated that the main conclusion of the theory regarding the mechanisms whereby enhancers reduce the barrier functions of the skin are as follows:

1) Most enhancers so far investigated appear to disturb intercellular lipid packing, increasing fluidity.

2) Many accelerants interact with intracellular protein.

3) A combination of processes 1 and 2 leads to effective enhancers.

4) Many small polar accelerants have the correct physicochemical properties to accumulate in the stratum corneum, altering its solubility properties, and thus promoting the partitioning of a drug, a co-enhancer, water, or a combination of two or three of these.

5) The permeability of the stratum corneum increases with raised water content, and any treatment that promotes hydration usually facilitates drug absorption (Barry, 1991).

A simple way of viewing the effects of enhancers in the skin is to regard the barrier as consisting of two parallel pathways (Cooper & Patel, 1990). The three suggested pathways for drug penetration through the skin are: polar, non-polar, and polar/non-polar. The enhancers act by altering one of these pathways. The key to altering the polar pathway is to bring about protein conformational change or solvent swelling. The key to altering the non-polar pathway is to alter the rigidity of the lipid structure and fluidise the crystalline pathway (thereby substantially increasing diffusion). The fatty acid enhancers will increase the fluidity of the lipid portion of the

stratum corneum. Some enhancers (binary vehicles) act on both polar and non-polar pathways by altering the multilaminate pathway for penetrants. Enhancers can increase the drug diffusivity in the stratum corneum by dissolving the skin lipids or by denaturing skin proteins. The type of enhancer employed has a significant impact on the design and development of the product (Shah, 1994). The polar pathway is thought to be hydrated protein that is quite sensitive to conformational changes induced by surfactants, heat, and the like. A marked contrast between the effects of surfactants on polar versus non-polar molecules serves to illustrate this point. Enhancers for the non-polar pathway are thought to fluidise the lipids. This concept could be quite feasible when considering diffusion processes in a continuous medium. The formulation of these enhancers is not a simple process because they often interact with emollients and these emollients, which are put into creams, can be rendered ineffective. Because of potential irritation problems with enhancers, they are most practical for short-term use. For prolonged application, more attention will have to be given to regulating cutaneous irritation (Cooper & Patel, 1990).

2.8.2.2.2 Considerations when using enhancers

Penetration enhancers usually increase the permeation of not only the drug, but also other formulation excipients. In addition, their own intrinsic skin diffusivity can be increased. These effects must be carefully evaluated to avoid toxicological implications, especially in terms of irritancy potential. Irritation caused by transdermal drug products appears to be a function of occlusion. It is linked to stratum corneum hydration and decreased diffusional resistance to formulation components such as enhancers. Enhancers should be evaluated for irritancy potential under conditions of long-term occlusion. Development of models by means of solubility parameters to predict drug-vehicle-skin interactions and flux rate may aid the optimal selection of an enhancer (Shah, 1994).

Absorption enhancers are broadly divided into six groups, as summarized in Table 2-2 providing some examples for each group (Lee *et al.*, 1991).

Table 2-2: Classification of penetration enhancers (Lee *et al.*, 1991).

Class	Examples
Chelating agents	EDTA, citric acid, salicylates and N-acyl derivates of collagen
Surfactants	Sodium lauryl sulfate and polyoxy-ethylene-9-lauryl ether
Non-surfactants	Unsaturated cyclic ureas and 1-alkyl- and 1-alkenylazacycloalkanone derivates
Bile salts and derivates	Sodium deoxycholate, sodium glucocholate and sodium taurocholate
Fatty acids and derivates	Oleic acid, caprylic acid, acylcarnitines, acylcholines and mono- and diglycerides

2.8.2.2.3 Terpenes

Terpenes, also known as terpenoids, are constituents of essential oils, which are the volatile and fragrant substances found mainly in flowers, fruits, and the leaves of plants. These compounds have been used as flavourings, perfumes, and medicines. Recently, these compounds have been shown to be effective penetration enhancers, with low skin irritancy and low systemic toxicity, for a number of hydrophilic and lipophilic drugs (Cornwell & Barry, 1994; Cornwell *et al.*, 1996; Godwin & Michniak, 1999).

Terpenes are a series of naturally occurring compounds that consist of isoprene (C_5H_8) units that are highly lipophilic and have large partition coefficients between octanol and water (Williams & Barry, 1991b).

It has been suggested that the mechanism of action of terpenes involves disruption of the intercellular lipids of the stratum corneum. It appears that, for hydrophilic drugs, the primary effect of terpene enhancer treatment is to increase drug diffusivity in the stratum corneum (i.e., to reduce the barrier properties of the skin) (Godwin & Michniak, 1999). The passage of terpene penetration enhancers into the lipid domain of the SC is essential for activity (Williams & Barry, 1991b).

Six monoterpene constituents of essential oils were chosen from the broad chemical classes of hydrocarbons, alcohols, ketones, and oxides (Table 2-2) for this study, namely: menthol, isomenthol, menthone, 1,8-cineole (eucalyptol), β-myrcene and limonene (Godwin & Michniak, 1999).

According to Gao & Singh (1997) hydrocarbon terpenes are effective to enhance the penetration of lipophilic drugs and oxygen-containing terpenes are effective to enhance the penetration of hydrophilic drugs. Hydrocarbon terpenes are poor enhancers and alcohol and ketone terpenes are more effective enhancers (Williams & Barry, 1991b). According to this, it is predicted that in this study the chances of menthol, isomenthol, menthone and 1,8-cineole to enhance 5-FU penetration through the SC would be the best, because these terpenes' chemical structures (Figure 2-7) show that they all are oxygen-containing terpenes. On the other hand, β-myrcene and limonene are hydrocarbon terpenes with no oxygen-containing groups, that are therefore more effective for lipophilic drugs. So if β-myrcene and limonene enhance the transdermal delivery of 5-FU through the SC at all, they are not expected to be the best penetration enhancers for 5-FU.

The characteristics of these terpenes are shown in Table 2-3 and their chemical structure in Figure 2-7.

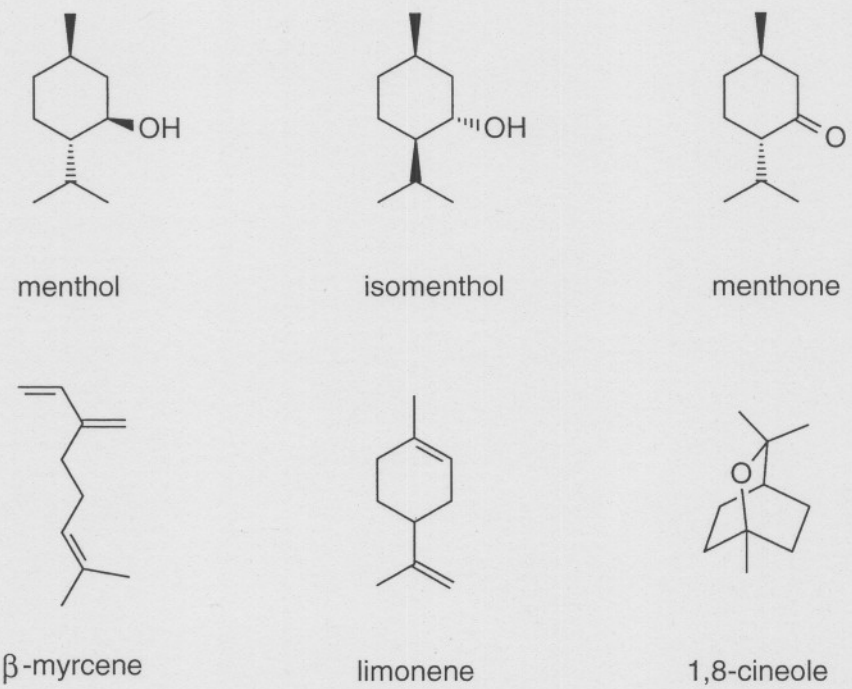

Figure 2-7: The chemical structures of the terpenes used in this study (Chemfinder, 2003).

Table 2-3: The characteristics of the terpenes used in this study (ACD software, Toronto, Canada).

TERPENE	CHARACTERISTICS			
	Molecular mass	**Solubility value in water**	**Solubility**	**Log P values**
Menthol	156.2674	0.092 g/l	Slightly soluble in water, soluble in alcohol	3.20 ± 0.2
Isomenthol	156.27	0.046 g/l	Soluble in alcohol	3.20 ± 0.2
Menthone	154.25	0.71 g/l	Slightly soluble in water, miscible with alcohol and oils	2.63 ± 0.26
B-myrcene	136.2364	2.6×10^{-3} g/l	Soluble in water, chloroform, ether and acetic acid	4.58 ± 0.27
1,8-cineole	154.25	0.41 g/l	Insoluble in water, soluble in alcohol	2.82 ± 0.27
Limonene	136.2364	2.7×10^{-3} g/l	Insoluble in water, soluble in alcohol	4.58 ± 0.24

2.9 CONCLUSION

The skin forms a complex barrier to the external environment, protecting us against harmful substances and maintaining bodily fluids within our system. The transdermal delivery of drugs' main advantage is the avoidance of hepatic first pass metabolism. The stratum corneum is the main barrier to the transport of molecules into the skin and has very good barrier properties, which could be taken as a disadvantage for transdermal delivery. The important step involved in transdermal delivery have been identified as the partitioning of the drug from the vehicle to

the stratum corneum, transport through the stratum corneum, partitioning from the lipophilic stratum corneum into the more aqueous viable epidermis, transport across the epidermis, and eventual uptake by the cutaneous microvasculature system followed by subsequent systematic distribution.

Although the process of transdermal delivery can be simplified, there are numerous factors that could have an affect on the permeation process of drugs. Physicochemical properties of importance in the selection of a drug for potential transdermal delivery is the lipid/water partition coefficient. This plays an important role because of the stratum corneum's lipid-rich intercellular channel through which a drug must penetrate. Another parameter to be taken into account is the solubility of the drug in the skin lipids.

The use of penetration enhancers provides an immense opportunity to reduce temporarily and reversibly the barrier properties of the skin and enhance and control the delivery of drugs through the largest and most easily accessible portal of entry to systemic circulation. However, it also presents tremendous challenges to control the delivery of drugs efficiently and without side effects and in therapeutic amounts from a reasonable small-sized transdermal delivery system (Büyüktimkin *et al.*, 1997).

The purpose of this study was to investigate the effect of selected terpenes on the transdermal delivery of the hydrophilic drug, 5-fluorouracil. The percentage of each terpene that penetrated through the SC was also determined.

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CHAPTER 3

EXPERIMENTAL METHODS AND THE EFFECTS OF TERPENES ON THE PENETRATION OF 5-FU

3.1 INTRODUCTION

5-Fluorouracil (5-FU) is a polar hydrophilic compound with pKa values of 8 and 13, and is therefore not a good penetrant through the stratum corneum. According to Flynn & Stewart (1988) and Williams and Barry (1991a) hydrophilic drugs have great potential for penetration enhancement, because of their low permeability coefficients. Selected terpenes (menthol, isomenthol, menthone, β-myrcene, limonene and 1,8-cineole/eucalyptol) were used as penetration enhancers in this study, their own penetration activity through the human SC were also determined. Terpenes are hydrocarbons with the general formula $(C_5H_8)_n$, and amongst them are also oxygen derivatives (as discussed in § 2.8.2.2.3). Essential plant oils are the main source of terpenes (Cal *et al.,* 2001). Penetration enhancers, with significant biological activity and the possibility of causing side effects, should not, or only in restricted quantities, penetrate through the skin (Cal *et al.,* 2001). Therefore, not only the promotion of penetration of 5-FU, but also the selected terpenes' own penetration, was examined on a semi-quantitative manner in this study.

Franz diffusion cells and human stratum corneum (prepared from human epidermal membranes) were used for all diffusion studies. A HPLC method was used for the analysis of 5-FU. HPLC validation procedure for 5-FU was done after the development of an analytical method that was reliable and sensitive enough to determine the concentration of 5-FU that penetrated through the skin (see 3.2.2). Gas chromatography (GC) was used for the analysis of the terpenes, in order to determine the cumulative percentage of terpenes penetrated through human SC over a period of 24 hours (see 3.2.3). The amount of terpenes that penetrated through the skin was calculated as a percentage of the applied dose. Since no actual amount was determined, this was a semi-quantitative analysis and no validation of the GC method was performed.

3.2 MATERIALS AND METHODS

3.2.1 MATERIALS

5-FU was obtained from Fluka (Steinheim, Switzerland). Terpenes used in this study were menthol, isomenthol, menthone, β-myrcene, 1,8-cineole and limonene. All these substances were obtained from Sigma-Aldrich (Steinheim, Germany). HPLC analytical grade acetonitrile (BDH Laboratory Supplies, Poole, England) and methanol (BDH Laboratory Supplies, Poole, England) were used in the experiments. Octane-1-sulphonic acid sodium salt was obtained from Romil Ltd (Cambridge, England). Sodium chloride (NaCl), sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$) and disodium hydrogen orthophosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$) were obtained from Merck Laboratory Supplies (Midrand, South-Africa). Orthophosphoric acid 85 % AR from Associated Chemical Enterprises (ACE) (Pty) Ltd (Southdale, England) was used. Absolute ethanol was obtained from Saarchem (Gauteng, South-Africa). Double distilled deionised water was prepared by a Milli-Q water purification system (Millipore, Milford, USA).

HPLC grade water was used throughout the study.

3.2.2 HIGH-PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

3.2.2.1 Apparatus

The HPLC system used for the analysis of 5-FU was an Agilent 1100 Series equipped with a variable wavelength UV detector, pump, injection device, and Chemstation Rev. A.06.02 data acquisition and analysis software or equivalent. The column used was a Synergi 4μ Hydro-RP 80A (250 X 4.6 mm) (Phenomenex, Germany). A HPLC Security Guard Cartridge System™ was used to prolong column life.

3.2.2.2 Chromatographic conditions

All analyses were performed at a flow rate of 1 ml/min and the mobile phase consisted of a mixture of 30 % acetonitrile, 1 % octane-1-sulfonic acid sodium salt and water, the pH (3.5) was adjusted with orthophosphoric acid. The mobile phase was filtered through a 0.45 μm HV filter (Millipore, Milford, USA) before use. The column temperature was held constant at 25 °C and

the effluent was monitored at a wavelength of 265 nm for 5-fluorouracil. The retention time for 5-fluorouracil was ± 4.7 minutes. The injection volume for all the samples was 10 µl.

3.2.2.3 Column maintenance

After each analysis, HPLC water was passed through the column for about 20 min at a flow rate of 1 ml/min, the column was then rinsed with HPLC water/methanol, 50/50, for 20 min at a flow rate of 1 ml/min in which it was also stored.

3.2.2.4 Preparation of standard solutions

Ten milligrams of 5-FU was transferred into a 100 ml volumetric flask and dissolved in water to produce a 100 µg/ml mother solution. Solutions with concentrations of 3; 1; 0.2; 0.04; 0.015 µg/ml 5-FU were prepared from the mother solution. These dilutions were used for the validation procedure.

3.2.2.5 Validation of HPLC procedures

3.2.2.5.1 Linearity

The assay of linearity for 5-FU was determined by performing linear regression analysis on the plot of the peak height *versus* concentration. Five standard solutions of each compound were prepared, as described in § 3.2.2.4, to obtain concentrations ranging from 0.015 to 3 µg/ml. The regression value was greater than 0.998 and the Y-intercept was 0.7587.

3.2.2.5.2 Precision

- *Intra-day precision*

Precision (repeatability) was determined by performing HPLC analyses (n = 3) of three different concentrations - low (0.04 µg/ml), intermediate (0.2 µg/ml) and high (1.0 µg/ml) – of 5-FU on the same day. The intra-day precision complied with pharmaceutical standards (see Table 3-1).

- *Inter-day precision*

The inter-day precision was determined by performing HPLC analyses (n = 3) of the three different concentrations - low (0.04 µg/ml), intermediate (0.2 µg/ml) and high (1.0 µg/ml) – of 5-FU on three consecutive days. The inter-day precision complied with pharmaceutical standards (see Table 3-2).

Table 3-1: The mean, standard deviation (SD) and percentage relative standard deviation (% RSD) for the percentage (%) of 5-FU recovered by analyzing three sets of three samples on the same day.

5-fluorouracil concentrations (µg/ml)	Mean % recovered	Standard deviation	% RSD
0.04	101.40	0.16	1.12
0.2	99.51	0.23	0.34
1.0	99.89	0.63	0.19

Table 3-2: The mean, standard deviation (SD) and percentage relative standard deviation (% RSD) for the percentage (%) of 5-FU recovered by analyzing three sets of three samples on three consecutive days.

5-fluorouracil concentrations (µg/ml)	Mean % recovered	Standard deviation	% RSD
0.04	104.93	0.40	2.84
0.2	98.82	0.46	0.71
1.0	100.04	5.36	1.63

3.2.2.5.3 Sensitivity

The sensitivity of an analytical method can be measured by determining the limit of quantification and limit of detection. The limit of quantification is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy (% RSD < 15 %). The limit of detection, on the other hand, is defined as the lowest concentration of analyte in a sample that can be detected, but not necessarily quantified. The limit of detection of 5-FU studied was 0.006 µg/ml and the limit of quantification for 5-FU studied was 0.03 µg/ml (% RSD < 15 %).

3.2.2.5.4 Selectivity

Selectivity is the ability of the analytical method to detect and analyse a component in the presence of other components. The compounds, mobile phase, phosphate buffer solution (PBS) pH 7.4 and ethanol were separately analysed by HPLC. This method was selective since there were no interfering peaks with the same retention time as 5-FU. There were no signs of interference at all with the peak of 5-FU, with a retention time of ± 4.7 min.

3.2.2.5.5 System repeatability

In order to evaluate the repeatability of the peak area and of the retention time, samples (0.1 µg/ml) of 5-FU was injected six times. The variation in the response (% RSD) of the detection system when six determinations of 5-FU were made on the same day, and under the same conditions, was found to be 0.21 % for the peak area and 0.11 % for the retention time. The system repeatability for 5-FU was well within acceptable criteria.

3.2.3 GAS CHROMATOGRAPHY (GC)

Gas chromatography (GC) was used to determine the percentage of terpenes diffused through the skin. There were no validation procedures done on the GC, due to the fact that the results was determined on a semi-quantitative level.

3.2.3.1 Apparatus

An Agilent 6890 Series Headspace GC (Hewlett Packard) equipped with a WCOT fused silica capillary column (Chrompak, Germany) was used in the analysis of the terpenes,

3.2.3.2 Chromatographic conditions

1) **Column:**

- Stationary phase used for the analyses was CP – wax – 52 CB
- Column length was 30 m with a inside diameter of 0.25 mm and an outside diameter of 0.39 mm. The film thickness was 0.25 mm.

2) **Operating temperatures:**

- The oven temperature was set at 70 ºC for 5 min then it was increased at 40 ºC/min, until the temperature reached a maximum of 180 ºC and it was held at this temperature for 6 min.
- The front injector temperature was 250 ºC and the front detector temperature was 260 ºC.

3) **Retention times:**

- The retention times for the various terpenes were: β-myrcene, 6.3 min; limonene, 7.0 min; 1,8-cineole, 7.4 min; menthone, 10.4 min; menthol, 11.9 min and isomenthol, 12.1 min.
- Terpenes were dissolved in absolute ethanol, and ethanol showed a retention time of 3.6 min.

The peak of ethanol didn't show any interference to those of the terpenes.

3.2.3.3 Preparation of standard solutions

Fifty mg of menthol and isomenthol were transferred into 100 ml volumetric flasks and dissolved in absolute ethanol to produce concentrations of 500 µg/ml as mother solutions. Then five standard solutions of each with concentrations between 0.976 µg/ml and 62.5 µg/ml were prepared by dilutions from the mother solution. In the case of menthone, β-myrcene, 1,8-cineole and limonene, 0.02 ml of each was transferred into a 10 ml volumetric flak to produce a 2 µl/ml mother solution. Dilutions from the mother solution were made (0.008 µl/ml - 0.8 µl/ml).

3.2.4 PREPARATION OF SATURATED SOLUTION OF 5-FLUOROURACIL AND AQUEOUS SOLUBILITY DETERMINATION

Phosphate buffer saline (PBS - pH 7.4) solution was used to prepare the saturated solution (SS) of 5-FU by transferring 4.4 g sodium chloride (NaCl), 2.1 g sodium dihydrogen orthophosphate ($NaH_2PO_4 \cdot 2H_2O$) and 9.2 g disodium hydrogen orthophosphate ($Na_2HPO_4 \cdot 2H_2O$) into a 1 000 ml volumetric flask and filled with water to 1 000 ml. A specific amount of PBS was used to make the SS. The 5-FU/PBS solution was equilibrated at a constant temperature of 37 ºC by vigorous stirring. The temperature was held constant by means of a water bath (Grant Instruments, UK). Vigorous stirring was affected with magnetic stirring bars to speed up the attainment of equilibrium. An excess of the solute (5-FU) was at all times present in the slurries. Equilibrium of 5-FU was reached within 24 hours. The pH of the SS was lowered to pH 6 with orthophosphoric acid, at this pH 5-FU is in its unionized form, in order to assure maximum permeation through the skin (Ritschel & Hussain, 1988) (Equation 3-1). Samples were filtered through 0.45 µm filters (Type HV, Millipore) and assayed by HPLC in order to determine the solubility of 5-FU.

3.2.5 PREPARATION OF 5 % TERPENE PRETREATMENT SOLUTIONS

2.5 g of menthol and isomenthol each were transferred into 50 ml volumetric flasks and were dissolved in absolute ethanol to produce a concentration of 50 mg/ml (5 % m/v). 2.5 ml of menthone, β-myrcene, limonene and 1,8-cineole each were transferred into 50 ml volumetric flasks and were dissolved in absolute ethanol to produce a concentration of 50 µl/ml (5 % v/v). Only 25 µl of the prepared solutions was used as pretreatment solution and applied to the skin half an hour before the diffusion studies.

3.2.6 DIFFUSION STUDIES

3.2.6.1 Skin preparation

The skin used in the permeation studies was obtained from the abdomen of female patients after cosmetic surgery. The full-thickness skin was frozen at -20 ºC for not longer than 24 hours after removal. Before preparation, the skin was thawed at room temperature, and all excess fat carefully removed. Epidermal layers were separated by immersing the skin in water at 60 ºC for 1 min. The epidermal layer was gently flayed from the remaining tissue with forceps. The skin sections (stratum corneum) were floated on to Whatman® filter paper and left to dry. The prepared samples were kept frozen at -20 ºC until used. Before a diffusion study was conducted, the frozen pieces of skin were thawed at room temperature and examined for defects. The skin was cut into circles with a diameter of ± 10 mm, before mounting them on the Franz cell (Figure 3-1).

3.2.6.2 Skin permeation method

Three different sets of diffusion experiments were done:

1) Saturated solution of 5-FU (pH 6) through untreated skin.

2) Saturated solution of 5-FU (pH 6) through skin that was pretreated with ethanol, to be able to exclude the penetration enhancing effect of ethanol from the final results (the enhancers were dissolved in ethanol for the pretreatment of the skin).

3) Saturated solution of 5-FU (pH 6) through skin that was pretreated with 25 µl of a 5 % solution of a particular terpene, where the particular terpene was dissolved in ethanol.

All samples were analysed by HPLC to determine 5-FU content.

In order to obtain the cumulative percentage of selected terpenes penetrated through the skin in the presence and absence of 5-FU, two sets of diffusion experiments were done:

1) Control: skin was pretreated with 25 µl of a 5 % solution of a particular terpene and PBS was added as donor compartment solution that diffused through the skin (PBS solution was used to prepare the saturated solution of 5-FU).

2) Saturated solution of 5-FU (pH 6) as donor compartment solution was added onto 5 % terpene pretreated skin (terpenes were dissolved in ethanol).

All samples were analysed by GC to determine the percentage of selected terpenes diffused through the SC.

Vertical Franz diffusion cells (Figure 3-1) with a receptor capacity of 1.9 to 2.5 ml and a 1.075 cm^2 diffusion area were used in the permeation studies. The epidermal layer was mounted on the lower half of the diffusion cell with the SC facing upwards. A small magnetic stirring bar was placed in each receptor compartment to accomplish stirring. The stirring magnet rotated at a speed of 500 rpm. Stirring was continued throughout the entire experiment. The donor compartment was placed on the lower half with the skin acting as a seal between the two halves and clamped together with a metal clamp. The receptor compartment was filled with 50/50 ethanol/water as receptor phase and the cells were placed in a water bath at 37 ºC. Care was taken to ensure that no air bubbles were trapped in the compartment or underneath the skin. After the receptor compartment was filled with the ethanol/water solution, the cells were equilibrated for 1 h before adding the drug-containing saturated solution. Pretreatment of the skin was performed by applying 25 µl of a 5 % solution of the particular terpene, or 25 µl of absolute ethanol to the skin. It was then left for 30 min to enable the ethanol to evaporate. 1 ml of saturated aqueous solution of 5-FU (pH 6) or PBS solution (control) was then applied onto the SC in the donor compartment. Samples were taken at 2, 4, 6, 8, 10, 12 and 24 hours by removing the total contents of the receptor compartment and replacing it with fresh 37 ºC receptor solution. This was done to ensure that sink conditions existed throughout the experiment. The amount of 5-FU diffused was determined by HPLC. The cumulative percentage of terpenes diffused after 6, 12 and 24 hours was determined by GC.

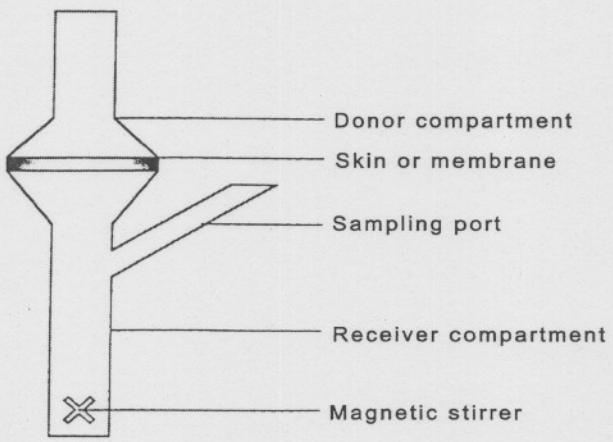


Figure 3.1: The vertical-type Franz diffusion cell (Roy, 1997).

3.3 DATA ANALYSIS

3.3.1 ANALYSIS OF 5-FLUOROURACIL PENETRATION

The percentage unionized drug was calculated with the Henderson-Hasselbach equation (Ritschel, 1988) (Equation 3-1).

$$\% \text{ ionised drug (for acidic compounds)} = \frac{100}{1 + \text{antilog } (pKa - pH)} \qquad \text{(Equation 3-1)}$$

The % unionized drug = 100 - % ionized drug.

The *in vitro* skin permeation data obtained was graphically plotted as the cumulative corrected amount of drug penetrated into the receptor phase as a function of time. The permeation profiles provided the following parameters: The slope of the straight line portions of this plot (at steady state) yielded the values of flux ($\mu g/cm^2/h$) (see Figure 3-2 as example) and the cumulative corrected receptor concentrations at 24 h.

The log P value of 5-FU was determined using ACD software program (Toronto, Canada).

Since K, D and h are unknown, the products Kh and D/h^2 were calculated by curve-fitting of the permeation data. The curve-fitting of data on Easyplot for Windows provided α and β values, where:

$$\alpha = Kh \text{ and } \beta = D/h^2$$

The product $\alpha\beta$ is equal to the permeability coefficient (k_p).

The individual diffusion experiments each provided estimates of α and β from which mean values were calculated. The flux ($\mu g/cm^2/h$) was determined after k_p was known:

$$\text{Flux} = k_p \times \text{saturated solubility}$$

The flux values determined in these two different ways were in good agreement with each other and didn't show any differences in the values.

The overall enhancer activity of each terpene was expressed as a ratio of the k_p value, including and excluding the effect of ethanol. Enhancement ratios (ER) were calculated using the following equation (Goodman & Barry, 1988):

$$ER = \frac{k_p \text{ with terpene pretreated skin}}{k_p \text{ without terpene pretreated skin}} \qquad \text{(Equation 3-2)}$$

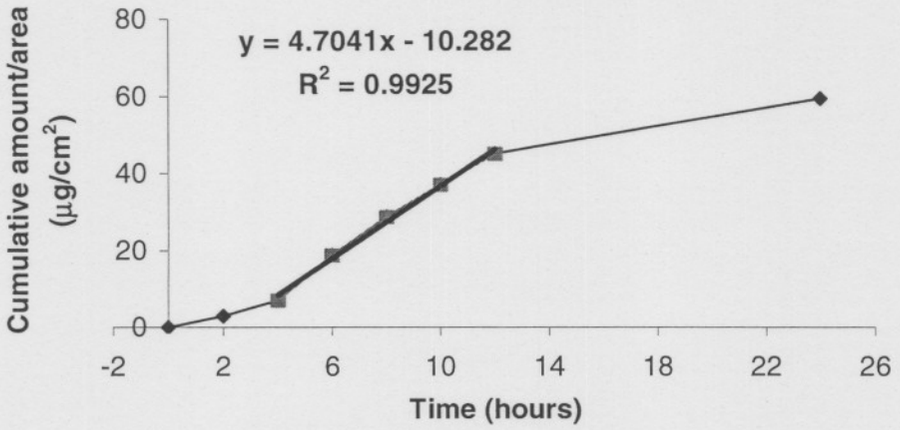


Figure 3-2: Determination of the steady-state flux.

3.3.2 ANALYSIS OF TERPENE PENETRATION

The analysis of the diffused terpenes was semi-quantitative. Standard solutions of the terpenes were prepared (see §3.2.3.3) and analyzed using GC. The data obtained was then graphically plotted as the area *versus* concentration and a straight-line was obtained. The sample data (area) from the diffusion experiments (analysed by GC) were used to determine the concentration of the terpenes diffused over a period of 24 hours by using the straight-line equation. The percentage of the applied amount of the terpene that penetrated through the SC was calculated. The average cumulative percentage of the terpenes penetrated at 6h, 12h and 24h in the presence of 5-FU was compared to the terpene penetration in the absence of 5-FU.

3.4 STATISTICAL ANALYSIS

3.4.1 ANALYSIS OF 5-FLUOROURACIL PENETRATION

Statistical analysis of the penetration of 5-FU, in the presence and absence of the terpenes was done to determine the effect of the terpenes on the penetration of 5-FU. The statistical methods used in order to state the differences in the flux values ($\mu g/cm^2/h$) of 5-FU with the various terpenes were the ANOVA procedure, followed by the Dunnett's test, to determine differences between each experiment and the two control experiments, where the skin was either untreated or pretreated with ethanol.

Statistically significant differences ($p < 0.05$) with a confidence interval of 95 % were indicated for the parameter flux (5-FU) for untreated skin in comparison to menthol, isomenthol and β-myrcene pretreated skin. There were no statistically significant differences ($p < 0.05$) in the comparison of the fluxes of 5-FU between untreated skin and menthone, limonene and 1,8-cineole pretreated skin.

Statistically significant differences ($p < 0.05$) with a confidence interval of 95 % were indicated for the flux, of 5-FU through ethanol pretreated skin in comparison to isomenthol, menthone and β-myrcene pretreated skin. There were no statistically significant differences ($p < 0.05$) between the flux of 5-FU through ethanol pretreated skin and menthol, limonene and 1,8-cineole pretreated skin.

3.4.2 ANALYSIS OF TERPENE PENETRATION

To obtain the final results for the percentage of every terpene diffused through the skin after 6, 12, and 24 hours, in the presence and absence of 5-FU, a repeated measure ANOVA (analysis of dependent variable and also for the interaction with time) was used.

The comparison between the parameter, which is the cumulative penetrated percentage of menthol and menthone in the absence and presence of a SS of 5-FU through the human SC (between the first time interval of 6 – 12 hours) showed a statistically significant difference ($p < 0.05$) with a confidence interval of 95 %. Therefore, 5-FU showed a statistically significant decreasing effect on the penetration of menthol and menthone. In the first time interval of 6 – 12 hours, 5-FU was capable of increasing the cumulative percentage of absorption for isomenthol, β-myrcene, limonene and 1,8-cineole. In comparison, where 5-FU was absent, only

the data of β-myrcene and 1,8-cineole could be determined with a statistically significant difference ($p < 0.05$) and with a confidence interval of 95 %.

In the second time interval (12 – 24 hours), the comparison between the cumulative % of the selected terpenes being penetrated (parameter) in the absence and presence of 5-FU, an increasing effect was caused by 5-FU for β-myrcene, limonene and 1,8-cineole and a decreasing effect for menthol, isomenthol and menthone. In this time interval only the compared values of menthol and isomenthol showed a statistically significant difference ($p < 0.05$) with a confidence interval of 95 %.

3.5 RESULTS AND DISCUSSIONS

3.5.1 TRANSDERMAL DELIVERY OF 5-FU

The saturated solubility of 5-FU in PBS was determined as 16 816 µg/ml. At this concentration 5-FU was capable to penetrate untreated human SC at a flux of 0.53 ± 0.24 µg/cm^2/h (Figure 3-3). When the skin was pretreated with ethanol (EtOH), the flux was 1.02 ± 0.26 µg/cm^2/h, an increase of approximately 1.9 times. The skin was pretreated with ethanol in order to exclude the penetration enhancing effect of ethanol from the final results (the terpenes were dissolved in ethanol for the pretreatment of the skin).

Figure 3-3 shows the flux of 5-FU from the saturated solution (SS) through untreated skin and through ethanol pretreated skin.

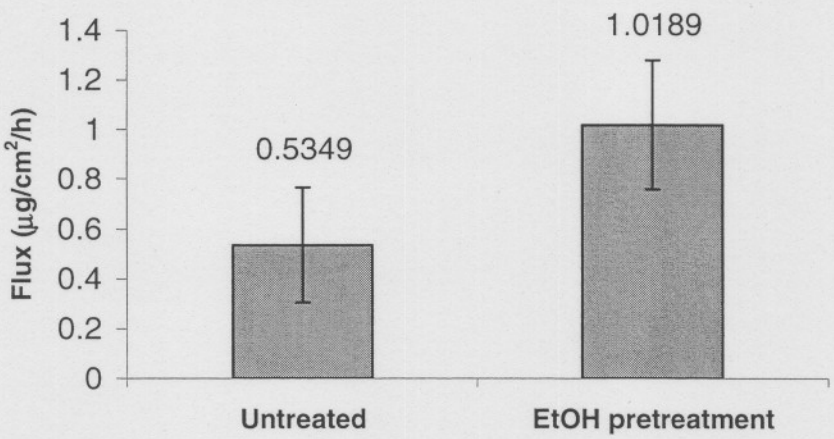


Figure 3-3: Flux of 5-FU from the saturated solution (SS) at 37 ºC (pH 6) through untreated and EtOH pretreated human SC. Mean ± SD, n = 6.

The percentage transport (using Equation 3-3) of 5-FU, after 24 hours, through untreated skin was found to be 0.00318 % and through ethanol pretreated skin 0.00606 % (Figure 3-4). Pretreatment of the SC with ethanol resulted in increased flux of 5-FU. Ethanol acts as a penetration enhancer for 5-FU.

$$\% \text{ transport} = \frac{\text{Flux}}{\text{Aqueous solubility}} \times 100\% \qquad \text{(Equation 3-3)}$$

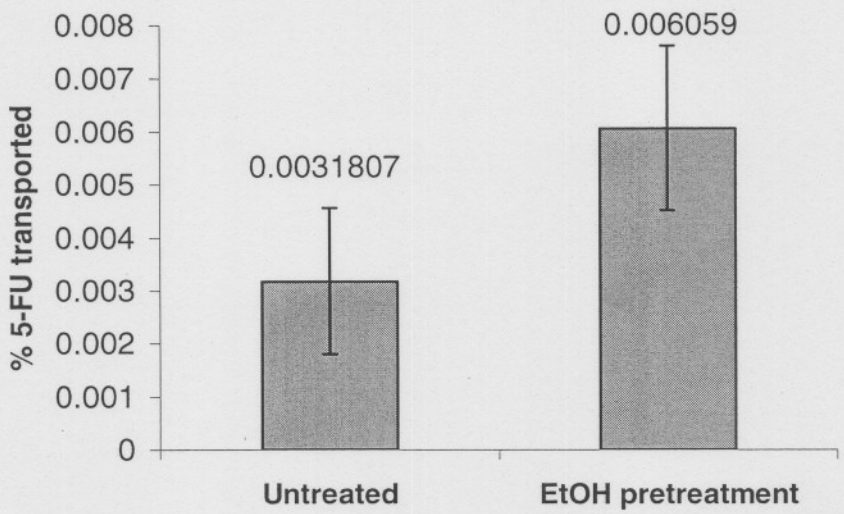


Figure 3-4: Percentage transport of 5-FU from the saturated solution (SS) through untreated and EtOH pretreated SC. Mean ± SD, n = 6.

Drugs should preferably have a balanced lipophilic/hydrophilic character and a drug with a log P value ~ 2.5 is considered to be a potential candidate for transdermal delivery (Potts & Guy, 1992). Since 5-FU has a log P value of -0.78 ± 0.31 (see 2.7.3.1) it is believed that 5-FU is not a good candidate for transdermal delivery. According to Goosen *et al.,* (1998) log P can only be used in combination with other indicative parameters to predict the transdermal absorption, which is discussed further on. Smaller molecules within a narrow range of molecular weight (200 – 500) penetrate the skin more rapidly than larger molecules. The pKa values of 5-FU are 8 and 13. The percentage of unionized moiety of 5-FU was calculated at pH 6 and can be seen in § 2.7.3.1. It would be expected that the unionized moiety of 5-FU would penetrate the skin best, together with the small molecular mass 5-FU has, which is 130.08. According to Beetge and co-workers (2000), the molecular mass, solubility constraint and the percentage unionized

moiety can only be used when combined with other properties in the prediction of possible transdermal drug delivery.

Since the SC is basically a lipophilic barrier, drug lipophilicity is regarded as one of the key parameters controlling drug skin penetration. It is therefore expected that more lipophilic drug derivates could show better partitioning and solubility into the SC, which could result in enhanced skin penetration of 5-FU. However, due to the biphasic nature of the skin, a balance should be seen in the lipid and water solubilities of drugs needed for enhanced transdermal delivery (Goosen *et al.*, 2002).

5-FU is capable of penetrating the SC (Figure 3-3) with a flux of 0.53 ± 0.24 μg/cm^2/h. When skin was pretreated with ethanol, 5-FU has a flux of 1.02 μg/cm^2/h. Hydrophilic drugs therefore, have great potential for enhancement because of their low permeability coefficients (Flynn & Stewart, 1988). Despite the fact that ethanol was not used as such in this study to prove the penetration enhancing effects of 5-FU, it can very well be seen in Figure 3-3, and therefore we can agree with Flynn & Stewart's (1988) statement on the penetration enhancing effect of ethanol.

In this study, only 25 μl ethanol was applied to the skin (with a diameter of ± 10 mm) as pretreatment, left for half an hour to evaporate, and according to Pendlington and co-workers (2001) ethanol does have a high rate of evaporation, and the rate of evaporation of a liquid is dependent on its surface area. The more thinly spread, the quicker it evaporates. Ethanol still has the potential to penetrate the skin of man or act as a penetration enhancer. Ethanol is one of the most promising enhancers and the concentration-dependent enhancing effect is well known: ethanol is able to enhance the skin penetration of a hydrophilic compound at a high concentration (Hatanaka *et al.*, 1995). Ethanol has an effect on the drugs penetrating the skin, probably due to new pore formation caused by the high concentration of ethanol being used. Ethanol also caused protein denaturation in the skin, causing it to behave like a porous membrane which is unable to distinguish drug polarity (Manabe *et al.*, 1996). There is a possibility that the drug solubility in the skin could be changed by the concentration of ethanol used as a donor solvent (Silvieri & DeAngelis, 1975). This can have an influence on the penetration of the drug. Ethanol can therefore be used as a penetration enhancer for 5-FU, although in this study its enhancing effect was excluded in order to determine the effects caused by the selected terpenes. Cornwell & Barry (1994) attempted to improve enhancer clearance by replacing the aqueous donor and receptor phase by ethanol/water (1:1) solutions. They found that ethanol increased the permeability coefficient for 5-FU 13-fold, demonstrating that, in aqueous solutions, it is a moderately potent penetration enhancer.

3.5.2 EFFECTS OF TERPENES ON THE TRANSDERMAL DELIVERY OF 5-FU

The flux of 5-FU through terpene pretreated skin in comparison with untreated and EtOH pretreated skin can be seen in Figure 3-5. Menthol caused a slight increase on the penetration of 5-FU, but a more profound increase can be seen with isomenthol pretreatment. Furthermore, menthone, β-myrcene, limonene and 1,8-cineole had no enhancing effect on the penetration of 5-FU, in fact, they all showed a decrease on the penetration of 5-FU, if compared to ethanol pretreated SC. However, 1,8-cineole also showed an increasing effect on the penetration of 5-FU when compared to untreated skin. The various mean flux values (± SD) can be seen in Table 3-3.

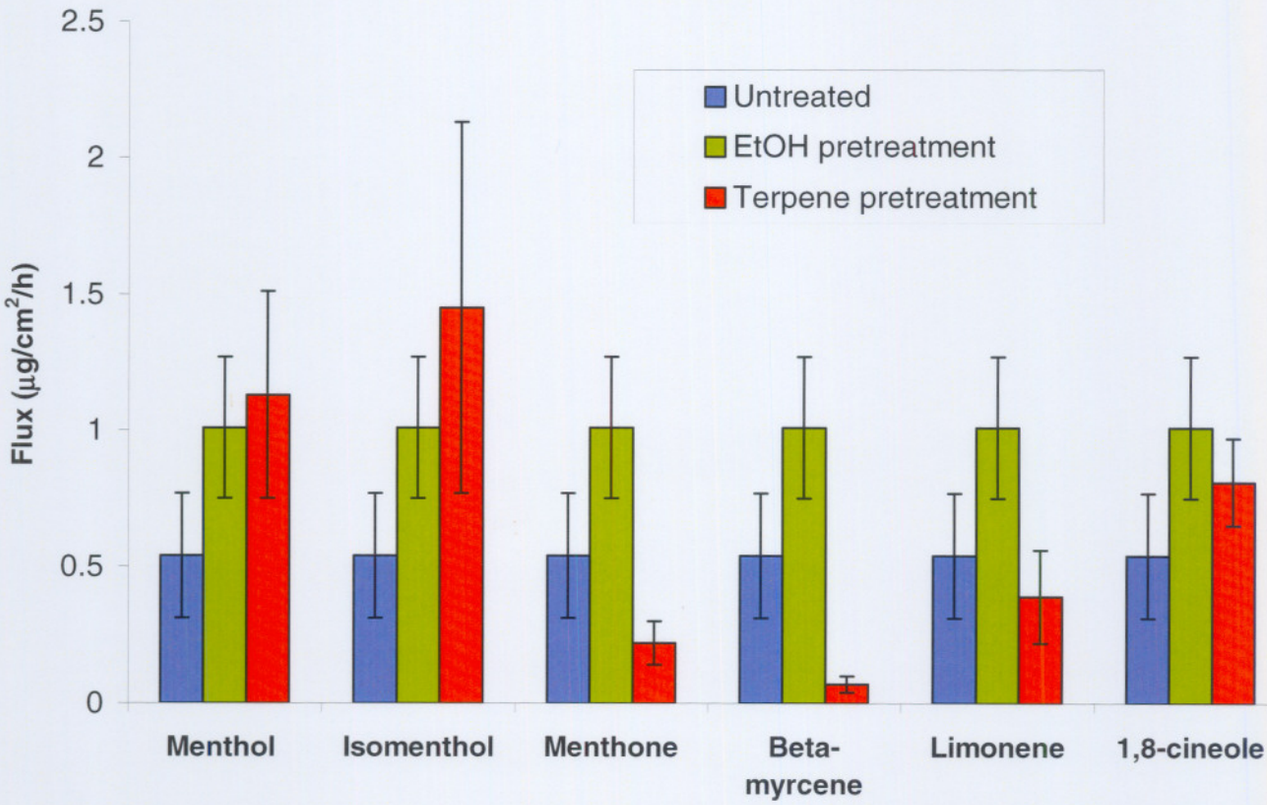


Figure 3-5: Comparison between the flux of 5-FU from the SS through untreated, ethanol pretreated and terpene pretreated SC. Mean ± SD, n = 6.

The permeability coefficients of 5-FU through terpene pretreated and untreated skin, excluding the effect of ethanol, were used to calculate the enhancers' activities, expressed as the enhancement ratios (ER) (Equation 3-2). Figure 3-6 shows the various ER values of the selected terpenes, excluding and including ethanol effects.

The ER values of 5-FU (excluding the effect of ethanol) with each of the selected terpenes are in the following increasing order: β-myrcene < menthone < limonene < 1,8-cineole < menthol < isomenthol. Menthol, isomenthol and 1,8-cineole showed values of 1.2181, 1.8193 and 0.6231, respectively, where menthone, β-myrcene and limonene showed negative values of -0.4673, -0.7511 and -0.1464, respectively, when ethanol effects of enhancement were excluded (Table 3-3). Menthone, β-myrcene and limonene had a decreasing effect on the penetration of 5-FU through human SC over a period of 24 hours. On the other hand, an increasing effect could be seen when skin was pretreated with menthol, isomenthol and 1,8-cineole. If the enhancing effect of ethanol was included, all the selected terpenes showed a higher ER value in comparison to their ER values where the effect of ethanol was excluded. Therefore, it can be concluded that ethanol has a synergistic effect on the enhancement activity of all the terpenes.

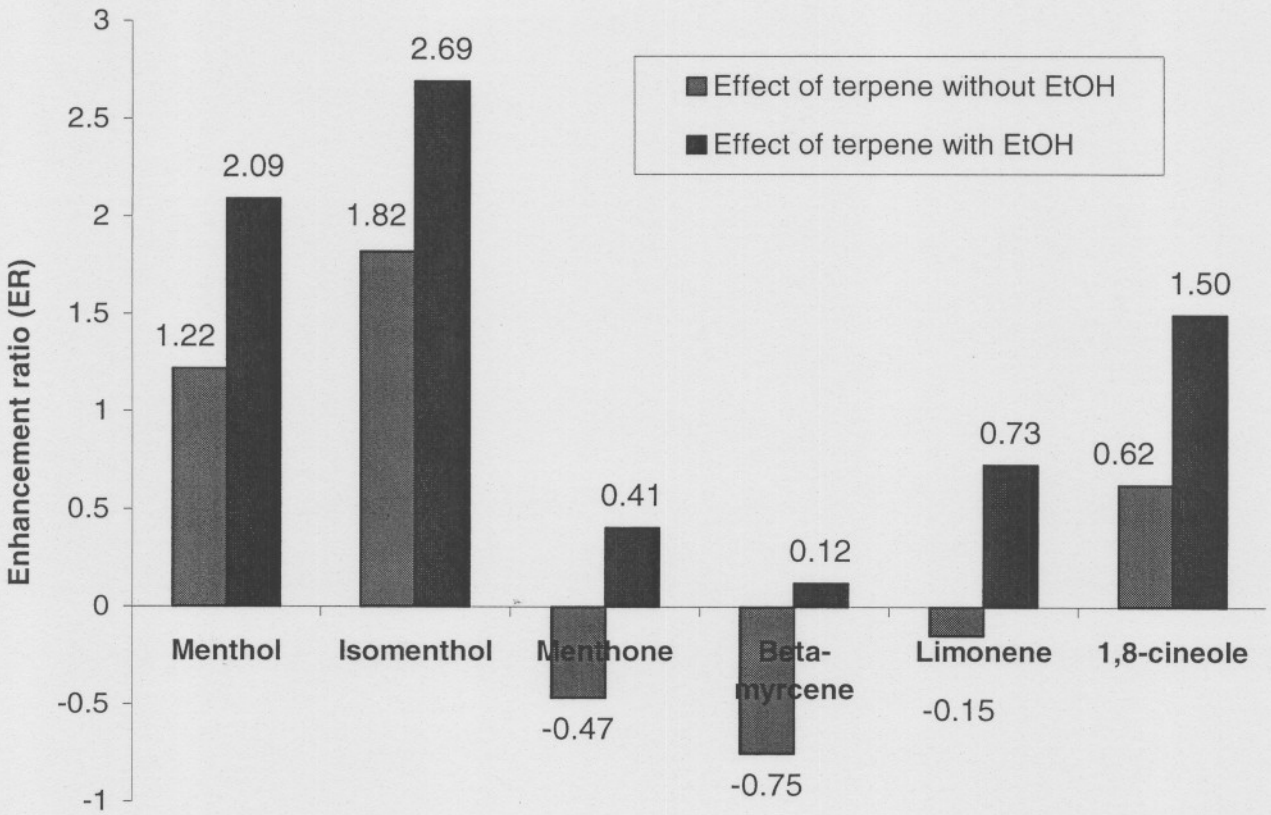


Figure 3-6: The enhancement ratios of the selected terpenes, excluding and including the enhancing effect of EtOH. Mean ± SD, n = 6.

A summary of the results is shown in Table 3-3.

Table 3-3: The effects of the selected terpenes on the penetration of 5-FU from its SS.

Skin	Flux ($\mu g/cm^2/h$)	Permeability coefficient (k_p) ($cm/h \times 10^5$)	Log k_p	Enhancement ratio (ER) excluding EtOH enhancing effects
Untreated skin	0.54 ± 0.23	3.21	- 4.49	N.a.
EtOH pretreatment	1.01 ± 0.26	6.01	- 4.22	N.a.
5 % menthol	1.13 ± 0.38	6.71	- 4.17	1.22
5 % isomenthol	1.45 ± 0.68	8.64	- 4.06	1.82
5 % menthone	0.22 ± 0.08	1.30	- 4.89	- 0.47
5 % β-myrcene	0.07 ± 0.03	0.389	- 5.41	- 0.75
5 % limonene	0.39 ± 0.17	2.33	- 4.63	- 0.15
5 % 1,8-cineole	0.81 ± 0.16	4.80	- 4.32	0.62

N.a. = Not applicable

According to Williams & Barry (1991b) (§ 2.8.2.2.3), oxygen-containing terpenes (menthol, isomenthol, menthone and 1,8-cineole) will give the best results in terms of enhancing the penetration of a polar hydrophilic drug. This was now once again proved through the results of this study. Menthol, isomenthol, menthone and 1,8-cineole are all oxygen-containing terpenes (Figure 2-7). These terpenes, except menthone, did enhance 5-FU penetration in comparison with 5-FU through untreated skin (Figure 3-5). ER values of 1.22, 1.82 and 0.63, respectively, for menthol, isomenthol and 1,8- cineole indicate that these three terpenes show the highest ER values (see Figure 3-6). The hydrocarbon terpenes (β- myrcene and limonene) showed a totally decreasing effect on the penetration of 5-FU as seen in Figure 3-5. Once again the results in this study confirmed that the hydrocarbon terpenes are effective penetration enhancers for lipophilic drugs and not for hydrophilic drugs, as was seen by Gao & Singh (1997).

The mean control value for k_p of 5-FU through untreated skin at 37 ºC is 3.21 x 10^{-5} cm/h (Table 3-3) and it is believed to be in good agreement with published data from Williams and Barry's investigation (1991a). The penetration enhancing activity of the terpenes are more clearly demonstrated in terms of the ER values (Figure 3-6). The hydrocarbon terpenes (β-myrcene and limonene) showed a decreasing effect compared to the oxygen-containing terpenes that showed enhancing effects on the penetration of 5-FU. The oxygen-containing terpene that showed the highest enhancement ratio value was isomenthol (ER 2.69). The ER value differences may possibly be due, in part, to conformational differences between the selected terpenes' chemical structures (Williams & Barry, 1991b). The differences might include firstly, the importance of the transcellular route for the penetration of a hydrophilic compound, such as 5-FU, through enhancer treated SC and, secondly, the partitioning behaviour of the compound and SC intercellular lipids (Moghimi *et al.,* 1996).

In this study 1,8-cineole showed a decreasing effect on the transdermal delivery of 5-FU. Williams & Barry (1991a) compared the ER of epoxides to the ER of 1,8-cineole and found that the ER of the epoxides were markedly lower. This may be due to conformational effects. Moghimi *et al.* (1996) studied the effect of 1,8-cineole on 5-FU (a model hydrophilic drug) and oestradiol (OE) (a model lipophilic drug). The partitioning ratio of 5-FU was lower with cineole (5 %) as compared to OE. They found that the matrix model effects of cineole towards the penetration of OE were better than towards 5-FU through SC. The reason for this could be the importance of the transcellular route for the penetration of hydrophilic drugs like 5-FU through enhancer treated SC and differences between the hydrophilicity and therefore partitioning of the models and SC intercellular lipids. Once again the results obtained in this study is in agreement with the studies done by Williams & Barry (1991a) and Moghimi and co-workers (1996).

The high log P values (octanol/water) of the terpenes imply that the penetration enhancers will not significantly modify corneocyte proteins (Williams & Barry, 1991b). This suggests that terpene penetration enhancers rather act by increasing diffusivity *via* SC lipid disruption. No significant protein interaction and no major partitioning alterations, which are the three major features of the LPP theory of penetration enhancer activity (as discussed in § 2.8.2.2.1), was observed. Cornwell and Barry's (1993) investigation on the treatment of human epidermis with terpene penetration enhancers showed increased electrical conductivity. The increase in ion transport suggests that terpenes open new polar pathways across the SC. A correleation between the increase in ion transport and the previously reported increase in 5-FU penetration suggests that terpene enhancers may create micro-pores in the intercellular lipids through which both ions and polar drugs, such as 5-FU, may pass.

The selected terpenes lipophilicities had a significant effect on the transdermal penetration of 5-FU. Using terpenes with increasing log P values, could be associated with an increase in drug penetration, through studies done by El-Kattan and co-workers (2001). The results in this study are not in correlation with the above statement. When the water solubility of the selected terpenes were taking in consideration, the results of this study seem to correlate with that of Goosen and co-workers (1998). They found that increasing lipophilicity/partitioning coefficient values alone did not result in a higher skin flux, but the highest skin flux through the skin was achieved by the more lipophilic analogue that showed the higest water solubility. This can be seen when looking at the water solubility and log P values of each terpene (Table 2-3) and comparing it with the results obtained in this study (Figure 3-5). Menthol, isomenthol and 1,8-cineole that showed the best enhancer activity towards 5-FU have the higest water solubilities with log P values of 3.20 ± 0.2, 3.20 ± 0.2 and 2.82 ± 0.27, respectively, towards the other terpenes with much lower water solubilities, and log P values, which are 4.58 ± 0.27 and 4.58 ± 0.24, respectively, for β-myrcene and limonene.

In a study done by Moghimi *et al.* (1996) the effect of limonene on OE and 5-FU was determined. They found the partitioning ratio for OE higher than that of 5-FU. They also found that limonene decreased the permeability coefficient of 5-FU through the model matrix at 32 °C, and therefore had a retarding effect on 5-FU. This was also proved through the results obtained from this study, where limonene showed a decreasing effect on the flux of 5-FU (Figure 3-5).

In conclusion, the terpenes which can be used as the most effective penetration enhancers for 5-FU would be menthol, isomenthol.

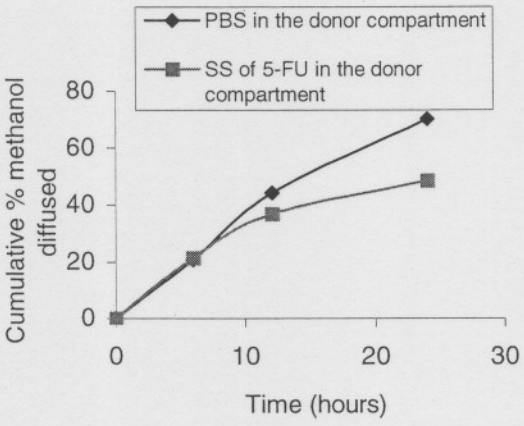
3.5.3 PENETRATION OF TERPENES THROUGH THE SC

The cumulative percentage of the applied dose (5 %) of the six selected terpenes which penetrated through human SC over a period of 24 hours, in the presence and absence of 5-FU, can be seen in Figure 3-7.

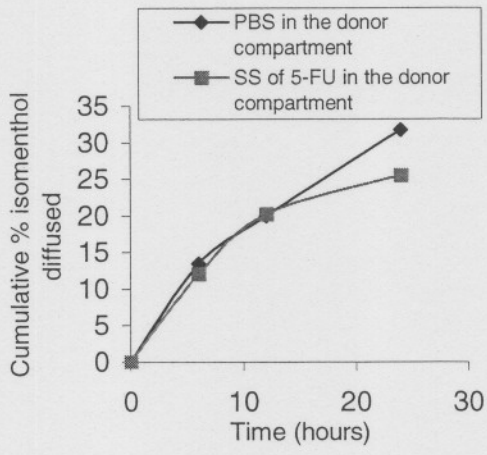
All the terpenes, applied as a 5 % particular terpene solution, were capable to penetrate through human SC in the absence of 5-FU. However, 5-FU had a retarding effect on the penetration of terpenes through the SC (Figure 3-7). When PBS was applied in the donor, 70.21 %, 31.66 % and 96.51 % of menthol, isomenthol and menthone, respectively, diffused through the SC. However, when a SS of 5-FU was applied in the donor, cumulative percentages of only 48.42 % (menthol), 25.52 % (isomenthol) and 62.77 % (menthone) were found.

5-FU had an increasing effect on the penetration of β-myrcene, limonene and 1,8-cineole through SC (Figure 3-7). When PBS was applied in the donor, 6.9 %, 2.92 % and 21.44 % of β-myrcene, limonene and 1,8-cineole, respectively, penetrated through the SC. However, when SS of 5-FU was applied in the donor, cumulative percentages of 10.5 % (β-myrcene), 8.51 % (limonene) and 33.78 % (1,8-cineole) were found.

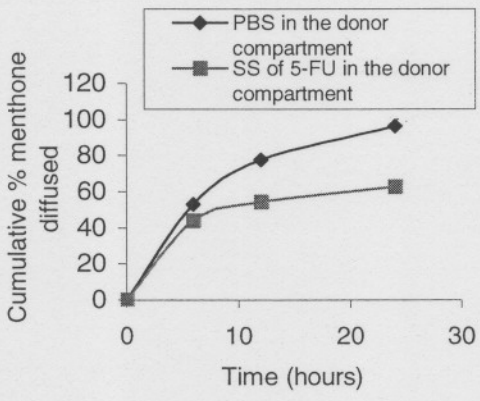
Looking at Figure 3-8 it is clear that all the terpenes did diffuse through the SC in the absence of 5-FU. However, 5-FU showed either an increasing or decreasing effect on the diffusion of the selected terpenes.



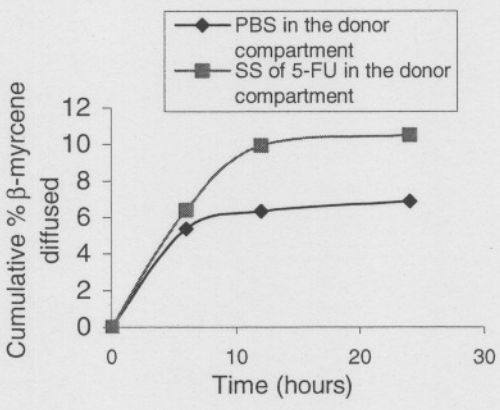
Menthol



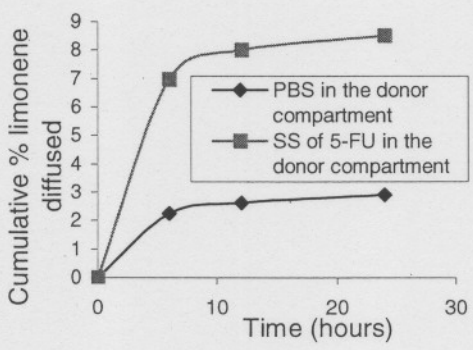
Isomenthol



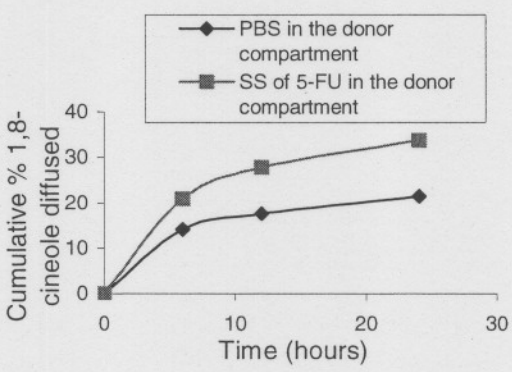
Menthone



β-myrcene



Limonene



1,8-cineole

Figure 3-7: The diffusion terpenes through human stratum corneum over a period of 24 hours in the absence and presence of 5-FU.

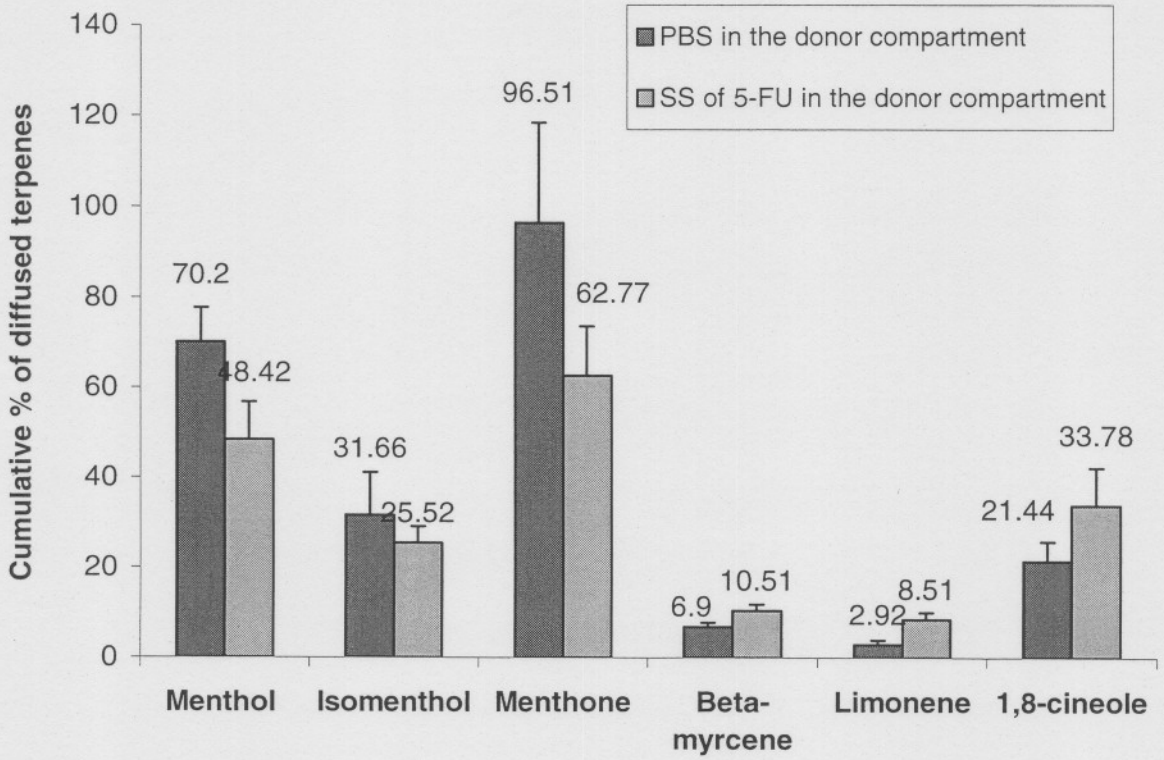


Figure 3-8: The cumulative percentage of selected terpenes diffused through the human SC after 24 hours in the absence and presence of 5-FU. Mean ± SD, n = 6.

It is clear that all the selected terpenes penetrated through the SC in the absence of 5-FU. In the presence of 5-FU, either an increasing or decreasing effect on the selected terpenes penetration could be seen. The cumulative percentage of terpenes that penetrated the skin were compared and the values are in the following order: limonene < β-myrcene < 1,8-cineole < isomenthol < menthol < menthone.

It is stated in the literature that neat terpenes penetrate the SC better than solutions thereof. Studies by Mackay *et al.* (2001) indicated that the uptake of terpenes through the SC depends only on its solubility in the membrane. Cornwell *et al.* (1996) proved that the SC uptake of 1,8-cineole and nerolidol saturated in propylene glycol was less than that from pure enhancer. They found that uptake of neat limonene and 1,8-cineole was 8.9 % and 26.2 % respectively. In this study, however, it was found that the uptake of these same terpenes from 5 % solutions was 2.92 % and 21.44 %, respectively, which compares well with the results of Cornwell and co-workers (1996). It must, however, be remembered that ethanol act as a penetration

enhancer itself (discussed in § 3.5.1). It can be justified that ethanol may exert part of its synergistic effects through increasing enhancer uptake into the skin. It can be justified that higher concentrations of the applied enhancer, would not showed better penetration of the terpenes through human SC.

Goosen and co-workers (1998) found that increased lipophilicity or partition coefficient values did not result in a higher skin flux, but the highest flux through the skin was achieved by the more lipophilic analogue that showed the highest water solubility. The above statement is in relation to the results obtained in this study. Menthone with a log P value of 2.63 ± 0.26, has the highest water solubility of the selected terpenes, at 0.71 g/l, and showed the highest penetration through the SC. Then menthol and isomenthol with log P values of 3.20 ± 0.2 for both, and water solubilities of 0.092 g/l and 0.046 g/l, respectively, showed the second and third highest penetration, respectively, through the SC. The results obtained in this study are in agreement with that of Goosen and co-workers' (1998) statement on the log P and water solubility values of analogues.

The results of this study are in agreement with that reported in the literature that the log P value for optimal transdermal delivery is in the region of 1-3 (Abdou, 1989; Guy, 1996; Potts & Guy, 1992). Terpenes with log P values (Table 2-3) around 2-3 (menthone, log P 2.63; menthol and isomenthol, log P 3.20 each) showed the highest penetration while those with higher log P values (limonene and β-myrcene, log P 4.58 each) showed lower penetration (Figurt 3-8) (Cal *et al.*, 2001; Potts & Guy, 1993; Guy, 1996).

3.6 CONCLUSION

It can now be concluded that the transdermal penetratin of 5-FU has the ability to be enhanced using certain terpenes as penetration enhancers. Menthol, isomenthol and 1,8-cineole showed the most promising results to enhance the penetration of 5-FU through human SC. These oxygen-containing terpenes were found to be better penetration enhancers of 5-FU than hydrocarbon terpenes. These penetration enhancing effects of the three terpenes may be due to their log P values and water solubilities. All the selected terpenes showed the ability to diffuse through the SC in the absence of 5-FU. However, 5-FU had an increasing or decreasing effect on the selected terpenes' penetration through the SC. The effect of 5-FU on the terpenes is not clear and will need further investigation.

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CHAPTER 4
SUMMARY AND FINAL CONCLUSIONS

Administration of drugs transdermally has several advantages over conventional techniques. It is possible to minimize first-pass metabolism, patient compliance is good and bioavailability problems inherent in gastrointestinal absorption are avoided (Guy & Hadgraft, 1985). The transdermal route of the delivery of drugs can be considered as not only one of the safest and most accessible, but also the most challenging route of administration. This can mainly be attributed to the excellent barrier properties of the skin and also the limited number of available drugs that exhibit physicochemical properties, making them suitable for transdermal delivery (Bonina *et al.,* 1995).

The aim of this study was firstly to determine the possible terpene penetration enhancers that were possible to enhance the penetration of 5-FU through human SC. The samples obtained from the studies were analysed by means of HPLC. Secondly the penetration of the selected terpenes through the SC was investigated. The samples from these studies were analysed using the gas chromatography method. All the experimental diffusion studies were carried out by using vertical Franz diffusion cells with human epidermal membranes.

Clinically, the SC is of great interest since it is usually the main rate-limiting barrier to drug absorption across the skin (Scheuplein, 1965 & 1967). The tissue is composed of highly flattened, keratin filled cells embedded in a lipid/water matrix. Intercellular lipids are arranged as stack of bilayers (Madison *et al.,* 1987). Drugs must diffuse through the intercellular lipid matrix, and to reduce reversibly the resistance of this pathway, researchers employ penetration enhancers (Williams & Barry, 1991a). As stated in Chapter 2, 5-FU doesn't appear to cross the human epidermis mainly through aqueous shunt routes (Cornwell & Barry, 1993). It is further suggested that 5-FU travels across the intercellular lipid bilayers in the SC. The effects of terpene pretreatment suggest that terpene enhancers may create new polar pathways in the SC through which 5-FU may pass.

In terms of the log P values of the selected terpenes, one could expect that the terpenes with a log P value near or lower as 3 and with a high solubility, will show the best penetration

enhancing effects towards 5-FU (Goosen *et al.*, 1998). The results obtained from this study are in agreement with that of Goosen and co-workers (1998). Menthol, isomenthol and 1,8- cineole (with log P values near 3 and high solubility values) (Table 3-3) are the best penetration enhancers for 5-FU. It could be concluded that menthol and isomenthol were the most effective penetration enhancers for 5-FU, although the extend of penetration enhancement is not large enough for clinical application.

Looking at the selected terpenes' chemical structures (Figure 2-7), as it was predicted in Chapter 2, oxygen-containing terpenes (menthol, isomenthol, menthone and 1,8- cineole) has a bigger chance to enhance the penetration of 5-FU through the SC than hydrocarbon terpenes (limonene and β-myrcene) (Williams & Barry, 1991b). Comparing this with the obtained results of this study shows the good correlation. The oxygen-containing terpenes did enhance the penetration of 5-FU through the SC much better than the hydrocarbon terpenes did.

A summary of the statistically significant effects of the selected terpenes on the transdermal delivery of 5-FU in the absence and presence of EtOH pretreatment, is given in Table 4-1.

Table 4-1: The statistically significant effects of the selected terpenes on the penetration of 5-FU.

5-FU penetration through terpene pretreated skin	5-FU penetration through untreated skin	5-FU penetration through EtOH pretreated skin
Menthol	↑	**No effect**
Isomenthol	↑	↑
Menthone	**No effect**	↓
B-myrcene	↓	↓
1,8- cineole	**No effect**	**No effect**
Limonene	**No effect**	**No effect**

↑ = statistically significant increased in flux

↓ = statistically significant decreased in flux

From the results obtained in this study on the penetration of the selected terpenes through the human SC over a period of 24 hours, it is clear that all the terpenes were penetrated, although in low percentages. It was also determined that 5-FU had either an increasing or decreasing effect on the absorption of the selected terpenes. An explanation for the specific effects of 5-FU on the penetration of the selected terpenes are not clear and need further investigation. The two terpenes that penetrated the best were menthol and isomenthol. It can be suggested that the good penetration is due to their chemical structure (oxygen-containing terpenes) (Williams & Barry, 1991b) and their log P values together with their solubilities (Goosen *et al,* 1998).

Terpenes are found in aromatherapeutic oils that are used in aromatherapy treatments, and according to this study results, it is clear that terpenes are absorbed through the skin and can cause systemic effects. It can now be understood why aromatherapy treatment is effective after a person had been messaged with terpene-containing aromatherapeutic oils. The results after such aromatherapy treatment can now be explained on the hand of the absorption of terpenes through human skin.

Should there be a continuation of this study, the following is proposed to be investigated:

- The enhancement effect of the selected terpenes in different concentrations, increasing from 1-5 % and with neat terpnes towards 5-FU.
- Absorption of selected terpenes through the SC and the effect of 5-FU on the penetration of the selected terpenes.

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