PERCUTANEOUS DELIVERY OF METHOTREXATE IN THE ABSENCE AND PRESENCE OF NATURAL PERMEATION ENHANCERS

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"I CAN DO EVERYTHING THROUGH HIM WHO GIVES ME STRENGH"

Philippines 4:13

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ABSTRACT

Title: The percutaneous absorption of methotrexate in the absence and presence of natural penetration enhancers.

The transdermal delivery of drugs has a lot of advantages above other routes of delivery, such as the avoidance of first-pass hepatic and intestinal metabolism, the non-invasive infusion of drugs, etc. However, the transdermal delivery of drugs, especially hydrophilic drugs, is limited due to the lipophilic nature of the stratum corneum. Methotrexate is a folic acid antagonist with antineoplastic activity and is used for the treatment of psoriasis and Kaposi's sarcoma. The permeation of methotrexate through the skin for systemic use is however limited due to its high molecular weight, the fact that it is mainly dissociated at physiological pH and its hydrophilic nature (Alvarez-Figueroa *et al.*, 2001). Thus, the aim of my study was to enhance the permeation of methotrexate with the use of terpenes. Terpenes are lipophilic in nature and have Log P values of around 2-4 (Godwin & Michniak, 1999). These characteristics make them excellent candidates as penetration enhancers. Terpenes are not only used for penetration enhancers, but in a huge number of other products, such as aromatherapeutic oils. For this reason the permeation of the terpenes through human skin and the effect of methotrexate on this permeation were also determined. The following enhancers were used in this study: menthol, menthone, isomenthol, limonene, β-myrcene, α-pinene and 1,8-cineole.

Five different sets of experiments were done in this study: a) a control experiment with methotrexate in the absence of the terpenes without ethanol; b) a control experiment with methotrexate in the absence of the terpenes with ethanol; c) experiments with methotrexate in the presence of the terpenes; d) control experiments with the terpenes in the absence of methotrexate and e) experiments with the terpenes in the presence of methotrexate. For this study only human female abdominal skin was used. A saturated solution of methotrexate in water:propylene glycol (50:50) with a pH between 4 and 5 (Vaidyanathan *et al.*, 1985) was used as the model drug and the receptor phase was PBS-buffer (pH=7.4) and water:ethanol (50:50) for HPLC and GC analysis respectively. The diffusion apparatus used consisted of Vertical Franz diffusion cells with a capacity of 2 ml

and a diffusion area of 1.075 cm^2. The cells were placed in a water bath (± 37 °C) on magnetic stirrers for the duration of the experiment. After the receptor phase was placed in the receptor compartment the cells were equilibrated for an hour before putting 25 µl of a 5 % terpene solution in absolute ethanol on the skin in the donor compartment. This was left for half and hour to allow evaporation of the ethanol. The saturated solution of the methotrexate was now placed on the skin in the donor compartment. The experiments for methotrexate stretched over a period of 12 hours and samples were collected every 2 hours. The terpene experiments were performed over a 24-hour period and samples were taken at 2,4,6,12 and 24 hours. The concentration methotrexate permeated was determined by using HPLC-analysis and terpenes by using GC-analysis.

The flux (µg/cm^2/h), k_p (cm/h), lag time (h) and enhancement ratio were calculated to compare the methotrexate permeation in the control and actual experiments. The results showed that α-pinene, β-myrcene and isomenthol enhanced the permeation of methotrexate most, although all the terpenes had an enhancing effect. They produced a 4-fold increase in the flux values of methotrexate. Due to the fact that the terpene experiments were only a semi-quantitative evaluation only the percentage terpenes that permeated was calculated. The experiments were done on all the terpenes except α-pinene. All the terpenes permeated the skin with menthol having the highest permeation. The results also showed that methotrexate did have an effect on the terpene permeation. Menthone and menthol's permeation was higher in the presence of methotrexate, while the other terpenes had a higher permeation in the absence of methotrexate. The reason for this is not clear.

In conclusion, the study revealed that the enhancers used did have an enhancing effect on methotrexate permeation. This could be due to the extraction or disruption of lipids by the terpenes (Zhoa & Singh, 2000) or an increase in diffusivity and partitioning. The terpene experiments also showed that the terpenes do permeate the skin and that methotrexate does have an effect on this permeation.

Key words: Percutaneous absorption, stratum corneum, hydrophilic, methotrexate, terpenes.

OPSOMMING

Titel: Die perkutaneuse absorpsie van metotreksaat in die afwesigheid en teenwoordigheid van terpene.

Die transdermale aflewering van geneesmiddels het baie voordele bo ander roetes van aflewering soos die vermyding van die eerste deurgangseffek van hepatiese en intestinale metabolisme, intraveneuse aflewering van geneesmiddels, ens. Ongelukkig is die transdermale aflewering van geneesmiddels, veral hidrofiele geneesmiddels, baie beperk as gevolg van die die lipofilisiteit van die stratum corneum. Metotreksaat is 'n foliensuur-antagonis met antineoplastiese aktiwiteit en word gebruik in die behandeling van psoriase en Kaposi se sarkoom. Die permeabiliteit van metotreksaat deur die vel vir sistemiese gebruik is egter beperk as gevolg van sy hoë molekulêre gewig, die feit dat metotreksaat by fisiologiese pH dissosieer en sy hidrofiliese natuur (Alvarez-Figueroa *et al.*, 2001). Die doel van my studie was dus om die penetrasie van metotreksaat deur die vel te verbeter deur van terpene as bevorderaars gebruik te maak. Terpene is lipofilies en het hoë Log P waardes van ± 2-4 (Godwin & Michniak, 1999). Hierdie einskappe maak hulle uitstekende kandidate vir penetrasiebevorderaars. Terpene word egter nie net as penetrasiebevorderaars gebruik nie, maar ook in 'n groot aantal ander produkte soos aromatiese olies. Om hierdie rede is die penetrasie van die terpene deur die vel ook bepaal asook die invloed van metotreksaat op die penetrasie. Die volgende terpene is in die studie gebruik: mentol, mentone, isomentol, limoneen, β-mirseen, α-pineen en 1,8-sineool.

Vyf verskillende stelle eksperimente is in hierdie studie gedoen: a) 'n kontrole-eksperiment van metotreksaat in die afwesigheid van die terpene sonder etanol; b) 'n kontrole-eksperiment van metotreksaat in die afwesigheid van die terpene met etanol; c) eksperimente van metotreksaatdeurlaatbaarheid in die teenwoordigheid van die terpene; d) kontrole-eksperimente van die terpeenpenetrasie in die afwesigheid van metotreksaat; e) eksperiment met terpene in die teenwoordigheid van metotreksaat. Vir hierdie studie is slegs menslike vroulike abdominale vel gebruik. 'n Versadigde oplossing van metotreksaat in water:propleenglikol (50:50) met 'n pH tussen 4 en 5 (Vaidyanathan *et al.*, 1985) is gebruik as die donorfase, die reseptorfase was PBS-buffer en water:ethanol (50:50) vir HPLC en GC-analise onderskeidelik. Die diffusie-apparaat het bestaan uit vertikale Franz selle met 'n volume van 2 ml en 'n diffusie-area van 1.075 cm^2. Die selle is in 'n waterbad

(± 37 °C) op magnetiese roerders geplaas vir die verloop van die eksperiment. Die reseptorfase is in die reseptorkompartement geplaas en die selle is toegelaat om vir 1hr te ekwilibreer waarna 25 µl van 'n 5 % terpeenoplossing in absolute etanol op die vel geplaas is en vir 'n halfuur laat staan is om verdamping van die etanol te bewerkstellig. Die versadigde oplossing van die metotreksaat is nou in die donorkompartement op die vel geplaas. Die metotreksaat eksperimente het oor 'n periode van twaalf uur gestrek, met ontrekkings elke 2 uur. Die terpeeneksperimente het oor 'n 24 uur periode gestrek, met ontrekkings op 2h, 4h, 6h, 12h en 24h. Die konsentrasie wat die vel gepenetreer het, is deur middel van die HDVC bepaal en die konsentrasie van die terpene deur middel van die gas-chromatograaf.

Die fluks (µg/cm^2/h), k_p (cm/h), vertragingstyd (h) en die bevorderingsverhouding is bereken om die metotreksaatpenetrasie deur die vel van die kontrole en die eksperimente met mekaar te vergelyk. Die resultate het aangetoon dat α-pineen, β-mirseen en isomentol die deurlaatbaarheid van metotreksaat die meeste verbeter het, al het die ander terpene ook 'n positiewe effek getoon. Bogenoemde drie terpene het 'n 4-voudige verhoging in die fluks van metotreksaat meegebring. As gevolg van die feit dat die terpeeneksperimente slegs 'n semi-kwantitatiewe bepaling was, is slegs die % terpene wat die vel deurgedring het, bereken. Al die terpene het die vel gepenetreer met mentol wat die hoogste permeabiliteit gehad het. Die resultate toon ook aan dat metotreksaat wel 'n invloed op die terpeenpenetrasie gehad het, waar mentol en mentone se penetrasie hoër was in die teenwoordigheid van metotreksaat. Die ander terpene se penetrasie was hoër in die afwesigheid van metotreksaat. Die rede hiervoor is nie duidelik nie.

Ten slotte het die studie getoon dat die bevorderaars wat gebruik is wel 'n bevorderende effek op die permeabiliteit van metotreksaat gehad het. Dit kan wees as gevolg van die ekstraksie (Zhoa & Singh, 2000) of verbreking van die lipiede in die stratum corneum of die verhoging in die partisie en diffusiwiteit van metotreksaat. Die terpeeneksperimente het ook gewys dat terpene wel die vel binnedring en dat metotreksaat hierdie penetrasie beïnvloed.

Sleutelwoorde: Perkutaneuse absorpsie, stratum corneum, hidrofiliteit, metotreksaat, terpene.

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STATEMENT OF PROBLEM

The skin is the largest organ of the body and acts as a protective barrier with sensory and immunological functions (Foldvari, 2000). The skin's barrier function is accomplished, entirely and quite remarkably, by the outermost few microns of the skin – the stratum corneum, a compositionally and morphologically unique biomembrane. 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 stratum corneum (Naik *et al.*, 2000).

The transdermal route offers several advantages over other routes for the systemic delivery of drugs. For example, the delivery is non-invasive, and no hospitalization is required (El-Kattan *et al.*, 2000). Unfortunately due to the barrier, the stratum corneum, which is lipophilic in nature the permeation of especially hydrophilic drugs through the skin, is hindered.

Methotrexate is a folic acid antagonist with antineoplastic activity and is used for the treatment of psoriasis and Kaposi's sarcoma. The permeation of methotrexate through the skin for systemic use is however limited due to its high molecular weight, the fact that it is mainly dissociated at physiological pH and its hydrophilic nature (Alvarez-Figueroa *et al.*, 2001). To overcome this problem terpenes (menthol, menthone, isomenthol, limonene, β-myrcene, α-pinene and 1,8-cineole) were used as penetration enhancers. They are a series of naturally occurring compounds that consists of isoprene (C_5H_8) units that are highly lipophilic and have large partition coefficients between octanol and water (Godwin & Michniak, 1999). Terpenes have also recently shown that they have an enhancing effect on drugs, such as 5-fluorouracil (Williams & Barry, 1991).

The aim of this study was the following:

- to determine the enhancing effect of the terpenes on methotrexate permeation;
- to determine if the terpenes do permeate the skin and
- if methotrexate has an influence on the permeation of the terpenes.

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PERCUTANEOUS ABSORPTION AND DRUG DELIVERY

CHAPTER 1

1.1. INTRODUCTION

The idea of delivering drugs through the skin is old. As far back as the sixteenth century B.C., the Ebers Papyrus recommended that the husk of the caster oil plant be crushed in water and placed on an aching head and "the head will be cured at once, as though it never ached" (Finnin & Morgan, 1999).

Delivery of drugs via the skin has many attractions, including increased patient acceptability (noninvasiveness) and avoidance of gastrointestinal disturbances and first-pass metabolism of the drug (Finnin & Morgan, 1999). The skin represents an extraordinary evolutionary feat. Not only does it physically encapsulate the organism and provide a multifunctional interface between our surroundings and us, but it is perpetually engaged in the assembly of a highly efficient homeostatic barrier to the outward loss of water. In so doing it furnishes a membrane that is equally adept at limiting molecular transport both from and into the body (Naik *et al.*, 2000).

Human skin is the most complex organ found in the body, judged by the number of cell types that it contains. Similarly, transdermal delivery is an intricate procedure, which is exemplified by considering just some of the features that are in operation during drug permeation from a patch (Barry, 1999). Research interests focus on the elucidation mechanisms across biological or synthetic membranes. Efficacious delivery of most pharmaceutically active agents requires that the drug permeate a biological membrane such as epithelia, cell envelopes, lipid bilayers, etc. Permeation mechanisms are dependent on the physicochemical properties of both the therapeutic agent and membranes of interest (Knutson, 2001). In order for a medicament to have a chance of being delivered transdermally certain suitable physicochemical properties must be present. That is, the drug must be stable, have a molecular weight less than 1000, a melting point less than about 93 degrees Celsius, a solubility in oil and water greater than 1 mg/ml and a ph of 5 to 9 in a supersaturated aqueous solution (Toppo, 1994).

Molecules traverse membranes either by passive diffusion or active transport. A passive diffusion process implies that the solute flux is linearly dependent on the solute concentration gradient; on the other hand, active transport processes typically involve a saturable mechanism. Transport across the skin occurs primarily by passive diffusion (Potts *et al.*, 1992). A major problem in attempting to control the drug flux arises from the impermeability of human skin and its biological variability (Barry, 1987).

Over the past decades there has been a general realisation that the bioavailability of topically applied drugs are very low (Hadgraft, 1999). In order to increase the effectiveness of drugs that are delivered topically or transdermally it is beneficial to identify safe and pharmaceutically acceptable penetration enhancers (Brain *et al.*, 1991). Vehicles designed to enhance drug delivery through the skin must incorporate specific elements that improve the ability of the delivery system to overcome the barrier posed by the stratum corneum (Foldvari, 2000).

The understanding of the mechanisms of absorption and enhancement has improved and the different determinants at a molecular level are beginning to be understood. This knowledge can be used in the design of better dermal and transdermal medicines and associated permeation enhancers (Hadgraft, 1999). Overcoming the barrier function then, for the purpose of transdermal drug delivery, has been a necessarily challenging task for the pharmaceutical scientist and one that boasts significant progress (Naik *et al.*, 2000).

1.2. THE ADVANTAGES OF TRANSDERMAL DRUG DELIVERY

Given that the skin offers such an excellent barrier to molecular transport, the rationale for this delivery strategy needs to be carefully identified.

The transdermal mode offers several distinct advantages:

- the skin presents a relatively large and readily accessible surface area for absorption
- the application of a patch-like device to the skin is a non-invasive (and thus a patient compliant) procedure that allows continuous intervention.

Further benefits of TDD systems have emerged over the past few years as technologies have evolved. These include the potential for sustained release (useful for drugs with short biological half-lives requiring frequent oral or parental administration) and controlled input kinetics, which are particularly indispensable for drugs with narrow therapeutic indices (Naik *et al.*, 2000).

1.3. THE STRUCTURE OF THE SKIN

The skin is the largest organ in the body, both by weight and surface area. In adults, the weight of your skin accounts for about 16 % of your total body weight (Altruis Biomedical Network, 2000). An average square centimeter of skin contains 10 hair follicles, 15 sebaceous glands, 12 nerves, 100 sweat glands, 360 cm of nerves, and three blood vessels (Asbill & Michniak, 2000). Human skin is, on average, 0.5 mm thick (ranging from 0.05 mm to 2 mm) and is composed of four main layers: the stratum corneum (SC), viable epidermis, dermis and subcutaneous tissue (Foldvari, 2000). The structure of the skin is represented in figure 1.1.

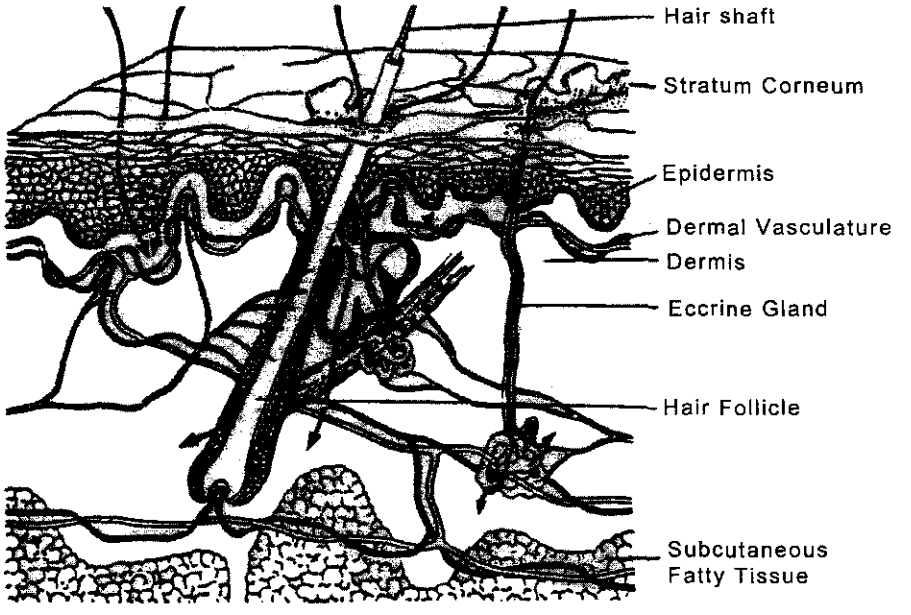

Figure 1.1: Schematic diagramme of cross-section of human skin (Roy, 1997).

1.3.1. Stratum Corneum

The thick (10-20 μm) surface layer, the stratum corneum or horny layer, is highly hydrophobic and contains 10-15 layers of interdigitated corneocytes, (figure 1.2) which are constantly shed and renewed (Foldvari, 2000). Exposure to moisture predominantly results in the swelling of the corneocytes in the vertical direction. The cells are composed mainly of insoluble bundled-keratins, surrounded by a cell envelope stabilised through covalently bound lipid and cross-linked proteins. Each corneocyte is mechanically attached to its neighbours by protein-rivet structures, desmosomes, which

contribute to the cohesion of the stratum corneum layer (Asbill & Michniak, 2000). Its organisation 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 (Foldvari, 2000). The intercellular lipids are mainly produced from the exocytosis of lamellar bodies, which occurs during the terminal differentiation of the keratinocytes (Asbill & Michniak, 2000). The lipid components of human abdominal horny layer are shown in table 1.1.

Table 1.1: Composition of the horny layer of human abdominal skin (Loth, 1991).

Polar lipids: (4.9%)	phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, sphingomyelin, lysolecithin.
Neutral lipids: (74.8%)	free sterols (14%), free fatty acids (19.3%), triglycerides (25.2%), sterol and wax esters (5.4%), squalene (4.8%), N-alkanes(6.1%)
Sphingolipids: (18.1%)	ceramides I (13.8%), ceramides II (4.3%), glucosylceramides I and II (traces)
Cholesterol sulphate (1.5%)	

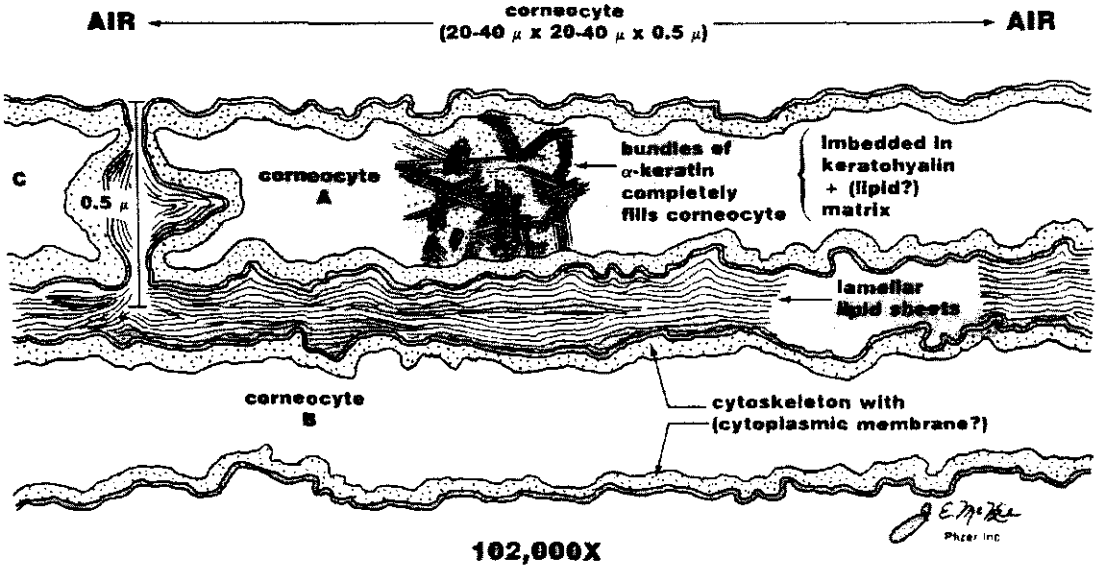

Figure 1.2: Schematic of the stratum corneum (Potts *et al.*, 1992).

The lipids of the SC are unique in a number of ways:

- they consist primarily of cholesterol, free fatty acids and ceramides and no phospholipids are present;
- the lipids of the stratum corneum are most abundant in the intercellular spaces
- the SC lipids exist in multilamellar arrays which form the only continuous medium from top to bottom of this tissue (Potts *et al.*, 1992).

The major component of the stratum corneum is represented by the keratins, which are relatively poor in cystine, rich in serine and glycine, and contain N-acetylserine at the amino terminus. The second component known as the corneocyte envelope is cornified cells of the stratum corneum bounded by an envelope produced in the final steps of terminal differentiation. The intercellular spaces of the stratum corneum are completely filled with broad, multiple lipid lamellae and represents the intercellular lamellae which is the last component of the stratum corneum (Wertz & Downing, 1989).

The SC however, has an exceptionally low permeability compared to other membranes. One explanation for this low permeability may be the highly tortuous nature of the extracellular lipid domain (Potts *et al.*, 1992).

It is well accepted that the stratum corneum is the major rate-limiting barrier to molecular diffusion through mammalian epidermis (Kurihara-Bergstrom & Good, 1987). A simple demonstration of this occurs when the layer is stripped with adhesive tape, and the permeability to water and other compounds dramatically increases (Asbill & Michniak, 2000). There are some exceptions to this – for instance for very lipophilic drugs the aqueous epidermal and dermal layers may provide a significant hindrance because of the clearance effect (Barry, 1987). 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 stratum corneum. The skin's barrier function appears to depend on the specific ratio of various lipids; studies in which non-polar and relatively polar lipids were selectively extracted with petroleum ether and acetone, respectively, indicate that the relatively polar lipids are more crucial to skin barrier integrity. Hence, the staggered corneocyte arrangement in a lipid continuum is suggested to bestow a highly tortuous lipoidal diffusion pathway rendering the membrane a 1000 times less permeable to water relative to most other biomembranes (Naik *et al.*, 2000).

Of the major SC species, it is the sphingolipids that are presumed to be of major importance for the epidermal barrier (table 1.2). Not only do they account for the most lipid by weight, but they also possess the majority of long-chain, saturated fatty acids and the majority of linoleic acid, which is ω-estrified at the termins of N-acyl fatty acids (Elias, 1992).

Table 1.2: Evidence for the role of sphingolipids in barrier function (Elias, 1992).

Accounts for up to 50% of lipid weight of stratum corneum.
Carriers of extremely long-chain, saturated fatty acids.
Replacement of long-chain fatty acids by short-chain fatty acids in marine mammals.
Principal repositories of linoleic acid.
Removal required to completely break the barrier.
Regulation of the water-holding capacity of the stratum corneum.

1.3.2. Viable epidermis

Directly below the stratum corneum is the viable epidermis, which consists of three layers: the stratum granulosum, spinosum and basale (Asbill & Michniak, 2000). The basal layer contains actively dividing cells, which migrate upwards to successively form the spinous, granular and clear layers. As part of this process, the cells gradually lose their nuclei and undergo changes in composition (Foldvari, 2000). The cells of the basal layer of the epidermis are columnar or cuboidal, with the long axis usually orientated perpendicularly to the surface. As these cells divide and ascend to the surface, they become progressively larger and of altered shape, oriented horizontally to the surface and increasingly flattened until they reach the SC, where their appearance becomes scale-like in form. The flattened elongated shape of the corneocyte may play a key role in barrier function (Potts *et al.*, 1992). In contrast to the lipoidal nature of the stratum corneum, the viable epidermis is of much greater aqueous character. Compounds which are absorbed through the stratum corneum, encounter this different environment as they diffuse toward the papillary dermis. For very lipophilic compounds, the greatest diffusional resistance may be encountered in the viable epidermal tissue. For less lipophilic compounds, it is the stratum corneum, which limits the rate of diffusion (Collier & Bronaugh, 1991).

Several other cells (e.g 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 (Foldvari, 2000). HMG CoA reeducates, the rate-limiting enzyme for cholesterol synthesis, exists in a phosphorylated and dephosphorylated state. Acute barrier disruption stimulates both an increase in enzyme content and a shift toward the activated state, which parallels the degree of barrier abrogation (Elias, 1992).

Keratinocytes, or dead cells, constitute about 95 % of the epidermal cells and function as a barrier, keeping harmful substances out and preventing water and other essential substances from escaping the body. The other 5 % of epidermal cells are melanocytes, which manufacture and distribute melanin, the protein that adds pigment to skin and protects the body from ultraviolet rays (Walther, 1995).

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 and cholesterol content simultaneously increase (Foldvari, 2000). The thickness of the epidermis varies with age, sex, and the location on the body of the skin. For example, the epidermis on the underside of the forearm is about 5 cell-layers thick and on the sole of the foot, the epidermis might be 30 cell-layers thick (Altruis Biomedical Network, 2000).

1.3.3. Dermis

The dermis, or the "true skin" is composed of gel-like and elastic materials, water, and, primarily, collagen (Walther, 1995). The dermis is largely acellular, but is rich in lymphatic vessels and nerve endings. The dermis is pervaded by a mass of arterioles, venules and capillaries. Permeants, which are transported through the SC and epidermis, are ultimately removed by this dermal vasculature (Potts *et al.*, 1992).

The dermis structurally supports the epidermis and, through its capillary circulation, provides oxygen and nutrients and removes waste products (and percutaneously absorbed material) by diffusion through the basement membrane. The dermal/epidermal junction is not a smooth interface. Rather it is marked by rete pegs, projections of epidermis into the dermis, and hair follicles, which are deeper invaginations of epithelium into the dermis (Collier & Bronaugh, 1991). An extensive network of dermal capillaries is linked 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 (Foldvari, 2000). The elasticity of the dermis is attributed to a network of protein fibers, including collagen and elastin, which are embedded in an

amorphous glycosaminoglycan ground substance. The dermis also contains scattered fibroblasts, macrophages, mast cells and leukocytes (Foldvari, 2000).

Like the epidermis, the hair follicle manufactures a keratin structure, hair. These follicles are found everywhere on the body except for the palms and soles. The sebaceous glands are attached to the hair follicles and through the follicles excrete an oily substance called sebum, which both lubricates and protects the skin (Walther, 1995). Sweat glands secrete mostly water, sodium chloride, urea ammonia and uric acid (Altruis Biomedical Network, 2000).

There are two distinctive sweat-producing glands, the apocrine and the eccrine. The apocrine gland is best known for producing body odor but otherwise has no known physiological function and is apparently a holdover from times past. The eccrine glands are an advanced and extensive system of temperature control. Several million of these glands are distributed over the entire body, with the highest concentration in the palms, soles, forehead, and underarms (Walther, 1995).

Nerve endings in the dermis are the source of the body's sense of touch. They sense heat, cold and pressure, providing both pain and pleasure (Walther, 1995).

1.3.4. The subcutaneous tissue (hypodermis)

The hypodermis is a layer of mesenchymally derived adipose cells that abut the connective tissue layer of the reticular dermis (Eckert, 1992). It is unevenly distributed over the body, and there are wide individual differences in distribution (Walther, 1995). The hypodermis is the innermost layer of skin and it functions to provide a cushion between the external skin layers and the internal structures such as bone and muscle (Eckert, 1992). It also synthesises and stores readily available high-energy chemicals (Danckwerts, 1991).

1.4. THE FUNCTIONS OF THE SKIN

The skin performs a complex role in human physiology. It protects us from potentially harmful external stimuli, water loss, friction wounds and impact wounds. Using its thermoreceptors the skin performs a very important role in regulating body temperature. Not only does the skin synthesize and metabolize compounds but it also disposes of chemical waste (Barry, 1983). When the skin is exposed to the sun's rays it also produces vitamin D in the epidermal layer (Altruis Biomedical Network, 2000).

1.5. THE PROCESS OF PERCUTANEOUS ABSORPTION

1.5.1. Mechanisms of percutaneous absorption

Percutaneous absorption involves the following sequence of events:

1. Partitioning of the molecule into the SC from the applied vehicle phase
2. Diffusion through the SC
3. Partitioning from the SC into the viable epidermis
4. Diffusion through the epidermis and upper dermis
5. Capillary uptake (Potts *et al.*, 1992).

The steps in the percutaneous absorption process are shown in figure 1.3 (Bucks & Maibach, 1999).

Hence, the movement of molecules through the skin involves transport through a number of resistances in series. It follows that the overall rate of transport is dictated by the least permeable barrier (Potts *et al.*, 1992).

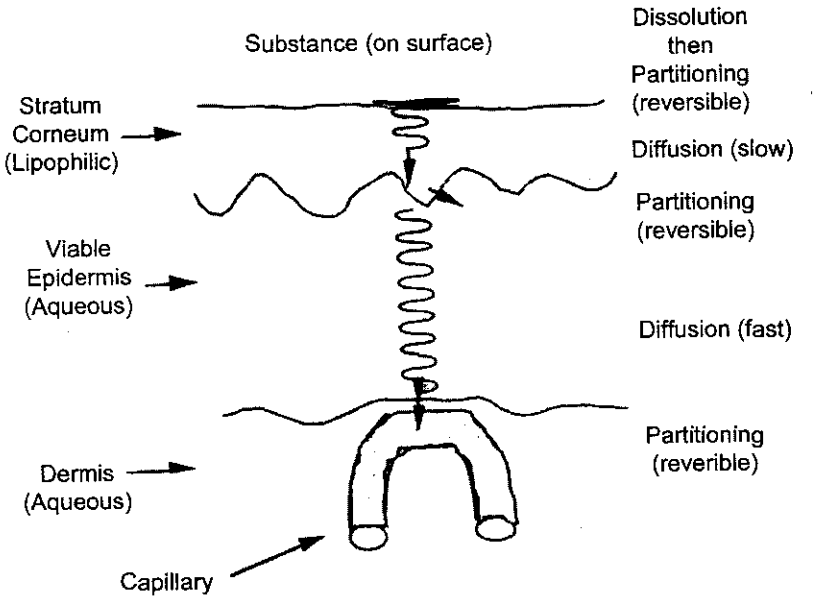

Figure 1.3: Schematic depiction of pecutaneous absorption (Bucks & Maibach, 1999).

1.5.1.1. Diffusion

In this process, transport across a cell membrane depends on the concentration gradient of the solute. Most drug molecules are transported across a membrane by simple diffusion from a region of high concentration to one of low concentration (Beers & Berkow, 2002). Topp (2000) stated that diffusion is the movement of mass due to a spatial gradient in chemical potential and as a result of the random thermal motion of molecules.

Diffusion within the confines of the SC has been modelled with the aid of three simplying modeling processes:

1. The particle (or molecule or ion) must pass through the vehicle (donor compartment) to the surface of the SC. The step controlling this process is diffusion, which obeys the Einstein $\sqrt{t}$ relationship.

2. The second step, passage into the SC, is controlled by the distribution coefficient K, which will be described in detail below.

3. In the third step, the permeant diffuses through the SC. This is generally the rate-determining step as shown by extensive experimentation in the study of skin permeation (Rieger, 1993).

It is generally agreed that the percutaneous absorption of most drugs is a passive diffusion process that can be described by Fick's first law of diffusion. Fick's laws include a diffusion coefficient, which is assumed to create a linear concentration gradient of the permeant within the SC. Gradients of permeants of the SC are, in fact, not linear, and one may infer that the diffusion coefficient for SC in vivo is not constant from top to bottom (Rieger, 1993).

Fick postulated that diffusive flow, which is flux (J), through a membrane should be proportional to the concentration differences ΔC between the two sides of the membrane and inversely proportional to the thickness l of the membrane. The proportionality constant is defined as the partition coefficient K; this relationship is known as Fick's First Law:

$$J = \frac{dC}{dl} \qquad \text{(Equation 1.1)}$$

$$J = P\Delta C \quad = \quad \frac{KD\Delta C}{l} \qquad \text{(Equation 1.2)}$$

The units of J are mole/cm^{2}sec, which clarifies the physical meaning: J is the quantity of solute passing through a unit of the membrane in unit time (Rieger, 1993).

Fick's first law is applicable only to membranes, which are homogeneous from one side to the other, which is equivalent to the statement that P (or $\frac{KD}{l}$) is invariate throughout the membrane's thickness. P must also be unaffected by the variable concentrations of sorbed substances which may include conditions of the vehicle as well as the permeating species.

The integrated form of Fick's First law describes a straight-line relationship. The existence of a linear correlation between Flux J and time at steady-state permeation has been established in vitro and in vivo for the passage of drugs through isolated SC, full-thickness epidermis, and whole skin.

The initial curvature of the flux vs. time curve, shown in figure 1.4, is typical of experimentally observed data. The straight-line portion begins at point X and can be

extrapolated to the time axis, where Q equals zero. The intercept on the time axis is called the lag time, t_L which can be used to compute the diffusion coefficient from $D = l^2/6t_L$. D (permeant dependent) and l (membrane dependent) play major roles in the shape of the flux vs. time curve (Rieger, 1993).

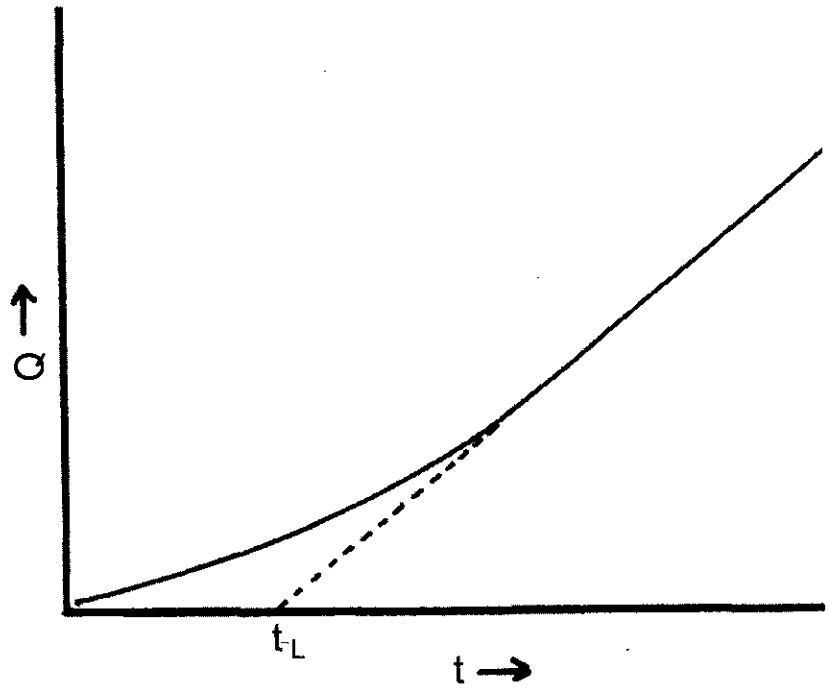

Figure 1.4: Time vs. permeation curve showing the approach to steady-state flux and the lag time tL (Rieger, 1993).

A slight modification of equation 1 is possible on the assumption that the concentration of the permeant is nil (sink condition) at the receptor interface and that the donor concentration is invariate (infinite dosing):

$$J = \frac{KDC_v}{l} \qquad \text{(Equation 1.3)}$$

This equation and related expressions describe the steady-state diffusion of a substance through a (homogeneous) barrier membrane. Since the skin is a multilamellar structure, the overall flux might be advantageously expressed as a sum of fluxes through multiple layers, with each layer exhibiting its own K, D and l (Rieger, 1993).

1.5.1.2. Partitioning

The partitioning criteria for percutaneous penetration are demanding. The molecule must be able to dissolve in the stratum corneum, and it must have a reasonably balanced affinity for this layer and the aqueous viable tissue (Guy & Hadgraft, 1989a). The importance of the partitioning step is implied by the dependence of percutaneous absorption with compound lipophilicity, as would be predicted if the skin behaved as a simple lipid membrane (Bucks & Maibach, 1999).

1.5.2. Penetration pathways through human skin

Chemicals permeate the skin either across the intact epidermis (transepidermal pathway) or via the sweat glands and hair follicles (appendageal or shunt route) (Moghimi *et al.*, 1999). A range of factors controls the relative importance of each route, including the physicochemical properties of the penetrant, diffusional time scale, follicle and gland desities, properties of the stratum corneum, vehicle effects, metabolism and hydration (Barry, 1991).

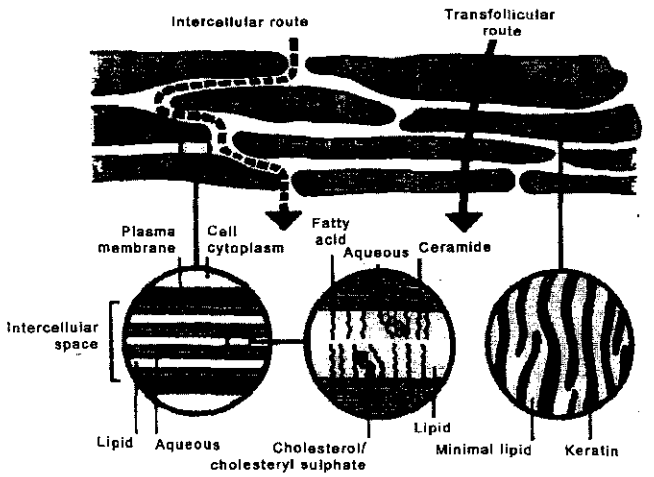

Figure 1.5: The different pathways through human skin (Loth, 1991).

1.5.2.1. The transepidermal pathway

Transepidermal permeation is a sequence of partitioning and diffusion in the SC, viable epidermis, and papillary layer of the dermis an infinite sink (Moghimi *et al.*, 1999). The major pathways in the stratum corneum for penetrants have been proposed for the transdermal delivery of drugs (figure 1.5): between the cells (paracellular route) or

through the protein-filled cells and across the lipid-rich regions (transcellular route) (Ghosh & Pfister, 1997).

1.5.2.2. The transappendageal pathway

The available diffusional area of the shunt route is approximately 0.1 % of the total skin area. Despite their small fractional area, the skin appendages may provide the main portal of entry into the subepidermal layers of the skin for ions and large molecules (Moghimi *et al.*, 1999). It has been deduced that, at steady-state, appendageal transport makes a negligible contribution to the overall percutaneous flux across human skin. Furthermore, there is a poor correlation between appendageal density and percutaneous absorption when different anatomical sites are compared. However, transport through the appendageal route has been shown to be significant during the initial (non-steady-state) period of percutaneous penetration (Potts *et al.*, 1992).

1.5.3. Factors that affect percutaneous penetration

1.5.3.1. Physicochemical Factors

1.5.3.1.1. Molecular weight

The diffusivity in liquids is expected to vary only slightly with increased molecular size (Roy, 1997). In general a drug having a molecular weight of more than 1000 daltons will be rather difficult to deliver passively through human skin without significant modifications of the stratum corneum (Roy, 1997). Relevant physicochemical parameters for the homologous series of n-alkanols and water are shown in table 1.3. The K values in table 1.3 reflect contributions from both the protein and lipid domains of the SC. It was assumed that the diffusion coefficient for each compound, D_m, could be described by the following relationship:

$$D_m = D_m^{\,0} MW^{-n} \qquad \text{(Equation 1.4)}$$

In which MW is molecular weight and both $D_m^{\,0}$ and n are constants that depend on the nature of the membrane (Zatz, 1993).

Table 1.3: Skin permeability coefficients and relevant physicochemical parameters for the homologous series of η-alkanols (Potts *et al.*, 1992).

	Log K_{oct}[a]	K[b]	K_p[c] (cm hr^{-1}) x 10^3	Molecular weight (Da)	Molecular volume ($Å^3$/molecul)
Water	-1.38	0.8	1.0	18	18
Methanol	-0.73	0.6	1.0	32	40
Ethanol	-0.32	0.9	1.0	46	59
Propanol	0.34	1.1	1.0	60	75
Butanol	0.88	2.5	2.0	74	92
Pentanol	1.4	5	6.0	88	108
Hexanol	2.03	10	13.0	102	124
Heptanol	2.49	30	32.0	116	141
Octanol	3.15	50	50.0	130	157
Nonanol	3.77	–	61.0	144	173

1.5.3.1.2. Molecular volume

According to Potts & Guy (1995) increasing the molecular volume increases the hydrophobic surface area and this will increase partitioning into, and hence, permeability through a lipid membrane. The diffusivity (D) in liquid media, in general, tends to decrease with an increase in the molecular volume (MV) and may be represented as follows (Roy, 1997):

$$D = MV^{\frac{1}{3}} \qquad \text{(Equation 1.5)}$$

1.5.3.1.3. Water solubility

The solubility parameter of the stratum corneum of human skin is about 10 $(cal/cc)^{1/2}$ and compounds with a solubility parameter close to this value are likely to have a high permeability coefficient (Roy, 1997). The importance of solubility is the reason a solvent carrier is typically used despite its reduction of the partition coefficient (Deus, 2000). The partioning behaviour of the drug will be linked with its solubility characteristics and is an important factor that must be taken into account in any assessment of the feasibility of transdermal or topical delivery (Hadgraft & Wolff, 1993).

1.5.3.1.4. Melting point

The lower melting point compounds, in general, would be more permeable through human skin because of their inherent higher solubility in the stratum corneum (Roy, 1997).

1.5.3.1.5. Water/octanol partition coefficient

Since it is experimentally difficult to obtain the appropriate lipid/water partition coefficient which is relevant for drug transport through the stratum corneum, many investigators have chosen to use the octanol/water partition coefficient (K_{oct}) as the index of lipophilicity. These values for the n-alkanols and water are listed in table 1.3 (Potts *et al.*, 1992). It is a measure of a given substance's relative affinity for octanol vs. water. The higher it is, the more it tends to be attracted to octanol and vice versa. To simplify, it is a measure of lipophilicity vs. hydrophilicity (Deus, 2000). The lipid/water partition coefficient of the drug is the basic determinant for specific drug permeability through the stratum corneum. A drug with a log P value of < 2 is considered to be a potential candidate for transdermal delivery (Guy & Hadgraft, 1989c). According to Guy (1996) compounds with a Log P value between 1 and 3, with relatively low molecular weights and modest melting points, are likely to have decent passive skin permeabilities.

1.5.3.1.6. pH and Osmolarity

The permeability of acidic and basic drugs through human skin is also affected by the hydronium ion concentration or pH of the media. Lipophilic species are more favourable for stratum corneum permeability, the net permeability coefficients of acidic and basic drugs will be dictated by the fraction unionised in the donor compartment or solution in direct contact with the stratum corneum surface. Optimization of transdermal formulation of weakly basis and acidic drugs with respect to pH of donor solution is worthwhile to

evaluate at the preformulation stage to identify preferable chemical species for maximal skin flux (Roy, 1997).

1.5.3.1.7. Drug lipophilicity

Essentially, the stratum corneum barrier is lipophilic, with the intercellular lipid lamellae forming a conduit through which drugs must diffuse in order to reach the underlying vascular infrastructure and ultimately to access the systemic circulation. For this reason, lipophilic molecules are better accepted by the stratum corneum. A molecule must first be liberated from the formulation and partition into the uppermost stratum corneum layer, before diffusing through the entire thickness, and must then repartition into the more aqueous viable epidermis beneath. Ideally, a drug must possess both lipoidal and aqueous solubilities; if it is too hydrophobic, the molecule will be unable to transfer into the stratum corneum; if it is too lipophilic, the drug will tend to remain in the stratum corneum layers (Naik *et al.*, 2000). It appears that the permeability of a molecule is directly related to its lipophilicity (which is often the key parameter) (Potts *et al.*, 1992).

1.5.3.1.8. Ionisation

Generally, drugs permeate through the skin better in their unionised form. One reason is the greater solubility on the unionised compound in the horny layer and the second is its poorer solubility in the aqueous donor solvent (Zatz, 1993). In a study reported by Kushla & Zatz (1991) the permeability coefficient for unionised lidocaine through hairless mouse skin was 15 times greater than for the ionised form.

1.5.3.2. Physicochemical properties of methotrexate

Amethopterin; N-[4-(2,4-diamino-6-pteridinyl-methyl] methylamino]benzoyl)-L-glutamic acid.

1.5.3.2.1. Structure, formula and molecular mass

Formula: $C_{20}H_{22}N_8O_5$

Molecular mass: 454.4 (The USP, 1999)

Because of its slightly high molecular weight, methotrexate's passive diffusion will be limited (Alverez-Figueroa *et al.*, 2001).

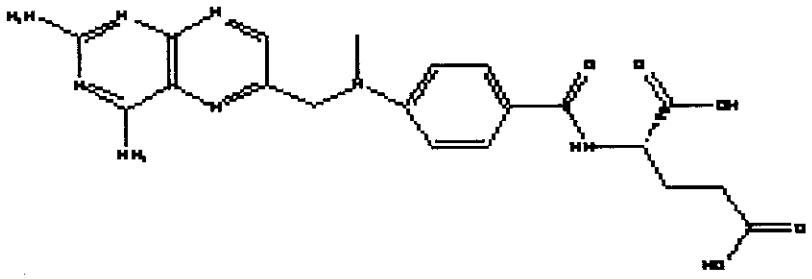

Figure 1.6: The chemical structure of methotrexate (Chemfinder.com., 2003).

1.5.3.2.2. Character and appearance

Methotrexate is a yellow or orange, crystalline, hygroscopic powder, practically insoluble in water, in alcohol and in methylene chloride. It dissolves in dilute solutions of mineral acids and in dilute solutions of alkali hydroxides and carbonates (European Pharmacopoeia, 2001).

1.5.3.2.3. Storage

Methotrexate should be preserved in tight, light-resistant containers (The USP, 1999). Methotrexate is irritant and care should be taken to avoid contact with skin and mucous membranes (Lund, 1994).

1.5.3.2.4. Physical Properties

1.5.3.2.4.1. Melting point

Methotrexate melts in the range 182° to 189° (Lund, 1994). As discussed in § 1.5.3.1.4 lower melting points will promote permeation of compounds through skin. Thus because of its high melting point methotrexate's permeation will be limited.

1.5.3.2.4.2. Dissociation constants

PK_a 3.8, 4.8, 5.6 (Lund, 1994)

1.5.3.2.4.3. Partition coefficients

The log P oct/water for methotrexate was determined at -1.85 (Alvarez-Figueroa *et al.*, 2001). According to Deus (2000) a Log P of ± 3 is desired, as Log P decreases, diffusion through intracellular lipids is hindered. This means that the Log P of methotrexate is a limiting factor in its permeation through the skin.

1.5.3.2.4.4. Solubility

Methotrexate is practically insoluble in water, in ethanol, in choroform, in 1,2-dichloro-ethane, and in ether. It dissolves in solutions of mineral acids and in dilute solutions of alkali hydroxides and carbonates. It is slightly soluble in 6M hydrochloric acid (Lund, 1994).

1.5.3.2.4.5. Crystal and molecular structure

Existence of different types of solid [preparation and characterization of two stable and metastable pseudopolymorphs and an amorphous form of methotrexate] (Lund, 1994).

1.5.3.2.5. Stability

1.5.3.2.5.1. Degradation pathways and kinetics

Methotrexate in solution is subject to photolytic and thermal degradation. In alkaline solutions (pH above 8) at 85°, first-order hydrolysis yielded the decomposition product N^{10}-methyl-folic acid (methopterin). When methotrexate solutions (pH 8.3) were kept under laboratory fluorescent light at room temperature the major degradation products were identified by ultraviolet spectrophotometry and HPLC as 2,4-diamino-6-pteridinecarbaldehyde, 2,4-diamino-6-pteridinecarboxylic acid, and *p*-aminobenzoylglutamic acid.

The temperature dependence of methotrexate degradation was examined in isotonic buffer-free solution (initially pH 8.5) over the range 65° to 95°, and an activation energy of 96.8 kJ/mol was established. The $t_{10\%}$ of this solution at 25° and at 4° was predicted to be 4.5 years and 88.7 years respectively (Lund, 1994).

1.5.3.2.6. The effect of pH and solubility on percutaneous absorption

It was found that for a 50 % v/v propylene glycol-water vehicle, a pH between 4 and 5 would appear to provide the most favorable environment for passive diffusion since the concentration of unionized methotrexate would be optimal (Vaidyanathan *et al.*, 1985).

The activity of propylene glycol is thought to be due to the solvation of α-keratin within the stratum corneum and the occupation of proteinaceous hydrogen bonding sites reducing drug-tissue binding and thus promoting permeation (Ho *et al.*, 1998).

1.5.3.2.7. Pharmacology

Methotrexate is a folic acid antagonist with antineoplastic activity. It competitively inhibits the enzyme dihydrofolate reductase, leading to inhibition of DNA synthesis (Chabner *et al.*, 1996). Since methotrexate inhibits mitotic activity, it is effective for the treatment of recalcitrant psoriasis (Vaidyanathan *et al.*, 1985).

The systemic use of this drug may provoke any of numerous side effects, including nausea, vomiting, fatigue, headache, dyspnea, leukopenia, anemia and hepatic toxicity. To reduce such effects, it would clearly be preferable to administer methotrexate topically, and to this end there have been studies of its administration in ointments, creams and gels (Vaidyanathan *et al.*, 1985).

1.6. PENETRATION ENHANCEMENT

The skin has an extremely good barrier function and to improve topical bioavailibility it is usually necessary to employ enhancement strategies (Hadgraft, 1999). To produce a systemic effect, transdermal drug delivery requires that suitable quantities of drug be transported through the skin. In addition to the potential for enhanced Transdermal drug delivery to improve transdermal delivery rate control, the main reason that drug delivery across the skin needs to be enhanced is because of the low permeability of most transdermal candidates across the skin (Finnin & Morgan, 1999). This has proved to be a challenge, and has led to the development of a large repertoire of 'penetration enhancer' compounds and physical techniques that, to different degrees, facilitate drug penetration across the skin (Foldvari, 2000). A penetration enhancer is a chemical which exhibits the only characteristic that it reversibly reduces the barrier nature of the stratum corneum without the accelerant damaging any viable cells. Thus the desirable attributes of an ideal penetration enhancer is as follows (Barry, 1991):

- The material should be pharmacologically inert and should possess no action of itself at receptor sites in the skin or in the body generally.
- 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 an excellent solvent for drugs.
- The material should spread well on the skin and it should possess a suitable skin "feel".
- The chemical should formulate in 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.

Table 1.4 lists the means that can be employed to facilitate the transport of drugs across the biological barriers (Hsieh, 1994):

Table 1.4: Examples of permeation enhancement technologies (Hsieh, 1994).

Chemical means
Permeation enhancers
Prodrug design
Physical means
Iontophoresis
Phonophoresis
Thermal modulation
Magnetic modulation
Mechanical modulation
Biological means
Receptors
Combinations

1.6.1. Mechanisms of action of penetration enhancers

Skin penetration enhancers are molecules which reversibly remove the barrier resistance of the stratum corneum. They allow drugs to penetrate more readily to the viable tissues and thus enter the systemic circulation (Barry, 1987).

According to Shah as quoted by Asbill & Michniak (2000), enhancers:

- increase the diffusivity of the drug in the skin;
- cause stratum corneum lipid-fluidization, which leads to decreased barrier function (a reversible action);
- increase and optimize the thermodynamic activity of the drug in the vehicle and the skin;

- result in a reservoir of drug within the skin;
- affect the partition coefficient of the drug, increasing its release from the formulation into the upper layers of the skin (Asbill & Michniak, 2000).

1.6.1.1. Possible site for accelerant action within the intercellular region

Within the intercellular route, it was proposed that accelerants might interact at the polar head groups of the lipids, within the aqueous region between lipid head groups, and between the hydrophobic tails of the bilayer. Within the corneocyte the major site of action would be the keratin fibrils and their associated water molecules. A penetration enhancer may also have a direct action whereby regions of the tissue change their bulk constitution. Thus with high concentrations of solvents such as propylene glycol, ethanol, the pyrrolidones or dimethylsulfoxide, so much solvent may penetrate into the tissue that it changes the partition coefficient for the drug (Barry, 1987).

1.6.1.2. Possible site for accelerant action within the intracellular region

The accelerant would interact with polar head groups on the keratin (the alkyl chains if surfactants could additionally interact with hydrophobic residues), relaxing the binding forces and altering the conformations of the helices and possibly, in extreme cases, forming pores through the tissue (Barry, 1991).

1.6.2. Chemical 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 membrane and through to the systemic circulation (Naik *et al.*, 2000).

The environs of the stratum corneum are thought to be the site of activity of the chemical penetration enhancers. Whilst the mechanisms by which these compounds are thought to promote permeation are now only beginning to be elucidated, their activity is thought to be a result of multiple effects within the diverse biochemical environments of this layer. We currently believe that most chemical enhancers are active by spatial disruption of the normally ordered arrangement of the intercellular molecules. It is the uniform, ordered nature of these biochemicals, especially lipid bilayers, that maintain and promote the diffusional resistance of the barrier (Walker & Smith, 1996).

1.6.2.1. Classification of chemical enhancers

The following classes of compounds have been tested for their enhancer action: water, hydrocarbons (alkanes and alkenes), acids, esters, alkyl amino esters, amides, ureas, amines and bases, sulfoxides, terpenes, steroids, dioxalanes, pyrrolidone and imidazole derivatives, laurocapram (Azone) and its derivatives (Asbill & Michniak, 2000). The structure of the enhancers is given is figure 1.8 (Barry, 1991).

1.6.2.1.1. Water

Water is perhaps the ideal enhancer, since hydrated skin is generally more permeable (Hadgraft, 1999). The level of hydration is a function of the water concentration gradient between the dermis and the surface of the skin as well as on the ability of the stratum corneum to bind water (Roberts & Walker, 1993). Water reacts with and forms hydration shells around the polar groups of ceramides and sphingolipids in the lipid double layer (Walters, 1989). This disturbs the packing configuration of the lipids that creates a more liquid and permeable hydrophobic route. This also causes a thickening of the hydrophilic layer with a resulting increase in mobility (Wiechers, 1989).

1.6.2.1.2. Sulfoxides

Decylmethylsulfoxide (DCMS) is thought to promote permeation enhancement as a result of protein-DCMS interaction creating aqueous channels, in addition to lipid interactions (Walker & Smith, 1996). Cooper postulated that DCMS acted as a surfactant, changing protein conformations and thus opening up aqueous channels (Barry, 1987).

1.6.2.1.3. Alcohols

Alcohols may influence transdermal penetration by a number of mechanisms. The enhancing ability of these alcohols appears to be related to their capability of extracting stratum corneum lipids and, in most cases, the increase in permeation rate is slight because only the polar lipids are significantly affected (Walters, 1989). The alkyl chain length of the alkanols is an important parameter in the promotion of permeation enhancement. Augmentation appears to increase as the number of carbon units increases, up to a limiting value. In addition, lower molecular weight alkanols are thought to act as solvents, enhancing the solubility of drugs in the matrix of the stratum corneum (Walker & Smith, 1996).

1.6.2.1.4. Polyols

They replace bound water in the intercellular spaces and enhance penetration of lipophilic drugs (Foldvari, 2000). The activity of propylene glycol is thought to result from solvation of α-keratin within the stratum corneum; the occupation of proteinaceous hydrogen bonding sites reducing drug-tissue binding and thus promoting permeation (Walker & Smith, 1996).

1.6.2.1.5. Fatty acids

Oleic acid has been found to decrease the phase transition temperatures of the skin lipids with a resultant increase in motional freedom or fluidity of these structures (Walker & Smith, 1996). Shorter chain (C10-12) and branched or unsaturated chain fatty acids are more effective than longer chain saturated fatty acids (Foldvari, 2000).

1.6.2.1.6. Esters

Esters such as ethyl acetate are relatively polar, hydrogen bonding compounds that may enhance permeation in a similar manner to the sulphoxides and formamides by penetrating into the stratum corneum and increasing the lipid fluidity by disruption of lipid packing (Walker & Smith, 1996).

1.6.2.1.7. Terpenes

Terpenes are constituents of essential oils, which are the volatile and fragrant substances found in mainly flowers, fruits and the leaves of plants. They are a series of naturally occurring compounds that consists of isoprene (C_5H_8) units that are highly lipophilic and have large partition coefficients between octanol and water (Godwin & Michniak, 1999).

Terpenes are promising, clinically acceptable enhancers because of their low systemic toxicity, high enhancement activity, and low cutaneous irritation at low concentrations (1-5 %) (Asbill & Michniak, 2000). Both the mono- and sesquiterpenes are known to increase percutaneous absorption of compounds by increasing diffusivity of the drug in stratum corneum and/or by disruption of the intercellular lipid barrier (Walker & Smith, 1996). It also increases electrical conductivity (Foldvari, 2000). For example results have shown that the combination of terpenes with propylene glycol can significantly increase the transdermal penetration of the hydrophilic drug caffeine and the polar steroid hydrocortisone (Godwin & Michniak, 1999). It has been well established that terpenes capable of hydrogen bonding are more effective penetration enhancers for

hydrophilic drugs than for lipophilic drugs (Godwin & Michniak, 1999). Zhao & Singh (2000) stated that terpenes increased the permeation of solutes by disrupting the highly ordered structure of intercellular lipids and improving the partitioning of solutes in the SC.

According to Barry (1987) and Goodman *et al.* (1989) quoted by Williams & Barry (1991) accelerants may act by one or more of three main mechanisms: disruption of the highly ordered lipid structure between the corneocytes, so increasing intercellular diffusivity, interaction with intracellular protein to promote permeation through the corneocyte and increased partitioning of the drug or co-enhancer into the tissue. In a study done by Yamane *et al.* (1995) DSC results showed that 1,8-cineole and menthone formulations acted as enhancers on 5-fluorouracil through lipid disruption at skin temperature as manifested by shifts in the lipid transition enthalpies and entropy changes which were related to increase in the propylene glycol content in the vehicles. In the same study (+)-limonene showed no evidence of lipid disruption or increased drug partitioning. Its mild enhancement effect might be due to heterogeneous distribution of the oils in the stratum corneum lipids.

Menthol is a popular enhancer for topical use. The mechanism responsible for the enhancement is attributed to its influence on the stratum corneum (Ho *et al.*, 1998). In another study done by Kunta *et al.* (1997) the enhancing effect of menthol on the model drug, propranolol, was attributed to the functional groups with hydrogen-bonding ability. A co-solvent system with a terpene has also shown to improve permeability, for example Zhao & Singh (2000) proved that menthone/50 % ethanol enhanced the permeability of propranolol by stratum corneum lipid extraction, and by improvement in the partitioning of the drug in the stratum corneum. Some of the characteristics and the chemical structures can be seen in table 1.5 and figure 1.7 respectively.

Table 1.5: The characteristics of various terpenes (Budavari, 1996).

TERPENES	*MOLECULAR FORMULA*	*MOLECULAR WEIGHT*	*APPEAREANCE*	*SOLUBILITY*
(-)Menthol	$C_{10}H_{20}O$	156.2674	Colourless to white crystals	Soluble in alcohol, chloroform only slightly soluble in water
Isomenthol	$C_{10}H_{20}O$	156.2674	Colourless to white crystals	Insoluble in water
β-Myrcene	$C_{10}H_{16}$	136.2364	Colourless oily liquid	Insoluble in water, soluble in alcohol and ether (Weast & Astle, 1980).
1,8-Cineole	$C_{10}H_{18}O$	154.2516	Colourless liquid	Insoluble in water (< 0.1 g/100 ml)
α-Pinene	$C_{10}H_{16}$	136.2364	Colourless to pale yellow liquid	Insoluble in water.
Menthone	$C_{10}H_{18}O$	154.2516	Colourless liquid	Insoluble in water
Limonene	$C_{10}H_{16}$	136.2364	Colourless liquid	Low water solubility.

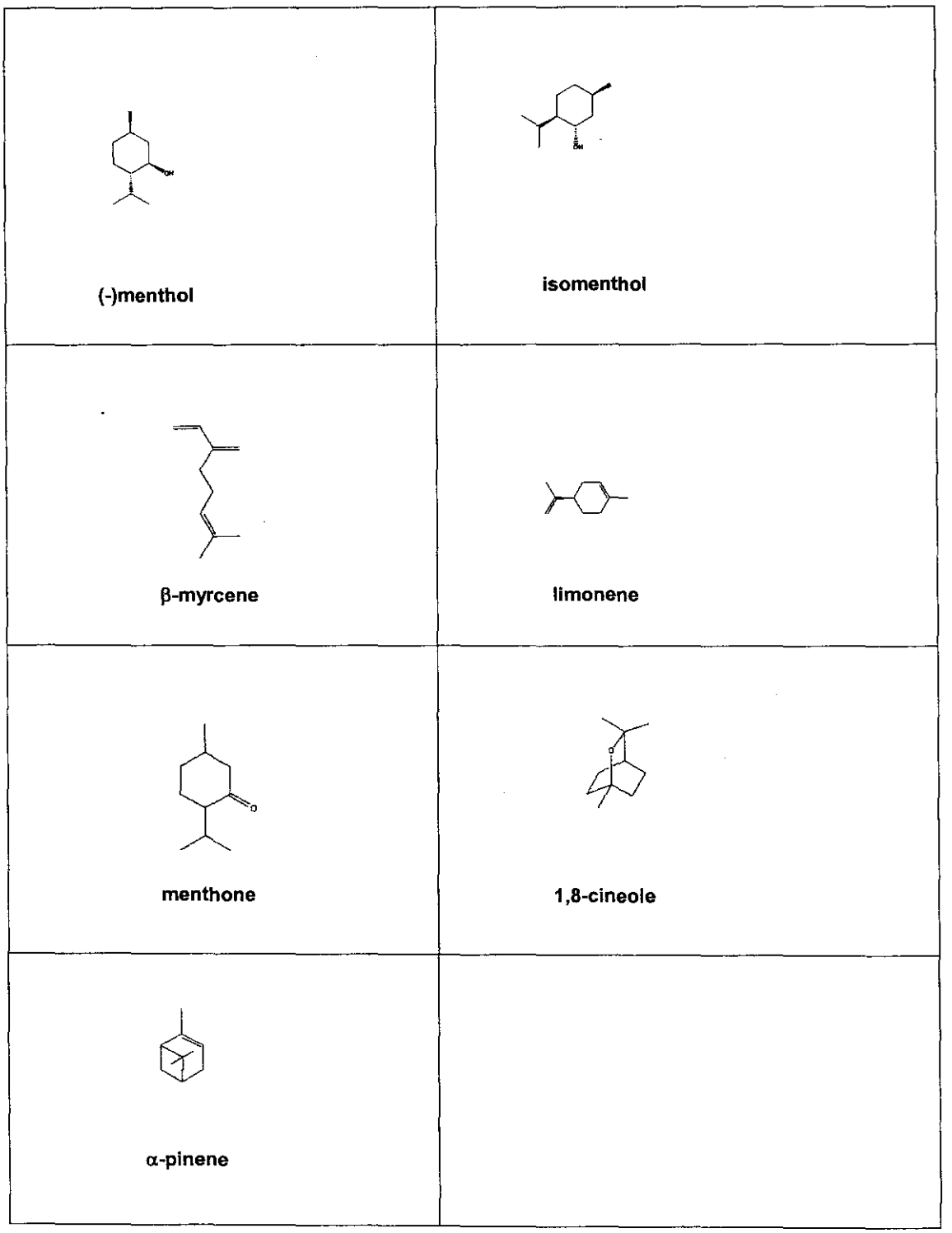

Figure 1.7: The chemical structures of various terpenes (Chemfinder.com, 2003).

1.6.2.1.8. Dimethylsulfoxide (DMSO)

Most current theories on DMSO action consider that it displaces bound protein water, thereby producing a looser structure (Barry, 1987). At higher concentrations it increases lipid fluidity and disrupts lipid packing and interacts with both the keratin and lipid component of the SC (Foldvari, 2000). It has been postulated that DMSO denatures the intercellular structural proteins of the stratum corneum, or promotes lipid fluidity by disruption of the ordered structure of the lipid chains (Walker & Smith, 1996).

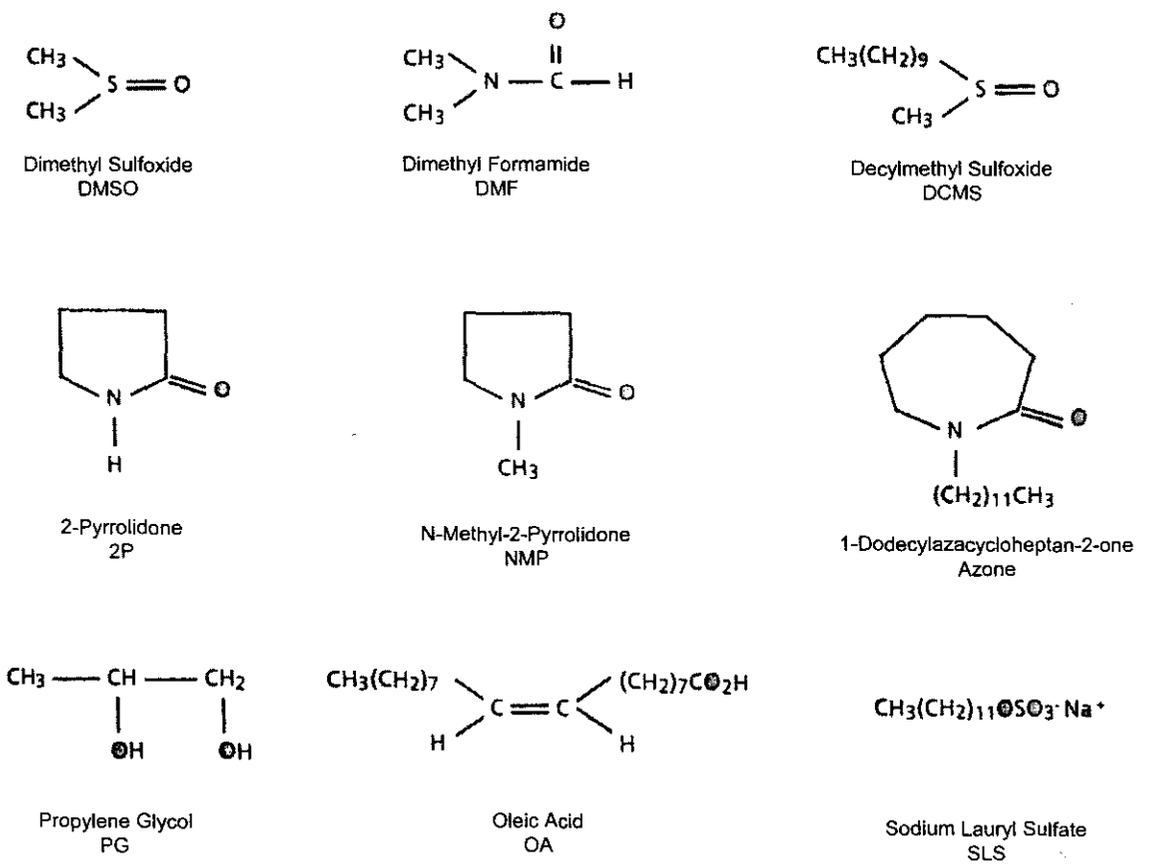

Figure 1.8: The structures of some common penetration enhancers (Barry, 1991).

1.6.2.1.9. Azone

Azone probably increases skin permeability by a different mechanism to that proposed for molecules such as DMSO (Barry, 1987). Azone disrupts lipids in both the head group and tail regions (Foldvari, 2000). It is effective at low concentrations and is thought to act by lipid fluidization as well as ion paring (Naik *et al.*, 2000).

1.6.3. Physical penetration enhancement

Although many different physical approaches to enhancing percutaneous absorption have been attempted, the most notable approaches are iontophoresis, ultrasound and electroporation. None of these enhancement methods is passive in that they require the input of energy to achieve their effects (Finnin & Morgan, 1999).

1.6.3.1. Iontophoresis

Iontophoresis is a process which causes an increased penetration of solute molecules into tissues by the use of an applied current through the tissue and which has therefore been employed for the transdermal delivery of drugs (Burnette, 1989). Charged species are repelled into and through the skin as a result of an electrical potential across the membrane; the efficiency of this process is dependent on the polarity, valency and ionic mobility of the permeant as well as on the composition of the delivery formulation and the current profile (Naik *et al.*, 2000).

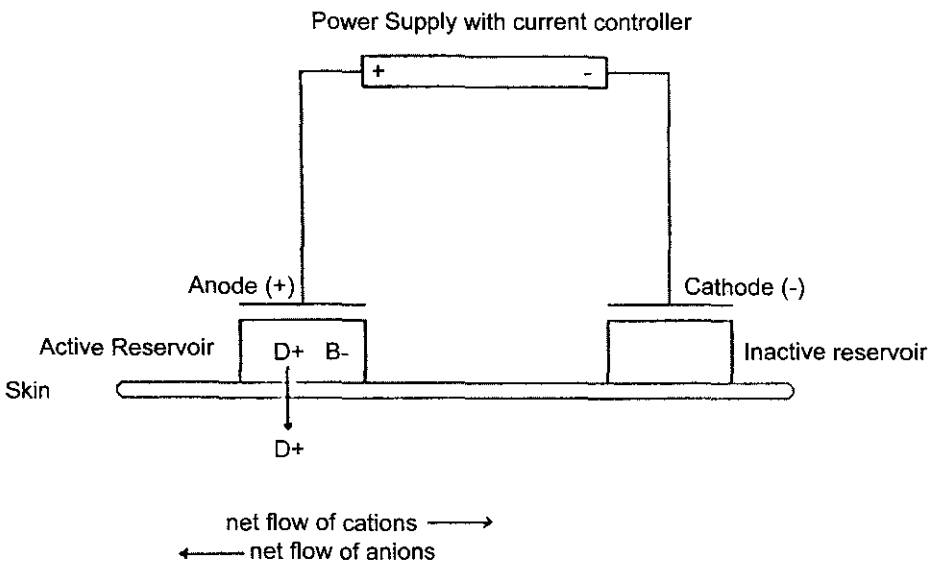

Figure 1.9: Schematic illustration of the basic features of an iontophoretic drug delivery system (Wong, 1994).

A basic design for an iontophoretic transdermal drug delivery device is shown in figure 1.9, which will be used to explain iontophoretic drug delivery. An iontophoretic drug delivery system comprises a power supply; an anode connected to the active or drug-

containing reservoir, which is in contact with the skin; and a cathode connected to the inactive reservoir, which contains buffers, ions, or other materials that can conduct electricity to complete the circuit. The inactive reservoir must also be in contact with the skin. The skin acts as an impedence barrier to drug transport (Wong, 1994).

Factors influencing iontophoretic drug delivery

1. The charge of the drug ion.
2. The applied current strength to be used.
3. The conductivity of the drug candidate.
4. The pH of the vehicle used in the drug reservoirs.
5. The competition of extraneous ions in the drug reservoir.
6. The drug concentration in the reservoirs.
7. The pK_a of the drug candidate.
8. The aqueous solubility of the drug candidate.
9. The molecular weight of the drug candidate.
10. The lipophilicity of the drug candidate.
11. The potential for skin irritation and sensitization.
12. The transport number of the drug candidate.
13. The effect of temperature.
14. Electrochemical stability of the drug candidate.
15. Electro-osmotic effects (Wong, 1994).

Advantages

1. Increased capability of delivering larger amounts of therapeutic agents compared to passive delivery systems.
2. Ability to deliver significantly higher amounts of relatively large molecular weight compounds.

3. Better control of the delivery profile, including nonzero-order profiles (Sarpotdar, 1993).

Limitations

1. Complexity of the delivery system.

2. Chemical stability of the therapeutic agent. In an iontophoretic patch, one also has to consider the effects of applied current of the compound.

3. Relatively unknown toxicology of prolonged exposure to current.

4. Cost. It is obvious that the costs of development and manufacture for the iontophoretic patch would be significantly higher than the passive transdermal patch (Sarpotdar, 1993).

1.6.3.2. Electroporation

Electroporation, by contrast, which uses high-voltage short duration pulses is thought to create localized regions of membrane permeabilization by producing aqueous pathways lipid membrane bilayers (Naik *et al.*, 2000).

1.6.3.3. Ultrasound (sonophoresis)

The use of ultrasound, defined as sound of frequency greater than 20 kHz, to compromise the skin's barrier function has also received considerable attention. Mechanistically, sonophoresis is considered to enhance drug delivery through a combination of thermal, chemical and mechanical alterations within the skin tissue (Naik *et al.*, 2000).

1.6.4. Supersaturation

In order to improve absorption it is possible to use supersaturated solutions which have chemical potentials greater than that of a saturated solution (Hadgraft, 1999). However, topical vehicles relying on supersaturation have major limitation of formulations instability, both prior to and during application to the skin (Finnin & Morgan, 1999). Stabilisation of supersaturated topical preparations can be achieved over limited periods using anti-nucleant polymers (Hadgraft, 1999) and anticrystal-growth agents (Finnin & Morgan, 1999). There appears to be an almost linear increase in drug flux with degree of supersaturation (Hadgraft, 1999).

1.6.5. Occlusion

A predominant effect of occlusion is to increase hydration of the stratum corneum, thereby swelling the corneocytes, promoting the uptake of water into intercellular lipid domains. The magnitude of increased stratum corneum hydration is related to the degree of occlusion exerted and dependent upon the physicochemical nature of the dressing (Bucks & Maibach, 1999). Faugermann *et al.* as quoted by Bucks & Maibach (1999) showed that occlusion a) increases the transepidermal flux of chloride and carbon dioxide b) increases microbial counts on skin c) increases the surface pH of skin from a preoccluded value of 5.6 to 6.7. Occlusion does not necessarily increase percutaneous absorption. Penetration of hydrophilic compounds, in particular, may not be enhanced by occlusion (Bucks and Maibach, 1999).

1.7. SUMMARY

The skin is one of the most complex organs of the human body. It plays an essential role in protection, thermoregulation, etc. Due to the skin's protective function it is mostly impermeable to even small molecules especially hydrophilic one's. This is mainly due to the lipophilicity of the stratum corneum, which is the rate–limiting barrier. In order to overcome the stratum corneum's barrier function, physical and chemical penetration enhancers have been developed. These penetration enhancers disrupt the lipids in the stratum corneum making it more permeable to drugs.

The above as well as physicochemical properties of the drug, biological factors and the diffusion model influences the topical delivery of drugs for systemic use. As transdermal drug delivery has many advantages above other delivery routes such as intraveneous it is very important that the barrier function of the skin be overcome. Thus, in this study, ways to overcome this barrier function by using terpenes as penetration enhancers will be investigated.

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HPLC VALIDATION OF METHOTREXATE AND EXPERIMENTAL PROCEDURE

CHAPTER 2

2.1. INTRODUCTION

Transdermal delivery of various drugs has many advantages above any other route of delivery. Due to methotrexate's tendency in causing numerous side effects such as nausea and vomiting after systemic administration it would be preferable to deliver it topically.

The object of the HPLC validation was to ensure that the analytical method was sensitive and reliable enough to determine the concentration methotrexate that permeated the skin. The percentage terpenes that permeated were determined with the use of Gas Chromatography but as it was only a semi-quantitative analysis no validation was done.

2.2. MATERIALS

Methotrexate was supplied by Fluka (Sigma-Aldrich, Steinheim). HPLC-grade methanol (BDH Laboratory supplies, England), di-Potassium hydrogen orthophosphate anhydrous (Saarchem, South-Africa) and double distilled deionized water [prepared by a milli-Q academic water purification system (Millipore, Milford, USA)] were used for the mobile phase. A Luna 5u C18 (2) 250 x 4.60 mm column and precolumn was supplied by Phenomenex (USA). The terpenes menthone (Fluka), 1,8-cineole (Sigma), limonene 97 % (Aldrich), β-myrcene 90 % (Sigma), (-)-menthol (Sigma), α-pinene 99+ % (Aldrich) and isomenthol 99 % (Aldrich) was used as received. Absolute ethanol that was used in the standard solutions of the terpenes was supplied by Saarchem, South Africa.

2.3. ANALYSIS OF METHOTREXATE IN METHANOL

2.3.1. The HPLC system

The Agilent 1100 Series HPLC (Hewlett Packard) was used in the validation of methotrexate:

- 1100 Series pumping systems
- 1100 Series autosamplers
- 1100 Series visible detectors
- 1100 Series fluorescence detectors
- 1100 Series LC/MSD
- 1100 Series GPC/SEC system
- 220 Microplate sampler
- Chemstation
- Chemstation Plus

Mobile phase: 6.96g di-Potassium Hydrogen orthophosphate anhydrous was weighed and dissolved in a 1000 ml of HPLC-water. The pH of the buffer solution was then adjusted to pH 7 with phosphoric acid. The mobile phase consisted of buffer solution:methanol (70:30). It was filtered through a 0.45 μm HV filter (Millipore, Ireland) and degassed.

Flow rate: The operating flow rate was 1.0 ml/min.

Wavelength: Methotrexate was monitored at 302 nm UV.

Stop time: Methotrexate had a retention time of 4.5 min so the stop time was set at 5 min.

2.3.2. Column maintenance

After each analysis HPLC-water was passed through the column for about 20 min at a flow rate of 1.0 ml/min. The column was then rinsed with HPLC-water:methanol (50:50) for 20 min at a flow rate of 1.0 ml/min in which it was stored.

2.3.3. Standard solutions

The standard solutions were made up as follows:

1. 10 mg Methotrexate was weighed and made up to a 100 ml in a volumetric flask with methanol. This solution had a concentration of 100 μg/ml.

2. 10 ml of this solution was transferred to a 100 ml volumetric flask and filled to volume with HPLC water. This solution had a concentration of 10 µg/ml.

3. Then 1 ml of the above solution was transferred to a 100 ml volumetric flask and filled to volume with HPLC water, the concentration of this solution was 0.1 µg/ml.

4. The 10 µg/ml and 0.1 µg/ml solutions was used to prepare standard solutions of 0.02, 0.04, 0.06, 0.08 and 0.1 µg/ml.

2.3.4. Experimental procedures

2.3.4.1. Linearity

Linearity is the capability of the analytical method to render results that are directly proportional to the concentration of the analyte before or after mathematical transformation. The linearity of methotrexate was determined by plotting the peak area ratio to concentration. The standard concentrations were made up as described in § 2.3.3. The regression value for methotrexate was 0.999 as can be seen in figure 2.1.

Table 2.1: The peak area ration vs. concentration of methotrexate.

Concentration (ug/ml)	Area 1	Area 2	Average	Std Dev	% RSD
0.02	6.78411	7.39235	7.08823	0.43009063	6.067673151
0.04	13.02011	13.1438	13.081955	0.08746204	0.668570086
0.06	19.91466	20.80536	20.36001	0.62982001	3.093416997
0.08	26.18039	26.79937	26.48988	0.43768496	1.652272322
0.1	32.79353	33.03428	32.913905	0.17023596	0.517215923

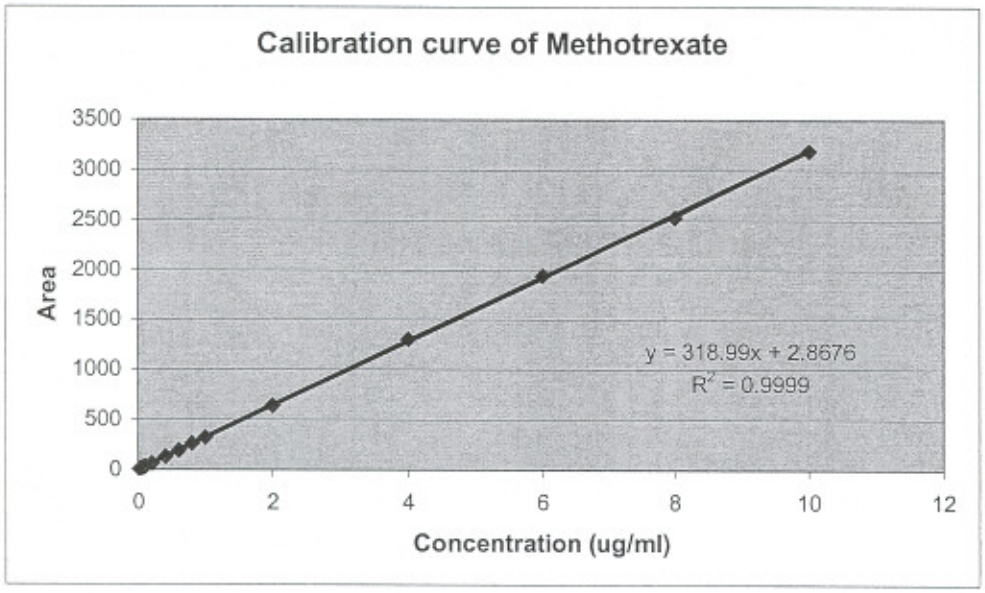

Figure 2.1: Linear regression curve of methotrexate.

Table 2.2: The regression value, y-intercept and slope of the regression curve of methotrexate.

R-squared (R^2)	0.999
Y-intercept	2.8976
Slope	318.99

2.3.4.2. Precision

The same standard solutions as described in § 2.3.3 were used in the determination of intra-day and inter-day precision.

2.3.4.2.1. Intra-day precision

The intra-day precision was determined by performing HPLC-analysis on five different concentrations of methotrexate on the same day. The results met the requirements of the USP as can be seen in table 2.3.

Table 2.3: The mean, standard deviation and % RDS of methotrexate for intra-day precision.

Concentration (ug/ml)		0.02	0.04	0.06	0.08	0.1
Area	Sample 1	7.52747	15.04525	22.44583	29.92644	37.34388
	Sample 2	7.53958	15.04498	22.41628	29.97473	37.47934
	Sample 3	7.55112	15.09885	22.62353	30.13892	37.66627
Mean		7.53939	15.0630267	22.4952133	30.01336333	37.49649667
Std Dev		0.011826145	0.03102421	0.11210342	0.111383744	0.161878323
% RDS		0.156858111	0.20596266	0.49834342	0.371113836	0.43171586

2.3.4.2.2. Inter-day precision

The inter-day precision was determined by performing HPLC-analysis on five different concentrations of methotrexate on three consecutive days. The inter-day precision of methotrexate was well within acceptable limits as can be seen in table 2.4.

Table 2.4: The mean, standard deviation and % RSD of methotrexate for inter-day precision.

Concentration (ug/ml)		0.02	0.04	0.06	0.08	0.1
Area	Day 1	7.53939	15.0630267	22.4952133	30.01336333	37.49649667
	Day 2	7.462573333	14.88993	22.33738	29.82191667	37.23981667
	Day 3	7.466366667	14.89305	22.2286833	29.6371	37.20512
Mean		7.489443333	14.9486689	22.3537589	29.82412667	37.31381111
Std Dev		0.043296645	0.09904903	0.13401775	0.1881414	0.159158645
% RDS		0.578102313	0.6625943	0.59953115	0.630836243	0.426540844

2.3.4.3. Limit of detection

The limit of detection is the lowest concentration of the analyte that can be detected but not necessarily quantified. The limit of detection of methotrexate was 0.01 μg/ml.

2.3.4.4. Limit of quantification

The limit of quantification is the lowest concentration of the analyte that can be determined with acceptable precision (% RSD < 15). The limit of quantification for methotrexate was 0.02 μg/ml.

2.3.4.5. Selectivity

Selectivity is the ability of an analytical method to determine the analyte selectively in the presence of other compounds that may interfere. The receptor phase (PBS-Buffer) was separately analyzed by the HPLC and were found not to have any interfering peaks.

2.3.4.6. System repeatability

A sample (10 μg) was injected six times to determine the repeatability of the peak area and the retention time. The % RSD for the peak area and the retention time were 1.168 and 0.094 respectively. The values were well within the accepted ranges.

2.4. GAS CHROMATOGRAPHIC PROCEDURES

2.4.1. The GC system

An Agilent 6890 Series Headspace GC (Hewlett Packard) was used in the analysis of the terpenes.

2.4.2. Chromatographic conditions

Column: WCOT fused silica capillary column (Chrompak)

Stationary phase: Cp-wax-52 CB

Column length: 30 m

Inside diameter: 0.25 mm

Outside diameter: 0.39 mm

Film thickness: 0.25 mm

Oven Temperature: 70°C for 5 min at 40°C/min up to a temperature of 180°C maintaining it for 6 min.

Front injector temperature: 250°C.

Front detector temperature: 260°C.

Retention times: *menthol:* 11.9 min

isomenthol: 12.1 min

β-myrcene: 6.3 min

1,8-cineole: 7.4 min

menthone: 10.4

limonene: 7.0 min

α-pinene: 4.5 min

Ethanol had a retention time of 3.6 min so it did not interfere in any of the above peaks.

2.5. TRANSDERMAL DIFFUSION STUDIES

Five different sets of experiments were done:

a) Control 1: methotrexate permeation in the absence of the terpenes;

b) Control 2: methotrexate permeation in the presence of ethanol but in the absence of the terpenes;

c) Methotrexate permeation in the presence of the terpenes;

d) Determination of the percentage terpenes that permeated the skin in the absence of methotrexate;

e) Determination of the percentage terpenes that permeated the skin in the presence of methotrexate.

2.5.1. Materials

Methotrexate was supplied by Fluka (Sigma-Aldrich, Steinheim). Double distilled deionized water was prepared by a Milli-Q Academic purification System (Millipore, Milford, USA). Saarchem, South Africa supplied propylene glycol, Sodium chloride, di-Sodium Hydrogen orthophosphate dihydrate and Sodium dihydrogen orthophosphate dihydrate. Menthone (Fluka), 1,8-cineole (Sigma), limonene (97 %) (Aldrich), β-myrcene (90 %) (Sigma), (-)-menthol (Sigma), α-pinene (99+%) (Aldrich) and isomenthol (99%) (Aldrich) was used as received. Saarchem, South Africa supplied the absolute ethanol that was used in the receptor phase.

2.5.2. Preparation of the PBS-buffer

Sodium chloride (4.4 g), di-Sodium Hydrogen orthophosphate dihydrate (9.2 g) and Sodium Dihydrogen orthophosphate dihydrate was weighed and made up to a 1000 ml in a volumetric flask with HPLC-water. The pH of the PBS-buffer was 7.4.

2.5.3. Methotrexate solubility

The excess of methotrexate was equilibrated in a propylene-water mixture (Vaidyanathan *et al.*, 1985) at a constant temperature of 37 °C and a pH of 4-5, with vigorous stirring using magnetic stirring bars. The temperature was held constant by means of a water bath (Grant Instruments, UK). Equilibrium was reached within 24 hours. At all times, an excess of the solute was present in the slurry. These samples were filtered through Whatman® Filter paper and assayed by HPLC. All experiments were carried out in triplicate.

2.5.4. Skin preparation

Human abdominal skin was obtained from "tummy tucks" that were done on white female patients. The excess fatty tissue was removed and the full-thickness skin was placed in water at 60°C for 1 min (Bronaugh & Collier, 1991). The epidermal layer was now gently removed by teasing it away with the help of forceps and was placed on Whatman® filter paper and left to dry. When dry it was wrapped in aluminum foil and stored in the freezer until further use.

2.5.5. Permeation studies

The object of the permeation studies was to determine if the terpenes had an effect on the permeation of the methotrexate and to see if the terpenes permeated the skin and what effect the methotrexate had on this permeation.

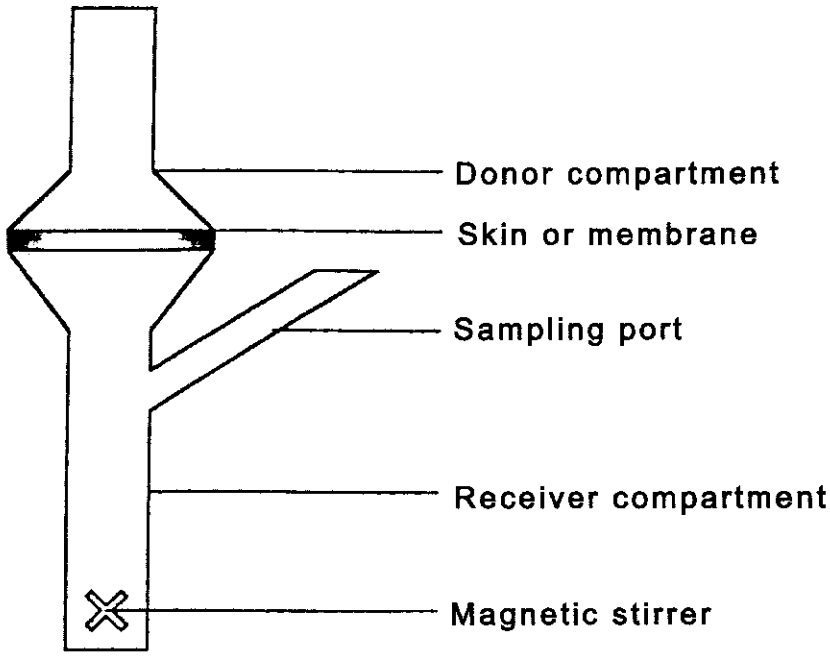

Figure 2.2: Vertical Franz diffusion cell.

Vertical Franz diffusion cells (figure 2.2) with a capacity of 2 ml and a diffusion area of 1.075 cm^2 were used in this study. The skin was clamped between the donor and receptor compartments also known as the "wet-wet" setup (Cleary, 1993). For the HPLC analysis PBS-buffer (pH 4.7) was used as a receptor phase and for the GC analysis the receptor phase was ethanol:water (50:50). The experimental procedure for all the experiments was the same. The receptor compartment was filled with the receptor phase, the Franz cell was then placed in a water bath (Grant Instruments, UK) at a temperature of ± 37°C (Cleary, 1993) on a magnetic stirrer and left for an hour allowing equilibration. 25 µl of a 5 % solution of the penetration enhancers in absolute ethanol was now placed on the skin surface in the donor compartment. This was left for half an hour to allow the evaporation of the ethanol. 1 ml of a saturated solution of methotrexate in water:propylene glycol (50:50) with a pH 4-5 (Vaidyanathan *et al.*, 1985) were placed in the donor compartment and immediately covered with Parafilm to avoid any evaporation of the volatile substances (Almirall *et al.*, 1996). The samples for the methotrexate experiments were taken by withdrawing the receptor phase and replacing it with fresh receptor phase every two hours for a period of twelve hours. For the terpene experiments the receptor phase was withdrawn and replaced with fresh receptor phase on the following times: 2,4,6,12 and 24 hours. The samples were analysed as discussed previously.

2.6. CONCLUSION

The HPLC method has been found to be reliable and sensitive enough for the determination of the concentration methotrexate. The experimental method for the permeation studies has been developed and will be used in the diffusion studies of methotrexate. In the following chapter the data obtained from the experiments will be analysed and discussed.

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CHAPTER 3

RESULTS AND DISCUSSION

3.1. INTRODUCTION

Kaposi's sarcoma is a HIV/AIDS related skin disease, which can be treated with methotrexate, but due to certain limiting factors there is currently no topical dosage forms commercially available (Alvarez-Figueroa *et al.*, 2001). Terpenes are natural substances that are found in aroma therapeutic oils and recent studies have shown that they increase the permeation of certain drugs.

Therefore in vitro diffusion cell studies were done to determine the enhancing effect of the terpenes on methotrexate. Terpenes are not only used as penetration enhancers but even more often are present in drugs and cosmetics as components of essential oils added for some other reason: inhalation, as rubefacient or analgesics (Cal *et al.*, 2001). Thus, the permeation of the terpenes was also determined using the in vitro Franz diffusion cell method.

3.2. MATERIALS

Methotrexate was supplied by Fluka (Sigma-Aldrich, Steinheim). Double distilled deionized water was prepared by a Milli-Q Academic purification System (Millipore, Milford, USA). Saarchem, South Africa supplied propylene glycol, Sodium chloride, di-Sodium Hydrogen orthophosphate dihydrate and Sodium dihydrogen orthophosphate dihydrate. Menthone (Fluka), 1,8-cineole (Sigma), limonene (97 %) (Aldrich), β-myrcene (90 %) (Sigma), (-)-menthol (Sigma), α-pinene (99+%) (Aldrich) and isomenthol (99%) (Aldrich) was used as received. Saarchem, South Africa supplied the absolute ethanol that was used in the receptor phase.

3.3. METHODS

In vitro permeation studies on methotrexate and the terpenes were done using the Franz diffusion cell method. The Franz cells had a capacity of 2 ml and a diffusion area of

1.075 cm^2. Human abdominal skin obtained from tummy tucks was used in the in vitro studies on skin permeation. The methotrexate and terpene diffusion experiments were done using the methods as discussed in chapter 2. The amounts methotrexate and terpenes that permeated were determined by assaying the receptor phase with HPLC and CG methods respectively.

3.4. DATA ANALYSIS

3.4.1. Analysis of methotrexate samples

The amount of drug that permeated through the skin at a given time was calculated from the following equation (3.1):

$$Q(t) = AKhC\left[D\frac{t}{h^2} - \frac{1}{6} - \frac{2}{\pi^2}\sum_{n=1}^{\infty}\frac{(-1)^2}{n^2}\exp\left(\frac{-Dn^2\pi^2 t}{h^2}\right)\right] \qquad \text{(Equation 3.1)}$$

where:

- Q(t) is the quantity passed through the membrane at a given time (t);
- A is the actual surface diffusion area;
- K is the partition coefficient between the membrane and donor vehicle;
- h is the membrane thickness;
- D is the diffusion coefficient of the permeant in the membrane and
- C is the concentration in the donor solution (Diez-Sales *et al.*, 1991).

As *t* approaches infinity the exponential terms become negligible and the linear steady-state expression is given by the following equation (3.2):

$$Q(t) = AKhC\left(D\frac{t}{h^2} - \frac{1}{6}\right) \qquad \text{(Equation 3.2)}$$

The cumulative concentrations (μg/ml) of at least 6 cells from the different experiments were plotted as a function of time (h). The products Kh and D/h^2 can be calculated by curve-fitting the permeation data on Easyplot for Windows. The curve-fitting provided a and b values, where:

$$a = Kh$$

$$b = \frac{D}{h^2}$$

The product of ab is equal to the permeability coefficient (k_p).

The flux ($\mu g/cm^2/h$) was determined from the slope of the linear portion of the graph cumulative concentration ($\mu g/ml$) against time (h).

The flux ($\mu g/cm^2/h$) can also be expressed as:

$$J_S = \frac{D_m \Delta C_m}{L} \qquad \text{(Equation 3.3)}$$

where:

- J_s is the steady-state flux;
- D_m is the membrane diffusivity
- ΔC_m is the concentration difference across the membrane;
- L is the length of the membrane.

This equation can be simplified to give the following:

$$J_S = k_p C_0 \qquad \text{(Equation 3.4)}$$

where:

- J_s is the steady-state flux ($\mu g/cm^2/h$);
- k_p is the permeability coefficient;
- C_0 is the donor compartment concentration (Bronaugh & Collier, 1991).

The enhancing effect of the terpenes on methotrexate permeation was calculated using the enhancement ratios of flux, that is the ratio of flux with the enhancer and that without the enhancer (equation 3.5) (El-Katton *et al.*, 2000).

$$ER = \frac{Methotrexate\ flux\ with\ enhancer}{Methotrexate\ flux\ without\ enhancer}$$ (Equation 3.5)

3.4.2. Analysis of terpene samples

The analysis of the amount of terpenes penetrated was only a semi-quantitative analysis thus the flux was not determined. Standard solutions of the terpenes were made and analysed using the GC. A graph where area as a function of concentration was plotted was used and a straight-line equation was determined. The average cumulative concentration was calculated from this equation. The average cumulative concentration of the terpenes permeated at 6h, 12h and 24h in the presence of methotrexate were compared to their permeation in the absence of methotrexate. The average percentage terpenes that permeated at 24h were also determined.

3.5. STATISTICAL ANALYSIS

The ANOVA (analysis of variance) procedure was applied to the mean flux ($\mu g/cm^2/h$) values using the Dunnett's t-test. The values were significant at $p < 0.05$.

The statistical analysis of the terpenes was also done using the ANOVA procedure. Differences in cumulative concentration with $p < 0.05$ were seen as statistically significant.

3.6. RESULTS

3.6.1. Methotrexate permeation through human skin

In this study the effect of various terpenes on the permeation of methotrexate was determined. The flux ($\mu g/cm^2/h$), k_p (cm/h) and the enhancement ratio (ER) of methotrexate before and after treatment with 5 % terpenes were calculated. As can be seen from the permeability parameters in table 3.1 methotrexate's permeation was promoted by all the terpenes. The flux ($0.011\pm0.003\ \mu g/cm^2/h$) of the control experiment after ethanol pretreatment was only slightly higher than the flux ($0.010\pm0.002\ \mu g/cm^2/h$) where no ethanol pretreatment took place (figure 3.1). β-Myrcene, α-pinene and isomenthol had a better enhancing effect when compared to the other enhancers. They produced a 4-fold increase in the drug permeation rate, while the others produced between a 2 and 3-fold increase. The enhancement ratios of methotrexate in the presence of the different terpenes are shown in figure 3.3.

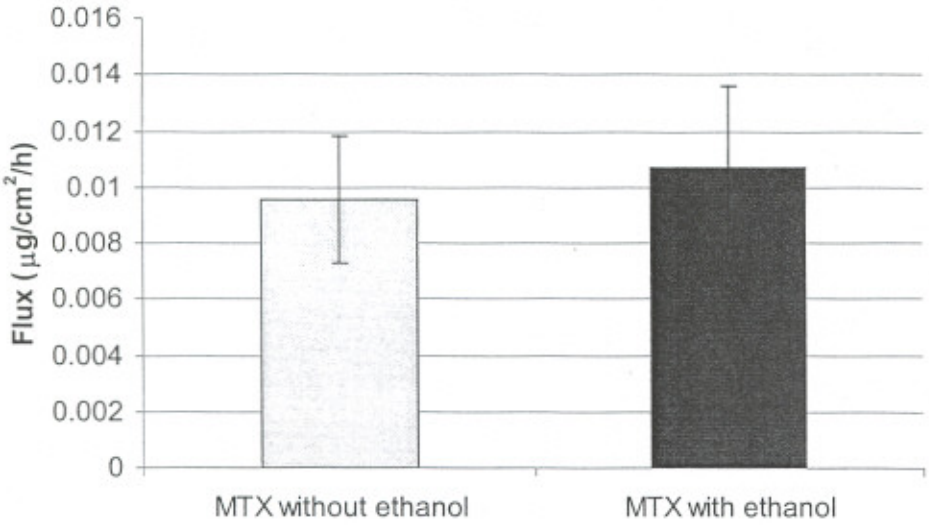

Figure 3.1: The effect of ethanol on methotrexate permeation. Means ± S.D., *n* = 6.

Table 3.1: Permeability parameters of methotrexate in the absence and presence of terpenes.

Methotrexate	Flux ($\mu g/cm^2/h$)	$k_p . 10^{-4}$	ER_{flux}	Lag Times (h)
Without ethanol	0.010±0.002	1.30	-----	0.20
With ethanol	0.011±0.003	1.50	-----	0.002
Menthone	0.027±0.012	3.46	2.77	0.64
1,8-Cineole	0.028±0.010	3.85	2.92	0.65
Limonene	0.030±0.002	4.20	3.17	0.002
α-Pinene	0.045±0.019	6.20	4.71	0.003
Menthol	0.038±0.004	4.96	3.99	0.92
Isomenthol	0.041±0.005	5.64	4.28	0.003
β-Myrcene	0.045±0.006	5.93	4.66	0.38

The statistical analysis of the flux values showed that all the terpenes had a significant enhancing effect on the methotrexate permeation ($p \leq 0.05$).

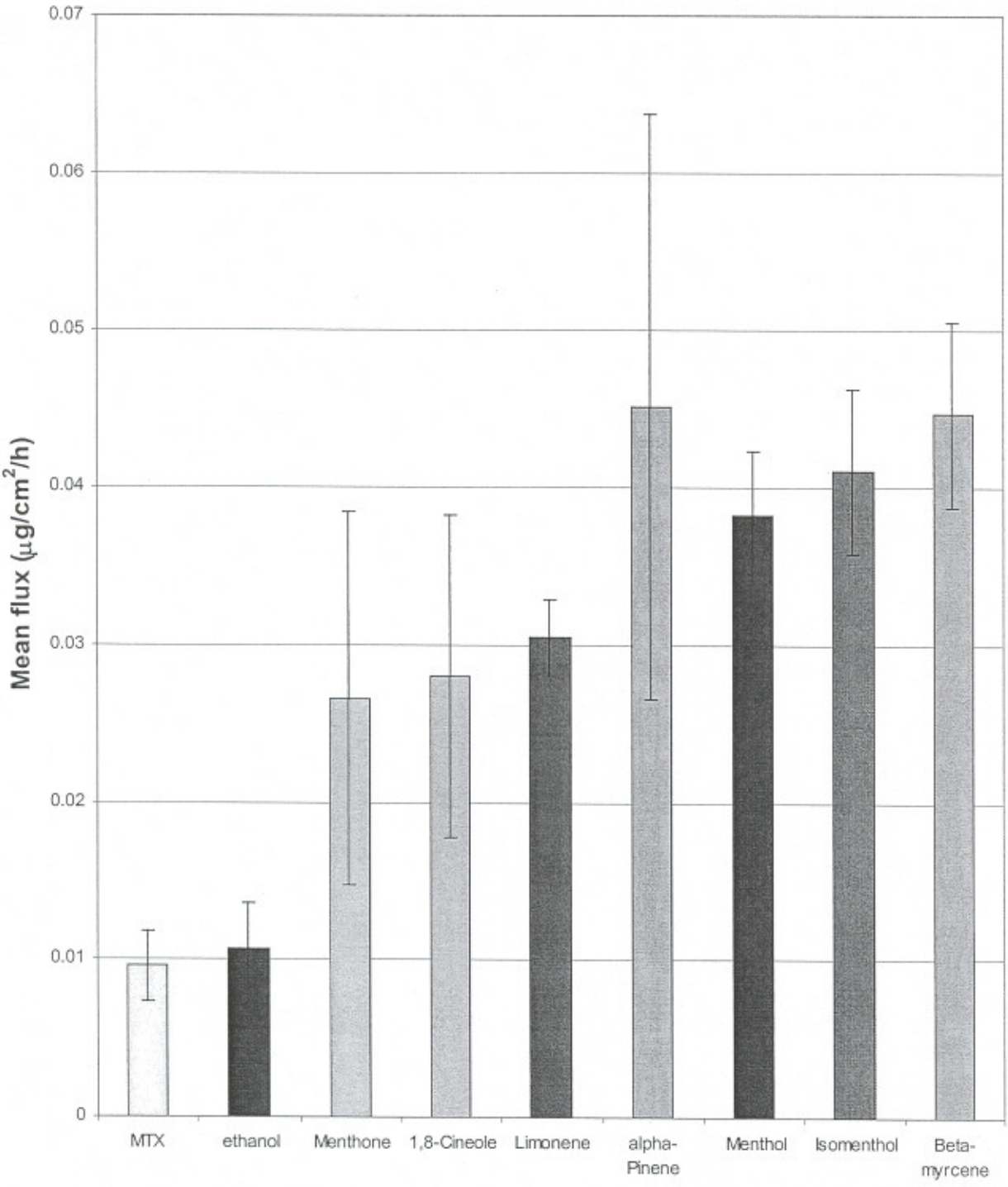

Fig 3.2: The average flux (µg/cm^2/h) of methotrexate before and after treatment with the terpenes. Means ± S.D., n = 6.

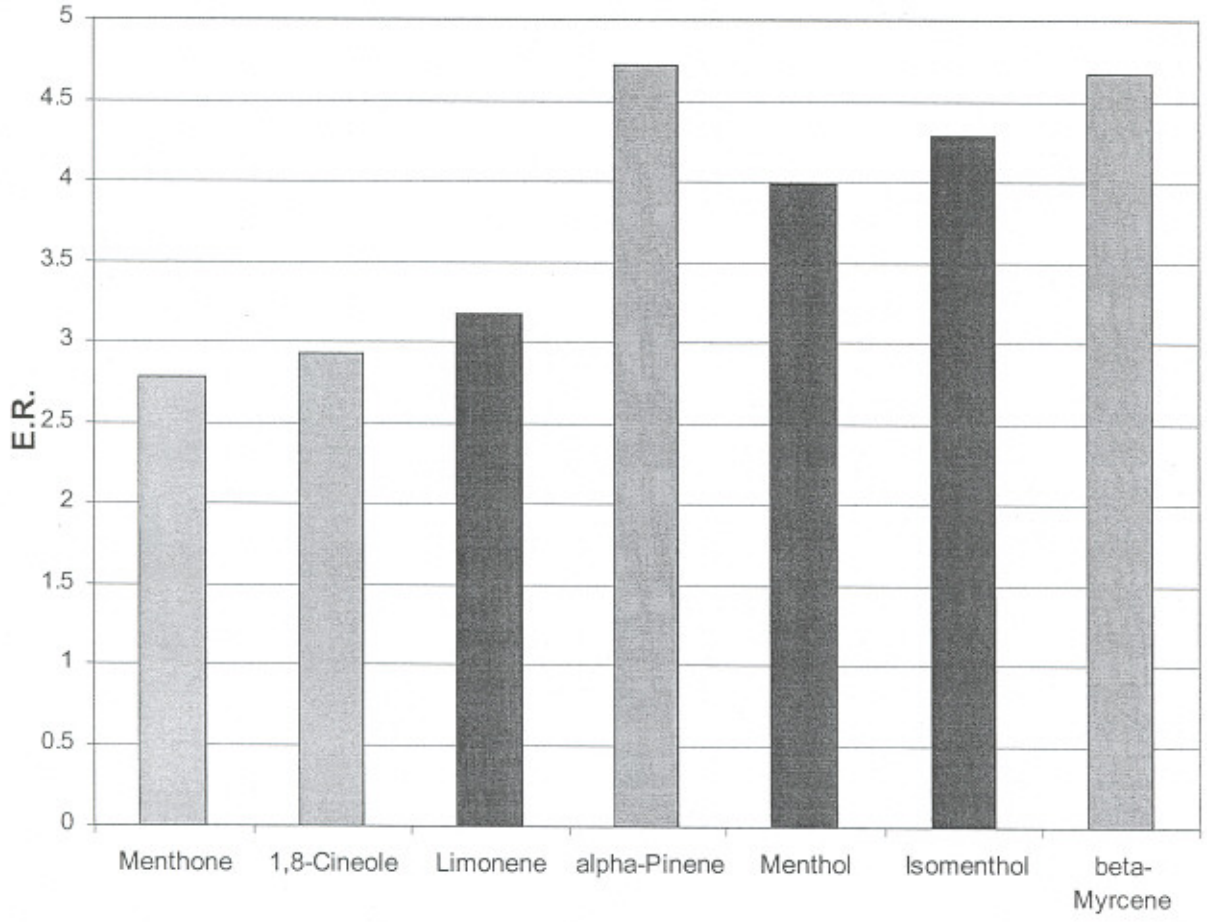

Figure 3.3: The enhancement ratios of flux after pre-treatment with the terpenes in the absence of ethanol.

3.6.2. Terpene permeation through human skin

The cumulative amount of terpenes that permeated the skin at 6h, 12h and 24h was determined. Due to the fact that this permeation was only a semi-quantitative analysis no permeability parameters were determined. The terpene permeation in the presence of methotrexate and in its absence was investigated to determine whether the methotrexate had an effect on their penetration through human skin. The mean cumulative concentrations of the various terpenes that permeated the skin at 6h, 12h and 24h can be seen in figure 3.4.

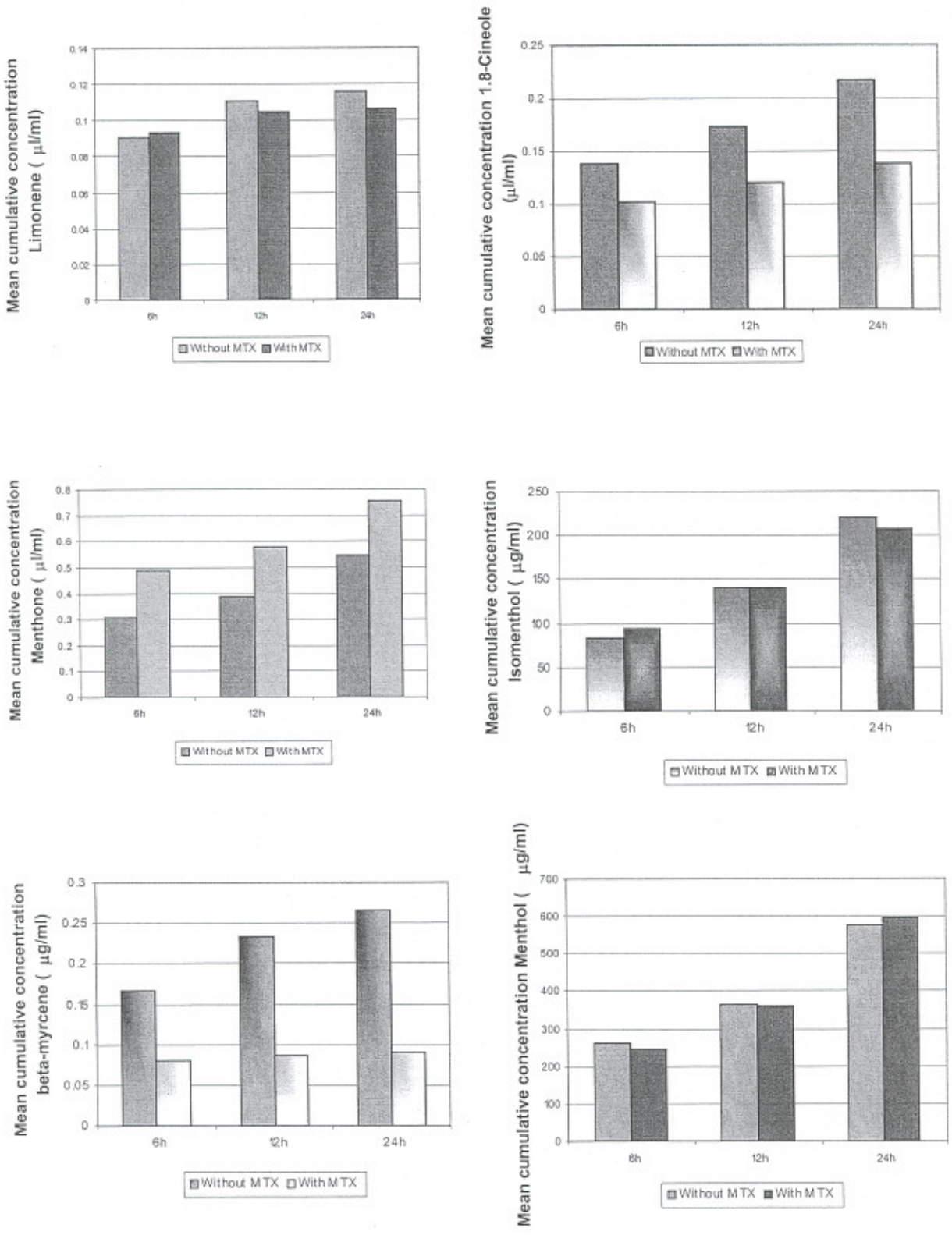

Figure 3.4: The mean cumulative concentrations of the terpenes at 6h, 12h and 24h.

The mean percentage terpenes that permeated the skin was also calculated, with menthone being the highest. The effect of the methotrexate on the terpene penetration can clearly be seen from figure 3.5. Menthone and menthol each had a higher permeation in the presence of methotrexate than in its absence. The other terpene's permeation was higher in the absence of methotrexate. Statistical analysis was done on the mean cumulative concentrations that permeated the skin between 6 and 24h and 12 and 24h. This showed that only the differences between the control and experiments of β-myrcene, 1,8-cineole and limonene were statistically significant.

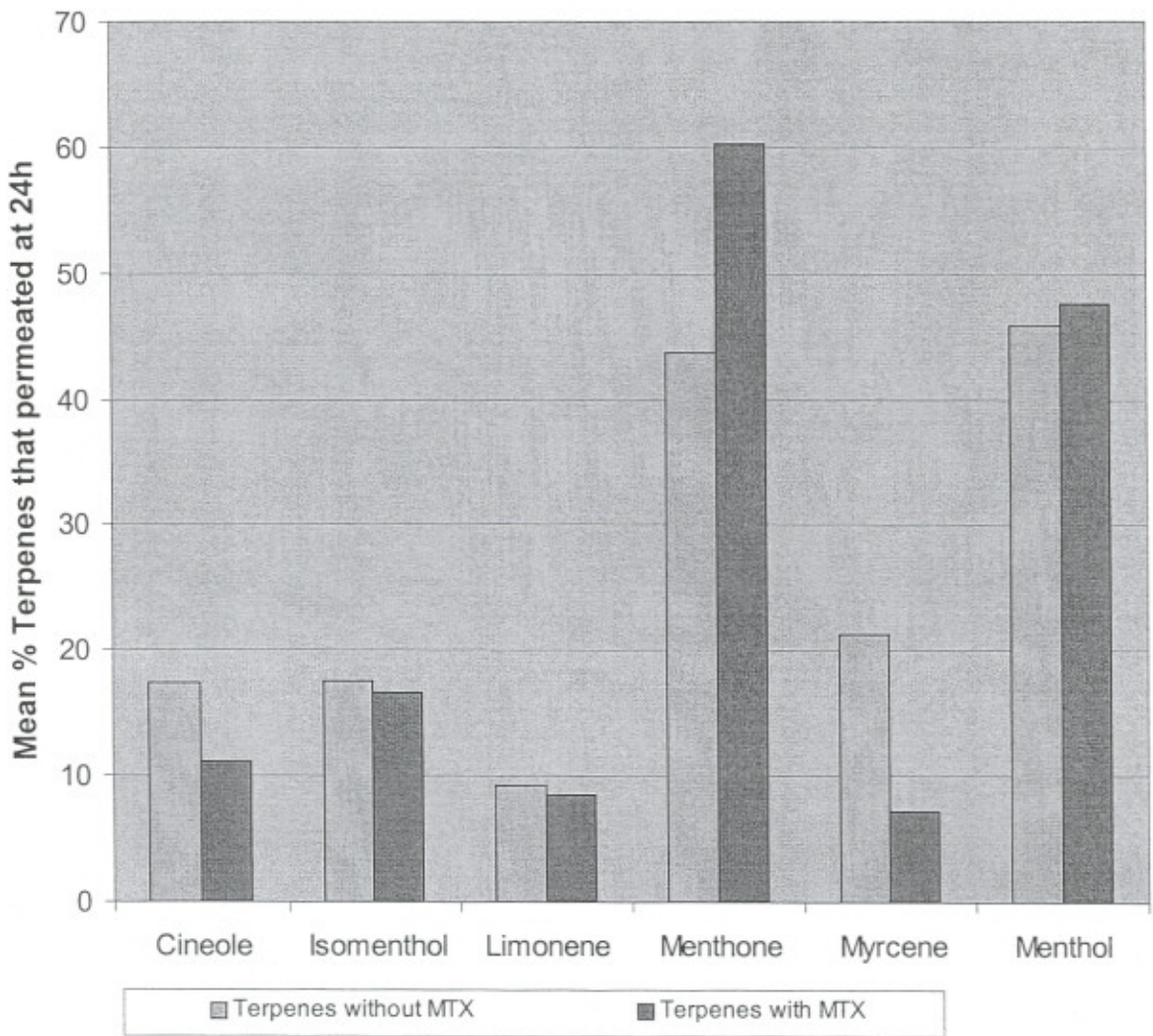

Figure 3.5: The mean % terpenes that permeated the skin at 24h in the presence and absence of methotrexate.

3.7. DISCUSSION

3.7.1. The effect of the terpenes on methotrexate permeation

Methotrexate is a hydrophilic compound with a molecular weight of 454.56 g/mol and a log P of -1.85 (Alvarez-Figueroa *et al.*, 2001). Thus the permeation of methotrexate through human skin will be limited. Looking at the flux results for methotrexate one can clearly see that the flux was higher in the presence of the terpenes than in their absence. In a study done by Brain *et al.* (1991) the effect of azone on the percutaneous delivery of methotrexate was determined. They found the flux to be 0.26 ng/cm^2/h, which is smaller than the flux values found in this study. The higher flux values could be explained by the use of propylene glycol as a co-solvent for methotrexate. Vaidyanathan *et al.* (1985) found that a saturated solution of methotrexate in a propylene glycol:water (50:50) vehicle provided the best system for passive diffusion. Barry (1987) has found that propylene glycol can act as a penetration enhancer. It probably works by solvating α-keratin and occupying hydrogen-bonding sites, thus reducing drug/tissue binding. It does not appear to influence horny layer lipid structure to any extent.

The effect of ethanol on methotrexate permeation should also be looked at as ethanol can act as a penetration enhancer (Yum *et al.*, 1994). In a study done by Magnusson (1997) the effects of ethanol and terpenes on the transdermal enhancement of tripeptide tyrotropin releasing hormone (TRH), it was found that ethanol had no or little effect on the flux of TRH. The effect of ethanol on drug permeation can be explained due to the fact that it slowly extracts lipid from the stratum corneum. To determine the effect of ethanol on methotrexate permeation, two control experiments were done: one with ethanol and one without. From figure 3.1 it can be seen that the ethanol had only a slight influence on the flux of methotrexate, thus the effect of ethanol is neglible. In this study absolute ethanol was used as a solvent for the terpenes, which might have been too high a concentration. Huang *et al.* (1999) found that the outer proportion of the stratum corneum is dehydrated after treatment of high ethanol proportions and this dehydration portion would have increased barrier properties of skin.

The effects of terpenes on the penetration enhancement of a number of compounds have been shown in recent years. For example Ceschel *et al.* (1999) found that limonene increased the permeability of chlorpheniramine maleate and Kunta *et al.* (1997) found that menthol increased the penetration of propranolol across excised hairless mouse skin. One should remember that hairless mouse skin is very thin and therefore much more permeable than human skin (Bronaugh & Collier, 1991). However, Williams

& Barry (1991) found that terpenes exert a negative effect on the partitioning of 5-FU into the tissue, thus having a retarding effect on 5-FU permeation.

The enhancement of methotrexate by terpenes could be the result of the various ways in which they act on the stratum corneum. Terpenes used in this study are lipophilic in nature and lipophilic characteristics may be involved in the extraction of intercellular lipids (Zhao & Singh, 2000). It also appears that for hydrophilic drugs, the primary effect of terpene enhancer treatment is to increase drug diffusivity in the stratum corneum (Godwin & Michniak, 1999). The permeation of methotrexate could be attributed to the above reasons. Hydrophilic terpenes (alcohol, ketone) are more effective in enhancing the permeation of hydrophilic drugs, whereas hydrocarbon terpenes (limonene) are more active towards lipophilic drugs (Moghimi *et al.*, 1997). Thus one would expect that menthol, isomenthol and menthone would have a better enhancing effect on methotrexate than limonene and α-pinene.

The enhancement ration (ER) of methotrexate with menthol and isomenthol were higher when compared to the other terpenes with exception of α-pinene and β-myrcene. The explanation for this could be in the fact that both of them are alcohol terpenes and contain hydroxyl groups. The stratum corneum consists of ceramides and neutral lipids, which are arranged, in a bilayer format (Foldvari, 2000). Between these ceramides there is a high degree of hydrogen bonding. Jain *et al.* (2000) suggests that menthol acts as an enhancer through disruption of hydrogen bonding between ceramide head groups. Isomenthol also has a hydroxyl group and its enhancement could also be explained on this principle.

In this study the flux of methotrexate in the presence of α-pinene and β-myrcene was higher than when the other terpenes were used. In a study done by Almirall *et al.* (1996) α-pinene had practically no effect of chlorpromazine and haloperidol's permeation profiles. Monti *et al.* (2002) studied the effect of α-pinene on oestradiol permeation through hairless mouse skin. The α-pinene had an enhancing effect through increasing the partitioning of oestradiol in the stratum corneum. The lag time of methotrexate in this study was lower in the presence of α-pinene than in its absence. The diffusivity of methotrexate will thus increase in the presence of α-pinene. The same observation was made by Williams & Barry (1991) in their study of the effect of α-pinene on 5-FU. Almirall *et al.* (1996) found that the lag times of chlorpromazine and haloperidol were unchanged in the presence of α-pinene, thus having no effect of the diffusion coefficient. There is not much data available on the effect of β-myrcene on drug permeability through skin

that I know of. However, β-myrcene is also a hydrocarbon like α-pinene and has similar flux values. Thus one could deduce that it might have the same action of acceleration as α-pinene, i.e. increasing the diffusivity of methotrexate in the stratum corneum.

Limonene is a hydrophobic terpene without a hydroxyl group (Kunta *et al.*, 1997) and because of this it's not able to break the hydrogen bonds between the ceramides. Zhao & Singh (2000) deduced that lipophilic characteristics may be involved in the extraction of intercellular lipids when they did a study on the enhancing effect of limonene 5 %/propylene glycol 50 % on tamoxifen penetration. In a study by Moghimi *et al.* (1996) the effect of limonene on oestradiol (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, thus having a retarding effect on 5-FU. Differential scanning calorimetry studies suggest that limonene does not interact with the stratum corneum lipids to disorganize them at skin temperature but may interact with the stratum corneum proteins. Small-angle X-ray diffraction studies showed that limonene reduces lipid bilayer periodicity in the stratum corneum (Cornwell *et al.*, 1996). Thus limonene affects both lipids and proteins of the stratum corneum and may increase the diffusivity of 5-FU in the stratum corneum through protein interactions (Moghimi *et al.*, 1996). Methotrexate is also a hydrophilic drug like 5-FU and limonene may exhibit the same action of acceleration on methotrexate as it did for 5-FU. Using DSC results in their study Yamane *et al.* (1995) suggested that limonene showed no evidence of lipid disruption or increased drug partitioning, but acceleration may be due to heterogeneous disruption of the oil in the stratum corneum lipids.

Menthone and 1,8-cineole also had an enhancing effect on methotrexate although this effect was the lowest of all the terpenes used. They both contain an oxygen group. The boiling points of 1,8-cineole (176 °C) and menthone (207 °C) are both lower than that of menthol (212 °C). This is an indication of weak cohesive or self-association of cineole and menthone molecules or in other words oxygen of functional ether and carbonyl group is in free form for interaction. Therefore the energy required for competitive hydrogen bonding in skin ceramides is relatively less for cineole and menthone than for menthol (Jain *et al.*, 2002). According to this the ER and flux values for cineole and menthone should be higher than that of menthol, which is not the case in this study. Methotrexate had a higher ER in the presence of menthol than it had with 1,8-cineole or menthone.

1,8-Cineole is a cyclic ether terpene and exists in the less thermodynamically stable boat conformation. As such the molecules are under considerable torsional and angular strain. Williams & Barry (1991) compared the ER of the epoxides to the ER of 1.8-cineole and found the ER of the epoxides markedly lower. This may be due to conformational changes. 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 (0.680) with cineole (5 %) if compared to oestradiol (1.71). They found the matrix models the effects of cineole toward permeation of OE through the stratum corneum better than that of 5-FU. This could be explained by the importance of the transcellular route for the permeation of hydrophilic drugs through enhancer treated stratum corneum and differences between the hydrophilicity and therefore partitioning of the models and stratum corneum intercellular lipids.

An important factor with enhancers is that their action on the skin should be reversible. The human stratum corneum is continually shed off and replaced by newly evolved stratum corneum layers. Thus the epidermis is a unique tissue with its programmed replacement and renewal of its barrier (Zhao *et al.*, 2001). Zhao *et al.* (2001) found that in the treatment of the skin with lower concentration terpenes (1 % & 2 %) the induced skin damage was reversible to a greater extent than with higher terpene concentrations (3 % & 5 %). Concentrations of 1-5 % were however found safe for use. Williams & Barry (1991) also found that the action of 1,8-cineole and menthone is reversible.

Siddique *et al.* (1985) found that a flux of about 0.06 $\mu g/cm^2/h$ is required from the stratum corneum to achieve the minimum effective concentration of methotrexate for the management of psoriase. The results of this study did however not meet the desirable skin flux as set by Siddique. Although an effective concentration was not achieved, the data in this study show that the terpenes did have an enhancing effect on methotrexate permeation. I also found that the hydrocarbon terpenes were more effective accelerants than the alcohol, ketone and cyclic ether terpenes. The main action of acceleration could be attributed to lipid disruption and extraction of lipid in the stratum corneum. An increase in partitioning and diffusivity is also possible.

3.7.2. The permeation of terpenes through skin in the absence and presence of methotrexate

The stratum corneum is highly hydrophobic in nature (Foldvari, 2000) thus lipophilic drugs will more easily permeate the skin than hydrophilic drugs. The terpenes are all lipophilic in nature and have low molecular weights which make their permeation through

skin easier. Their Log P values (table 3.2) are all in the range of 2 – 5. According to Guy (1996) compounds with a Log P value between 1 and 3 are likely to have decent passive skin permeabilities.

Table 3.2: The Log P values of the different terpenes (ACD Labs).

Terpenes	Log P
Menthol	3.20±0.20
Isomenthol	3.20±0.20
β-Myrcene	4.58±0.27
Limonene	4.58±0.24
Menthone	2.63±0.26
1,8-Cineole	2.82±0.27

The cumulative concentration terpenes that permeated the skin were compared and the values increase in the following order: limonene < 1,8-cineole < isomenthol < β-myrcene < menthone < menthol. Thus menthol had the highest permeation, which is strange if one takes into consideration that it has a lower Log P value than limonene and β-myrcene. Menthone has a Log P of 2.63, which is the lowest, and its permeation was second highest. Therefore in this study a comparison in permeation and Log P values cannot be made.

Menthol and isomenthol are both alcohol terpenes, which could mean that their permeation results could look the same. In this study it is not the case and the reason for this could be in the stereo–chemical structure of the molecules. In a study done by Cal *et al.* (2001) the permeation of 1,8-cineole was better than that of limonene, which agrees with the data in this study. A reason for the higher permeation of 1,8-cineole could be its Log P value of 2.82.

3.7.3. The effect of methotrexate on the permeation of the terpenes

All the terpenes were influenced by methotrexate's presence although not in the same way. Menthol and menthone's permeation were higher in the presence of methotrexate

than in its absence, while the other terpenes had higher permeations in the absence of methotrexate.

Menthol has a hydroxyl group and menthone an oxygen group. The competitive hydrogen bonding of the molecules to the hydrogen headgroups of the ceramide in the stratum corneum could explain their higher permeation in the presence of methotrexate. The methotrexate could disrupt the hydrogen bonding, seeing that it also has hydroxyl groups, between the ceramides and could lead to higher menthol and menthone permeation.

3.8. CONCLUSION

Methotrexate was introduced in 1948 and still remains the most widely used compound in the antifolate class of antineoplastic agents (Chatterjee *et al.*, 1997). The problem with the systemic delivery of methotrexate via oral, subcutaneous or intramuscular route is the occurrence of side effects such as nausea, vomiting, hepatic toxicity, etc. There have been a few documented studies on the delivery of methotrexate via the transdermal route to avoid such side effects. For example Brain *et al.* (1991) did a study on the effect of azone on the percutaneous delivery of methotrexate and Chatterjee *et al.* (1997) also used azone as an enhancer.

In this study the effect of terpenes on the percutaneous absorption of methotrexate was determined. Terpenes are naturally occurring oils that are derived from plants and have low cutaneous irritancy at low concentrations (1-5 %) (El-Kattan *et al.*, 1999). They are also used in aromatherapy. For this reason the permeability of the terpenes and the effect of methotrexate on this permeability was also determined. The following terpenes were used: menthol, isomenthol, menthone, 1,8-cineole, limonene, α-pinene and β-myrcene.

The permeability of methotrexate and the terpenes through human abdominal skin was determined using the in vitro Franz diffusion cell method. The flux of the methotrexate permeated was determined. It was found that there was a statistical difference ($p < 0.05$) in the flux from the control experiment (without terpenes) and the flux from the experiments with the terpenes. From the results could be seen that the terpenes had an enhancing effect on the percutaneous absorption of methotrexate with α-pinene having the highest enhancement. The average flux of methotrexate was howevere lower than reported before and ways in bettering this should be investigated. In determining the terpene absorption the cumulative concentration terpene that permeated was calculated.

The results showed that the terpenes did permeate the skin and that this permeation was influenced by the presence of methotrexate. The reason for the terpene permeation could be their low molecular weight and high Log P values. The effect of the methotrexate on their permeation is however not clear and will need further investigation.

In conclusion the following should be considered:

- transdermal delivery holds a lot of advantages above other routes of delivery and should be further investigated;
- ways in bettering the percutaneous delivery of methotrexate should be looked at especially the further investigation of the effect of terpenes on methotrexate absorption;
- the ways in which terpenes permeate the skin should also be investigated to a fuller extent.

3.9. REFERENCES

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