Optimization of transdermal permeation of non-steroidal anti-inflammatory agents and sunscreens

Vikram Sarveiya

A thesis submitted to the University of Manitoba in partial fulfillment of the requirements of the degree of Doctor of Philosophy

Faculty of Pharmacy, University of Manitoba

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To
My parents - Poonamchand and Savita
Sisters - Pragati and Sonal
Brother – Ketan
And my friend - Subuddhi Kulkarni

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Abstract

In recent years there has been a great deal of interest worldwide in the dermal and transdermal delivery of drugs. Many drugs have been formulated into transdermal systems and many others are examined for the feasibility of their delivery in this manner. Current study deals with optimization of transdermal delivery of ionic hydrophilic as well as lipophillic drugs. It assesses the percutaneous absorption and optimization of percutaneous absorption for increased efficacy.

Ion pair formation has been utilized by many researchers to improve the permeation of various drugs through the skin, other biological and synthetic membranes. This technique is further being explored to increase the permeation of two well known non-steroidal anti-inflammatory drugs (NSAID's) - ibuprofen and benzydamine. Nuclear magnetic resonance (NMR) spectroscopy was used to demonstrate the existence of ion pairs and to rationalize the permeation behavior in terms of molecular interactions. The permeation of ibuprofen sodium was studied at various pH values and it was found that the flux across PDMS membrane increased significantly with increase in pH from 4.0 to 7.0. The permeability coefficient increased with the increase in the amount of unionized acid. To study the effect of ion pairing on the permeability of ibuprofen, ibuprofen was paired with different counter amines and highest flux was measured from ibuprofen triethylamine.

Similarly, the flux for benzydamine hydrochloride increased significantly with the amount of unionized base. Permeation of benzydamine hydrochloride across excised human skin showed similar profile as through PDMS. The octanol/water partition coefficient was directly related to steady state flux. To study the effect of ion pairing on penetration, benzydamine was paired with ibuprofen, benzoate or octane sulphonate ions. The NMR chemical shift changes were more significant when benzydamine was paired with ibuprofen or benzoate, which suggested that benzydamine was able to interact more strongly with ibuprofen and benzoate rather than with octane sulphonate. This was also reflected in the permeation characteristics, where ibuprofen and benzoate ions significantly increased the permeation of benzydamine.

On the other hand, penetration of lipophilic sunscreens across human skin was assessed. A liquid chromatographic method was developed in order to facilitate the volunteer study to quantify sunscreens in different biological matrices. Reverse-phase high-performance liquid chromatographic assay was developed for quantifying four common sunscreen agents namely oxybenzone, octylmethoxycinnamate, octylsalicylate and homosalate in a range of biological matrices. This assay was further applied to study the skin penetration and systemic absorption of sunscreen filters after topical application to human volunteers. Separation was achieved utilizing a Symmetry C-18 column with methanol:water as the mobile phase. The assay permits analysis of the sunscreen agents in biological fluids, including bovine serum albumin (BSA) solution, plasma and urine, and in human epidermis. The HPLC assay and extraction procedures developed are sensitive, simple, rapid, accurate and reproducible.

A volunteer study was done in order to assess the penetration of sunscreens into and across the skin after topical application for commercial sunscreen formulation. Further, the effect of anatomical site on percutaneous absorption of sunscreen was measured. The volunteer study confirmed the systemic absorption of oxybenzone. Small amounts of oxybenzone (<130 ng/mL) were observed in the plasma, whereas other sunscreens were below the limits of detection. Up to approximately 1% of the applied dose of oxybenzone and its metabolites was detected in the urine. Appreciable amounts were also detected in the stratum corneum through tape stripping. A comparison of the skin penetration of sunscreen at different anatomical sites (face and back) demonstrates that as much as twice amount is present or retained in facial stratum corneum. This suggests that less amount of sunscreen would be required to produce the same protection as would be required on the back.

Since minimal skin penetration of sunscreens and insect repellents is highly desirable for optimum efficacy. Further, studies were done to reduce the percutaneous absorption of oxybenzone. Effect of hydroxypropyl-β-cyclodextrin (HPβCD) on the release and permeation of the sunscreen agent oxybenzone was investigated. The interaction between oxybenzone and HPβCD was studied by phase solubility analysis, thermal analysis and nuclear magnetic resonance spectroscopy. UV transmittance studies indicated that the presence of HPβCD did not suppress the UV absorbing

properties of oxybenzone. The release and membrane permeation of oxybenzone was significantly reduced in the presence of equimolar, 2 times molar and 1, 2 and 4% of HPβCD. It is concluded that HPβCD can reduce the release/membrane diffusion of oxybenzone whilst retaining its efficacy as a sunscreen agent. This formulation strategy may be useful in controlling skin penetration of topically applied sunscreens and other chemicals.

This work demonstrates the importane of ion pair technique and molecular interactions to optmize the transdermal permeation of non-steroidal anti-inflammatory drugs and sunscreens.

List of Publications

1. Sarveiya V, Templeton JF, Benson HAE. [2004] Ion pairs of ibuprofen : increased membrane diffusion. *J Pharm Pharmacol* 56(6):717-24.

2. Sarveiya V, Risk S, Benson HAE. [2004] Liquid chromatographic assay for common sunscreen agents: application to *in vivo* assessment of skin penetration and systemic absorption in human volunteers. *J Chromatogr B* 25;803(2):225-31.

3. Sarveiya V, Templeton JF, Benson HAE. Inclusion complexation of the sunscreen agent oxybenzone (2-hydroxy-4-methoxy benzophenone) with hydroxypropyl-β-cyclodextrin: effect on percutaneous absorption. *J Inclusion Phenom* [in press].

4. Sarveiya V, Templeton JF, Benson HAE. [2004] Influence of ion pairing on topical delivery of benzydamine. In (KR Brain & KA Walters, eds): Perspectives in Percutaneous Penetration, volume 9a, STS Publishing, Cardiff.

Conferences

1. Sarveiya V, Risk S, Benson HAE [2004]. Skin penetration and systemic of sunscreens after topical application. 62nd Annual Meeting, America Association of Dermatology, Washington, DC Feb 6-11; P288.

2. Sarveiya VP, Hall A, Benson HAE. [2003] High-performance liquid chromatographic assay for common sunscreen agents: application to *in vivo* assessment of skin penetration and systemic absorption in human volunteers. *J Pharm Pharmaceut Sci* 6(2): 101-188 (57).

3. Jiang P, Burczynski F, Sarveiya V, Campbell CJ, Briggs CJ. [2003] Flavonoids and antioxidant activities in two cultivated buckwheat species. *AAPSPharmSci vol 5, No 4, Abstract M1117.*

4. Sarveiya VP and Benson HAE. [2002] Inclusion complexation of the sunscreen agent oxybenzone (2-hydroxy-4-methoxy benzophenone) with hydroxypropyl-β-cyclodextrin: effect on percutaneous absorption. *AAPSPharmSci Vol. 4, No. 4, Abstract R6049.*

5. Kasichayanula S, Burczynski FJ, Sarveiya VP, Gu X. [2002] *In vitro* evaluation of percutaneous penetration of benzophenone-3 and N,N-Diethyl-m-toluamide (DEET). *AAPSPharmSci Vol. 4, No. 4, Abstract R6067.*

6. Sarveiya VP and Benson HAE.[2002] Effect of ionization on penetration of ibuprofen through polydimethylsiloxane membrane. AFPC conference.

7 Kasichayanula S, Burczynski FJ, Sarveiya VP, Gu X. [2002] Preliminary assessment of percutaneous penetration of benzophenone-3 and n,n-diethyl-m-toluamide *in vitro.* AFPC conference.

8. Sarveiya VP, Templeton JF, Benson HAE. [2001] Increased membrane diffusion of Ibuprofen by ion pair formation. *AAPS PharmSci 2001;* 3 (3) *AAPS Annual Meeting Supplement.*

9. Metge CJ, Vercaigne L, Carrie A, Sarveiya V, Zhanel GG. [2000] A Canadian clinical and economic evaluation of new fluoroquinolones (fq) for treatment of community-acquired pneumonia (cap) [abstract 117]. In: *Program and abstracts of the 40th International Conference on Antimicrobial Agents and Chemotherapy. Toronto, ON: American Society of Microbiology.*

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Chapter 1. Introduction

Human skin is the largest multifunctional organ of the body, accounting for more than 10% of the body mass. The skin has many essential functions, including protection, thermoregulation, immune responsiveness, biochemical synthesis, sensory detection, and social and sexual communication. As a protective interface between the internal organs and the environment, the skin encounters a host of toxins, pathogenic organisms, and physical stress.

The skin can be described as an evolutionary masterpiece of living body. It determines the local and systemic availability of any drug or chemical that accidently or deliberately comes in contact with the skin surface. Therefore the knowledge of structure and function of the skin is essential to clinicians and researchers (Wester and Maibach 1992; Guzzo *et al.* 1996). The skin can be structurally viewed as a series of layers; the four main divisions are the stratum corneum (non-viable epidermis), the remaining layers of epidermis (viable epidermis), dermis and subcutaneous tissue (Figure 1.1.).

The outer most layer is the stratum corneum (non-viable epidermis) or horny layer, which consists of compacted, dead, keratinized cells in stratified layers. Because of the dense layer of the stratum corneum, values of diffusion coefficients in this tissue are a thousand or more times smaller than any other skin tissue, resulting in high resistance and contributing to the relative impermeability of the skin. The stratum corneum is approximately 10 to 20μm thick, consisting in a given cross section of 15 to 25 flattened, stacked, hexagonal and cornified cells. Each cell is approximately 40μm in diameter and 0.5μm thick. Each stratum corneum cell is composed mainly of insoluble bundled keratins (~70%), a tough inert protein (Tregear 1966; Marieb 1992), and lipid (~20%). The major lipids present in the stratum corneum are ceramides, free fatty acids, cholesterol, and triglycerides (Anderson and Cassidy 1973; Bouwstra and Gooris 1997). The stratum corneum is the rate-limiting barrier that restricts the inward and outward movement of chemical substances. This impermeability of the stratum corneum is due to its lipophilic nature and the highly ordered structure of the lipid domains (Monash 1957; Scheuplein and Blank 1971).

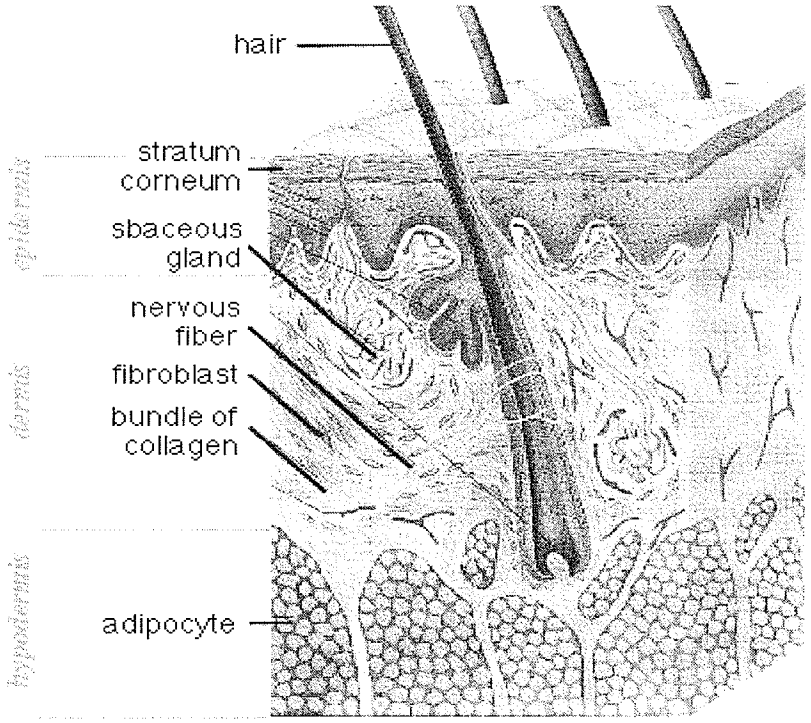


Figure 1.1. Structure of human skin

Beneath the stratum corneum are the metabolically active layers of the epidermis, collectively known as the viable epidermis. The basal or germinal layer (stratum basale) lies immediately above the dermis. Epidermal cells begin their mitotic journey upward to the surface; the cells flatten and shrink as they slowly die due to lack of oxygen and nutrition (Marieb 1992). During this journey the basal layer cells undergo differentiation into malphigian layer (stratum spinosum), and then into granular layer (stratum granulosum). The granular layer contains a waterproofing glycolipid that is secreted into the intercellular space to slow water loss across the epidermal layers (Marieb 1992). There are some important functions associated with the viable epidermis, including metabolism of substrates, synthesis of melanin from melanocytes for skin pigmentation and sun protection (Roberts and Walters 1998). The basal layer contains abundant melanosomes that may serve to protect the prominent nucleus from ultraviolet (UV) radiation (Wertz and Downing 1989). Other cells associated with the viable epidermis are the langerhans' cells (important in recognizing the presence of

antigens and facilitating an immune response), Merkel cells (with a role in sensory perception), and keratinocyte cell bodies (in contact with nerve fibres by membrane - membrane apposition) (Bressler and Bressler 1989; Salmon *et al.* 1994; Bartak and Arenberger 1996; Roberts and Walters 1998). The entire architecture of the epidermis constitutes a dynamic system in which each cell type changes continuously during its passage from the basal layer, where it is formed, to the surface of the horny layer, where it is discarded (Wertz and Downing 1989).

The dermis or corium is the next distinctive histological layer of the skin. It is 0.1 to 0.5 cm in thickness and not only provides the nutritive, immune, and other support systems for the epidermis but also plays a role in temperature, pressure, and pain regulation. It has a rich supply of nerve fibres, blood vessels and lymphatic vessels. Cutaneous receptors, glands and hair follicles also reside within the dermis (Marieb 1992). The dermis consists of collagenous fibres, providing support and cushioning, and elastic connective tissue, providing elasticity, in a semigel matrix of mucopolysaccharides (Idson and Lazarus 1986; Roberts and Walters 1998). The dermal vascular supply is very important for absorption of drugs and chemicals creating a sink condition and hence maintaining the concentration gradient across the skin layers.

Lastly, below the dermis is a layer of subcutaneous tissue, providing the necessary insulation and cushioning for deep tissue, muscle and bone.

1.2. Drug applications to the skin

In recent years there has been a great deal of interest worldwide in the dermal and transdermal delivery of drugs. Many drugs have been formulated in transdermal systems, and others are being examined for the feasibility of their delivery in this manner (e.g., nicotine, antihistamines, beta-blockers, calcium channel blockers, non-steroidal anti-inflammatory drugs, contraceptives, anti-arrhythmic drugs, insulin, antivirals, hormones, alpha-interferon, and cancer chemotherapeutic agents). Transdermal drug delivery offers advantages of avoiding local gastrointestinal irritation and the hepatic first-pass effect providing controlled plasma levels of potent drugs and improved patient compliance (Ranade 1991)

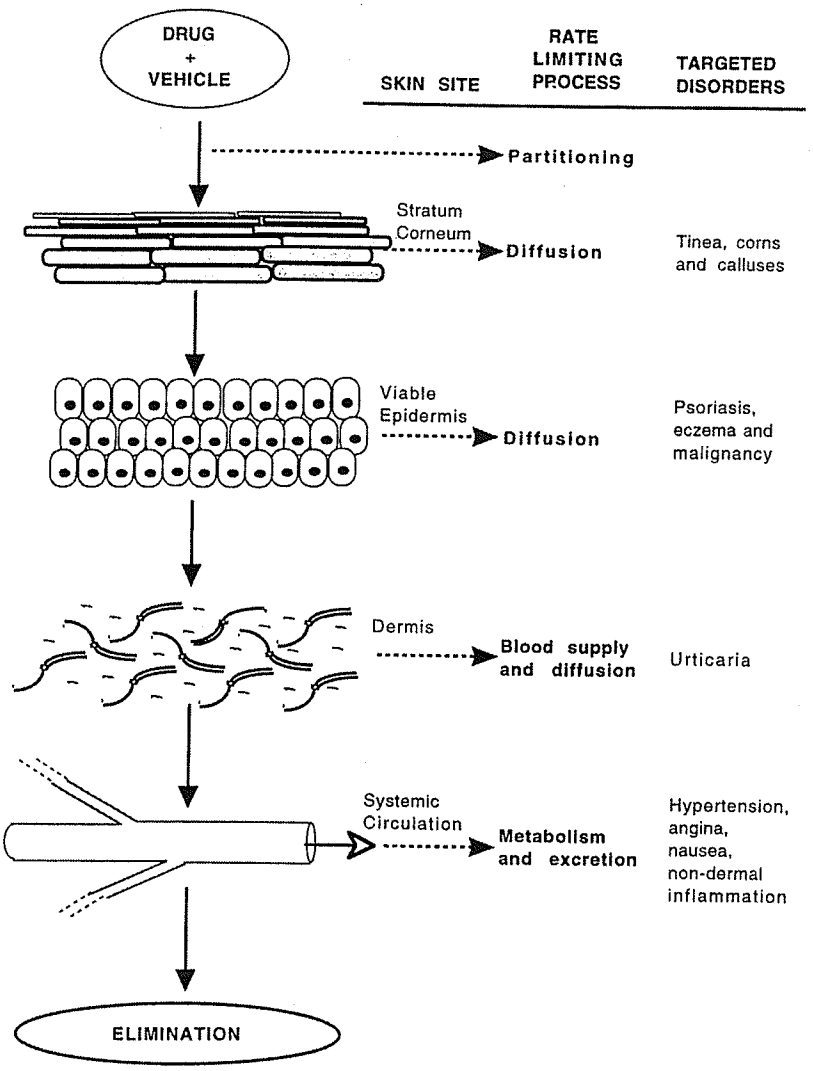


Figure 1.2. Sites of solute action below the skin after topical application. (Whitehouse and Roberts 1998)

In addition dermal drug application can be used to treat diseases of the upper skin parts or to reach deeper tissues whilst avoiding or minimizing systemic blood circulation and potential side effects. Figure 1.2. describes the various sites of solute action below the skin after topical application.

Therapeutic targets within the skin and underlying tissues vary according to disease localization and other factors. For instance, penetration of active ingredient only

to the depth of stratum corneum, is desired for sunscreens and insect repellants (Moody *et al.* 1992; Qiu *et al.* 1997; Qiu *et al.* 1998; Riviere *et al.* 2002). Treatment of acne requires drug targetting of pilosebaceous structures; in psoriasis, therapeutic drug levels should be attained in deeper epidermal layers; certain local musculoskeletal inflammations and osteoarthritis require drug levels as deep as underlying muscles, etc. The area of deep tissue penetration after topical application has, however, received limited attention. It will be clinically useful if deeper penetration can concentrate more active compound in local affected muscular tissue than can alternative administration routes. Better therapy may thus be possible with minimum systemic distribution of drug and associated side effects (Guy and Maibach 1983).

The topical delivery of local anesthetics to deeper layers of skin will be most useful when patients are averse to use of hypodermic needles, particularly in the pediatric patient population.

Skin can also be used to extract drugs from the blood by electroosmosis or reverse iontophoresis. This process has been used to monitor blood glucose levels with recently marketed Glucowatch from Cygnus Inc, USA (Tierney et al. 2001) and urea (Degim *et al.* 2003).

1.3. Barrier properties of the stratum corneum

The major source of resistance to penetration and permeation through the skin is the stratum corneum. The two-compartment ("bricks and mortar") model for the stratum corneum illustrates the differing roles, which structural proteins (keratins) and lipids play in barrier function (Michaels *et al.* 1975; Anderson and Egelrud 1992). The bricks represent the dead, flattened cells of the stratum corneum, the corneocytes, which contains a minor amount of lipid. The corneocytes hold the major protein portion fraction of the stratum corneum and the protein is composed of intracellular keratin filaments. These keratin filaments are crosslinked by intermolecular disulphide bridges (Sun and Green 1978; Walters 1989; Steinert *et al.* 1994). The mortar consists of structured complex lipid bilayers surrounding the keratin rich interiors. Current thinking suggests that the corneocyte protein envelope, which consists of insoluble and chemically resistant crosslinked protein complex, plays an important role in the

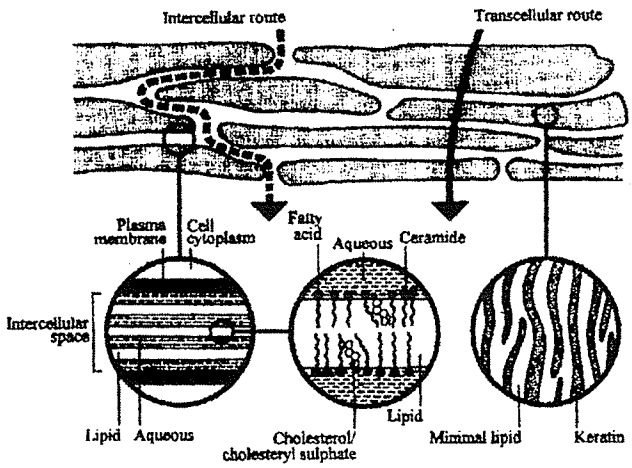


Figure 1.3. Simplified diagram of stratum corneum and two microroutes of drug penetration. (Barry 2001)

structural assembly of the intercellular lipid lamellae of stratum corneum (Roberts and Walters, 1998). It has been demonstrated that the corneocyte possesses a chemically bound lipid envelope comprised of N-(ω-hydroxylacyl) sphingosines which are covalently linked to the glutamate side chains of the envelope protein (Swartzendruber *et al.* 1987; Wertz and Downing 1989). Six classes of ceramides (designated ceramide 1 to 6) have been identified in the human stratum corneum and it is possible that the ceramide 1 acts as a "molecular rivet" in the intercellular lipid lamellae of the stratum corneum (Roberts and Walters 1998). The chemicals link between long chain ceramides and glutamate residues on the corenocyte protein envelope and further stabilize the intercellular lipid lamellae. Overall, the intercellular lipid lamellae appear to be highly structured and very stable, constituting a highly effective barrier to chemical penetration and permeation (Roberts and Walters 1998).

1.4. Pathways of permeation across the skin and properties of the penetrant

Chemicals permeate the skin either across the intact epidermis (transepidermal pathway) or via the sweat glands and hair follicles (appendageal or shunt route) other than the intercellular route. These routes provide barriers in parallel; therefore the

overall flux across the skin is the sum of the individual fluxes through each pathway, which itself depends on different physicochemical and geometrical properties (Barry 1983). Parallel permeation paths were also suggested by Scheuplein and Ross (1974) and Flynn *et al.* (1974). In short the absorption of drugs is possible via three routes: intercellular diffusion across the lipid matrix; transcellular diffusion through the corneocytes; and via the sweat pores and the hair follicles.

Transport along the sweat pores and hair follicles is regarded as being relatively insignificant because the orifices of the follicles occupy only 0.1% of the skin area and diffusion along the sweat ducts would be against an outward aqueous flow (Pugh *et al.* 1998). The skin appendages may provide the main portal entry into the subepidermal layers of the skin for ions (Tregear 1966; Scheuplein *et al.* 1969; Grimnes 1984). The intercellular pathway, which is hydrophobic in nature, is associated with the intercellular bilayers: a more tortous path through which a chemical diffuses within the intercellular lipid phase only (Michaels *et al.* 1975; Houk and Guy 1988). This is therefore the simplest model of penetration. On the other hand, if the molecule passes through the corneocyte (intracellular), it must pass through the corneocyte-lipid boundaries (Yotsuyanagi and Higuchi 1972; Roberts *et al.* 1977; Jetzer *et al.* 1986; Roy and Flynn 1989). The question of which route predominates is unresolved. Present opinion generally favors the continuous lipid route (Potts and Guy, 1995; Abraham *et al.* 1995; Roberts *et al.* 1996).

Figure 1.4. represents the flux arising from an ointment suspension, and illustrates the complexity of percutaneous absorption. In order to diffuse towards the vehicle/stratum corneum interface, the drug particles must first dissolve in the vehicle. They then partition into the stratum corneum and diffuse through it. After this the drug molecules partition into the viable epidermis. For a lipophilic drug this partitioning process can be unfavorable, because of the hydrophilic nature of the viable epidermis and the drug may form a depot in the stratum corneum. After passing into the dermis, the drug moves into the blood for systemic removal. Simply the skin can be considered as a trilaminate structure consisting of the outer, dense keratinized layer known as stratum corneum, the underlying viable hydrophilic epidermis, and the dermis which supports the vasculature. As has been shown, however, the skin is not a pure lipid

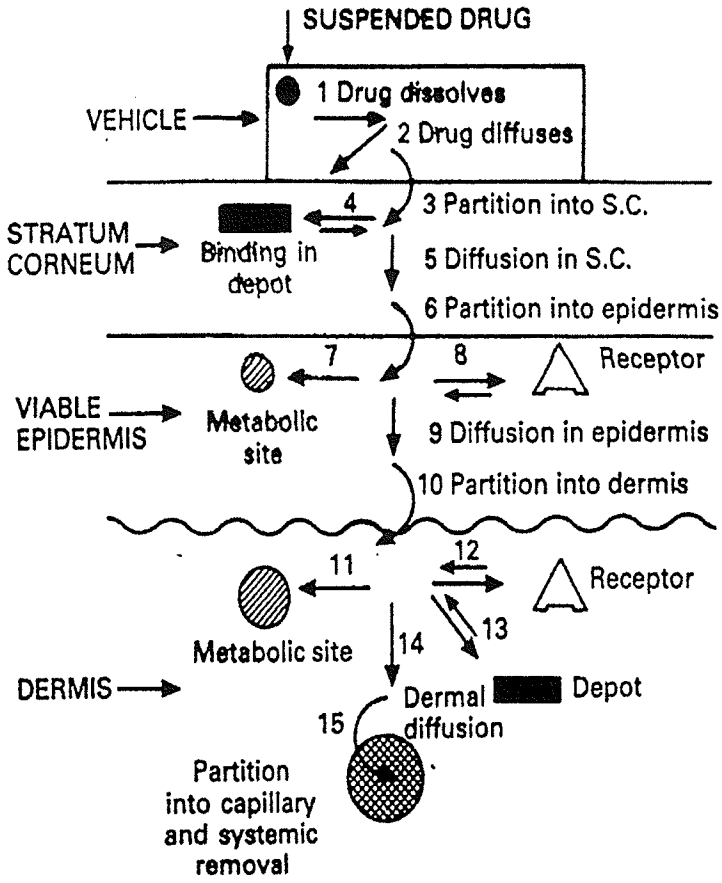


Figure 1.4. Some stages in percutaneous absorption from a suspension ointment. (Barry 1988)

membrane, and the log of permeability 'k_p' rate versus permeant lipophilicity appears sigmoidal (Figure 1.5.), which reflects the existence of hydrophilic barriers. As the alkyl chain length/lipophilicity of the permeant increases the permeability through the skin increases until it attains a plateau. This plateau is attributed to the barrier of hydrophilic nature. In other words, for hydrophilic penetrants, partioning into and diffusion within the stratum corneum is rate determining, whilst for highly lipophilic drugs partitioning out of the stratum corneum into the viable epidermis becomes important (plateau

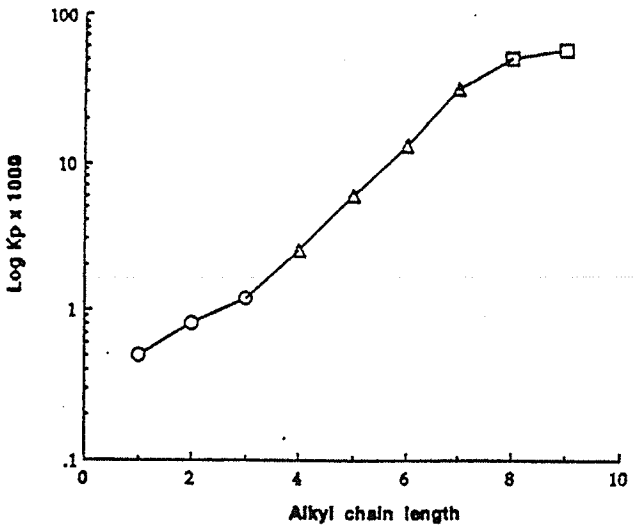


Figure 1.5. Effect of permeant lipophilicity on the rate of skin permeation (Scheuplein and Blank 1973)

region). A vast majority of drugs, except a few sunscreens, are hydrophilic and stratum corneum represents the major barrier for penetration. Since most drugs are weak acids or bases, they exist as charged species at physiological pH and their partitioning into the skin is hindered by their intrinsic charge. Passive diffusion of charged molecules across a membrane as lipophilic as stratum corneum is a slow process (Green *et al.* 1988). It is possible to reduce the resistance to transdermal flux either by disrupting the skin structure itself or by increasing the lipophilicity of the ionized permeant e.g. through ion pair formation (Barker and Hadgraft 1981). Log octanol/water partition coefficient or log P has been correlated with the lipophilicity of the drug. Yano *et al.*(1986) studied the absorption of many drugs and showed that maximum absorption was observed with drugs that had a log $P_{oct/water}$ of between 2 and 3. A similar parabolic relationship was observed between the log of the permeability coefficient for a group of non-steroidal anti-inflammatory drugs (NSAIDs) across human epidermal membrane *in vitro* and their respective log $P_{oct/water}$ values with an optimal log P of around 3 (Singh and Roberts 1994). However, a better correlation for skin permeability was reported if log $P_{oct/water}$ was corrected by a solute volume term (Abraham *et al.* 1995). Charge on the species is also considered to be one of the major factors in permeation of drug through the skin. The majority of drugs are weak acids or bases, and are ionized under normal

physiological conditions. The human stratum corneum acts as a significant barrier for the skin penetration of these hydrophilic ionizable drugs. Charged species are known to be poor penetrants across skin, other biological membranes and nonporous polymers. Their permeation coefficient has been estimated to be about 10^4 times smaller than for the respective uncharged species (Swarbrick *et al.* 1984). Melting point of a drug has also been shown to be a determinant in the flux of a drug across the skin. (Kaplun-Frischoff and Touitou 1997)) and (Stott *et al.* 1998; Stott *et al.* 2001) considered the permeant melting point depression on transdermal penetration in terms of the concept that lower the melting point of a substance the greater its solubility in a given solvent, including skin lipids. Melting point depression and increased flux was also recently reported for vasoconstrictors (Cross *et al.* 2003). However, a higher melting was also correlated with both higher polarity, and therefore less favorable partitioning, and higher molar volume (Hu and Matheson 1993).

1.5. Strategies to alter the Barrier Function

So far the relative impermeability of the stratum corneum barrier has been discussed. But the stratum corneum barrier can be altered to a certain extent using various strategies summarized in figure 1.6. Some of the factors which influence the barrier function are as follows:-

1.5.1. Effect of solvents/ vehicle/ chemicals on skin barrier properties.

The enhancing and delipidizing effects of solvents on the skin are well known. Skin permeability can be modified either by altering natural conditions of the skin such as hydration and disrupting lipid and protein structure of the stratum corneum using penetration enhancers. Although, the mechanisms are not yet fully understood, lipid perturbation and modification of partitioning of the drug into the tissue were found to be major factors for altered barrier functions (Barry 1987; Brain and Walters 1993; Harrison *et al.* 1996). Blank *et al* (1967) observed that methanol, ethanol, acetone, dimethylsulfoxide, ethylether, chloroform, and carbon tetrachloride extract lipids from

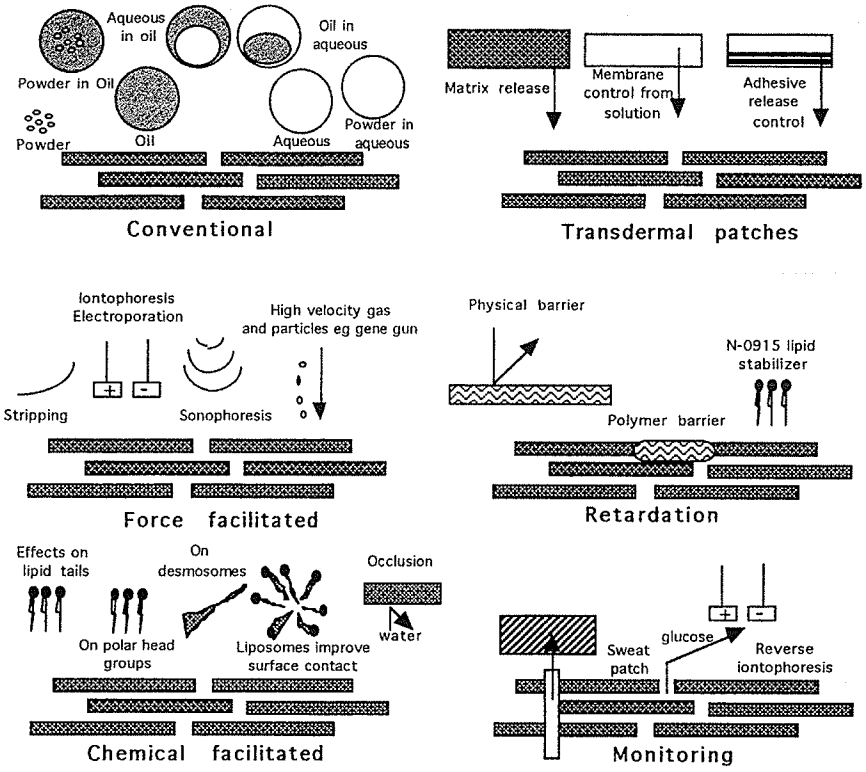


Figure 1.6. Strategies to alter percutaneous absorption

epidermis. Delipidization has also reported due to phenol, di-n-amyl-ether, di-2-ethyl hexylamine, and n-butyric acid. The kerotolytic action of salicylic acid, resorcinol, sulfur, and urea was proposed to be through protein denaturation. Scheuplein and Ross (1970) suggested that strong binding of surfactants to proteins have led to reversible $\alpha \leftrightarrow \beta$ conversion of keratin accompanied by uncoiling of the filaments. Other mechanisms for enhancement of skin penetration include swelling of epidermal components by plasticization of lipids by solvents such as octanol or by osmotic or hydroscopic related effects by polar solvents such as water, dimethyl sulfoxide, dimethyl formamide, urea, and propylene glycol.

1.5.1.1. Effect of hydration

The state of hydration of the stratum corneum is one of the major factors influencing the percutaneous absorption of a drug. The stratum corneum water content, at normal relative humidity, is between 15 and 20% of dry weight. Soaking, occlusion

and high humidity may increase the water content further up to 300-400% of the dry weight after extensive soaking. Increased water content results in increased elasticity and permeability of the stratum corneum, whereas reducing the water content will lead to the opposite effect (Roberts and Walker 1993). Water is strongly bound to the keratin in the stratum corneum. Bound water has been defined as " that part of the total

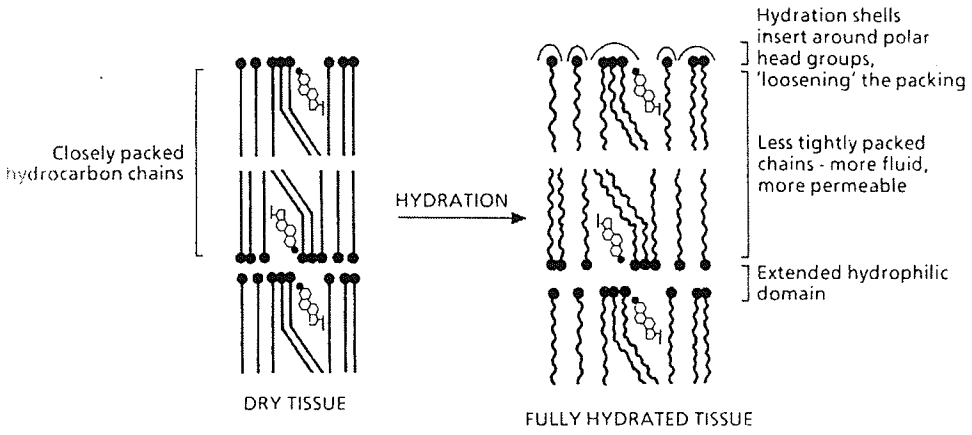


Figure 1.7. How hydration increases the fluidity of the stratum corneum lipids by insertion of water molecules between polar head groups (Barry 1987).

which is not available to dissolve water-soluble non-electrolytes". It is generally accepted that unbound or free water accounts for 70-80% of the total water found in the stratum corneum (i.e., 20-30% of the water is bound). It was found that the amount of "bound water" in fully hydrated stratum corneum can be as much as five times the dry weight of the skin tissue (Scheuplein and Morgan 1967) and was considered to act as an endogenous plasticiser (Blank 1952). The penetration of certain alcohols and cortisone across the fully hydrated skin was up to 10 and 15 times higher than across the dry skin, respectively (Scheuplein and Blank 1973; Scheuplein and Ross 1974). In other studies, hydration greatly increased the absorption of glycol salicylate and methyl salicylate (Wurster and Kramer 1961). One of the proposed mechanisms for the transport is by water being absorbed into the stratum corneum where it acts as a plasticiser in its bound state. Other studies suggests that stratum corneum not only swells, but also develops multiple folds, resulting in a 37% increase in surface area (Roberts and Walker 1993).

Insertion of water molecules between polar head groups of stratum corneum lipids leads to increased fluidity (Barry 1987; Menon *et al.* 1994), and is presumed to cause swelling of the compact structures in the horny layer (Florence and Attwood 1988), which facilitates flux. Increase in water content of the stratum corneum is associated with a decrease in lipid/ protein phase transition temperature and hence, induction of lipid disruption and protein denaturation (Potts 1989). Low temperature lipid phase transitions occuring near physiological temperature was suggested for mechanical alterations of the stratum corneum, whereas transitions occuring near 70° C were primarily responsible for barrier properties (Potts 1989).

In contrast to increased permeation for many drugs, hydration does not always enhance percutaneous absorption as the effect is dependent on the polarity of the drug, Poorly and moderately lipophillic molecules such as 5-methoxypsoralean showed increased absorption with increased hydration, while for molecules with a strong lipophilic character there was no effect on the absorption rate.

Occlusive dressings, ointments and oils are some of the ways to reduce to water loss to the atmosphere and to increase the binding of water to the stratum corneum, thus maintaining the skin hydrated state (Roberts and Walker, 1993).

1.5.1.2. Alkyl sulfoxide

Of the alkyl sulfoxides, dimethyl sulfoxide (DMSO) is well known as a penetration enhancer. Postulated mechanisms for the penetration enhancement include extraction of lipid, lipoprotein, and nucleoprotein structures (Embery and Dugard 1971; Creasey *et al.* 1978), protein denaturation, and lipid fluidization (Chattaraj and Walker 1995). It was reported that DMSO increases lipid fluidity by disrupting tightly packed lipid chains, which resulted in an interaction between the polar head groups of the lipids via hydrogen bonding (Chattaraj and Walker 1995). DMSO induced penetration enhancement was also found to be related to keratin conversion (Naik and Guy 1997). Interaction with intercellular lipids and cellular keratin is shown in figure 1.8. The modulation of both protein and lipid domains by alkyl sulfoxide was elegantly demonstrated using IR spectroscopy, which showed that similar soaking treatments of human stratum corneum *in vitro* with DMSO caused reversible α-helical to β-sheet conversions, of a fraction of keratin, probably by displacement of bound water.

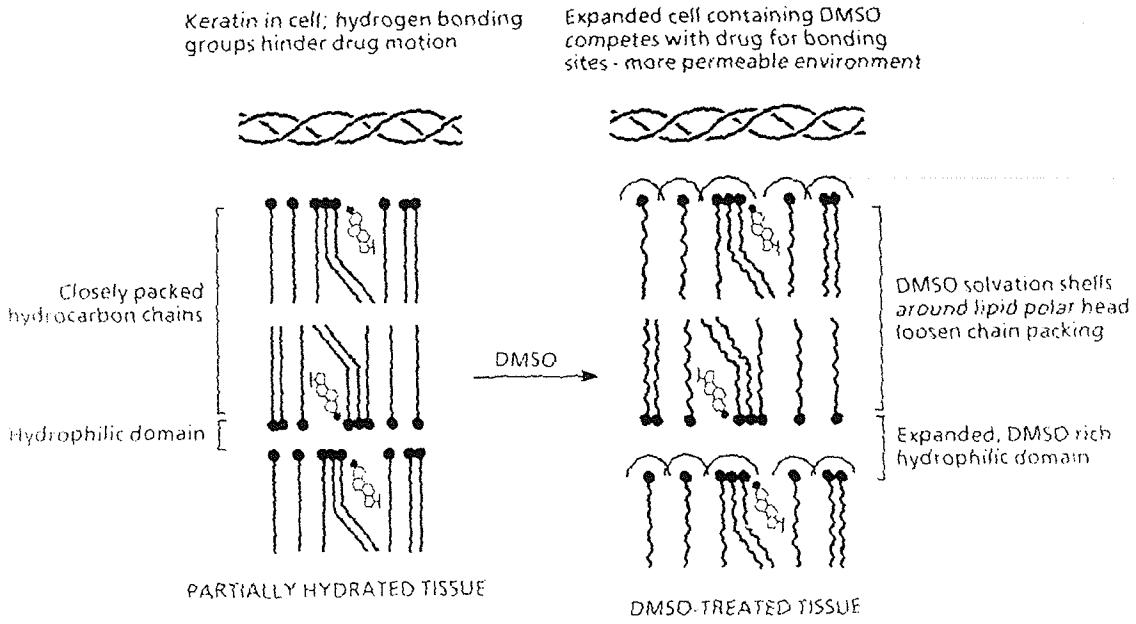


Figure 1.8. Effect of dimethylsulfoxide on the permeability of human stratum corneum interaction with intercellular keratin (Barry 1987).

The more hydrophobic homolog, decylmethyl sulfoxide (DCMS), has been shown by FTIR studies to produce a greater level of α- to β-keratin conversion, compared to DMSO (Naik and Guy 1997). In addition, DSC studies have revealed that DCMS has a similar effect to DMSO on the stratum corneum thermogram, but at a relatively lower concentration (Barry 1987).

1.5.1.3. Alkanols and phenyl alcohols

The inclusion of ethanol in commercially available transdermal systems has naturally provoked curiosity concerning its role as penetration enhancer in human skin. The penetration enhancing effect of this alcohol and others in the homologous series (C_2-C_{12}) was examined for the polar and poorly permeable solute, nicotinamide (Kai *et al.* 1990). The degree of enhancement varied parabolically with alkanol chain length, with n-hexanol generating maximal enhancement. In similar studies by Chien et al (1988), the transdermal permeation rate increased with the increasing chain length of alcohol, exibiting a maximum rate with six carbon atoms and then decreased as the

number of methylene group increased to eight. Severe lipid extraction observed with alkanols, plays an important role in the enhancement action (Naik and Guy, 1997). Indeed, a mechanism involving lipid "fluidization" has also been suggested for the enhancing action of ethanol (Ghanem *et al.* 1987). The effect of ethanol on nitroglycerin was found to reach a maximum at an ethanol concentration of around 70%, where uptake of ethanol into the stratum corneum or the delipidized stratum corneum is optimal (Berner and Liu 1995). Therefore, the ability of ethanol to increase penetration of lipophillic compounds was suggested to be via increased solubility of the compounds in the stratum corneum by cosolvency or lipidisation of the stratum corneum.

Phenol disrupts both the protein and lipid structures of the skin, and hence acts as a penetration enhancer. Phenyl alcohols have been observed to act as percutaneous penetration enhancers for hydrophillic drugs such as 5-flourouracil. Phenyl alcohols may act by modifying intercellular lipids, thus disrupting their highly ordered structure to increase diffusivity (Lopez *et al.* 1997).

1.5.1.4. Propylene glycol

A number of reports have described propylene glycol as a penetration enhancer for the transport of various chemicals (Portnoy 1965; Mollgaard and Hoelgaard 1983; Barry 1987). Sheth et al (1986) demonstrated the penetration enhancing effect of propylene glycol for triflourothymidine. The reaction of propylene glycol is reported to vary for possibly related difference in drug solubility. Various theories have been proposed for the mechanism of action of propylene glycol on skin permeability. For example, as propylene glycol penetrates into the layers of the skin, a "carrier" mechanism has been postulated in which propylene glycol carries the drug through the barrier layer (Sheth et al., 1986). Others have hypothesized that propylene glycol alter the barrier properties of stratum corneum (Idson 1975).

1.5.1.5. Fatty acids

The fatty acids have received extensive interest as potential penetration enhancer (Naik and Guy, 1997). In general, unsaturated long chain fatty acids are more effective penetration enhancers compared to their saturated counterparts. Attempts for transdermal drug delivery have led to extensive research of these compounds on their

skin penetration enhancement (Cooper *et al.* 1985; Green *et al.* 1988; Aungst 1989; Scheider *et al.* 1996). The mechanism by which fatty acids increase permeability appears to involve disruption of the densely packed lipids that fill the extracellular spaces of the stratum corneum (Aungst 1989). Oleic acid creates gaps in the packed lipid structure and thus reduces diffusional resistance (Barry 1987) [see figure 1.9.].

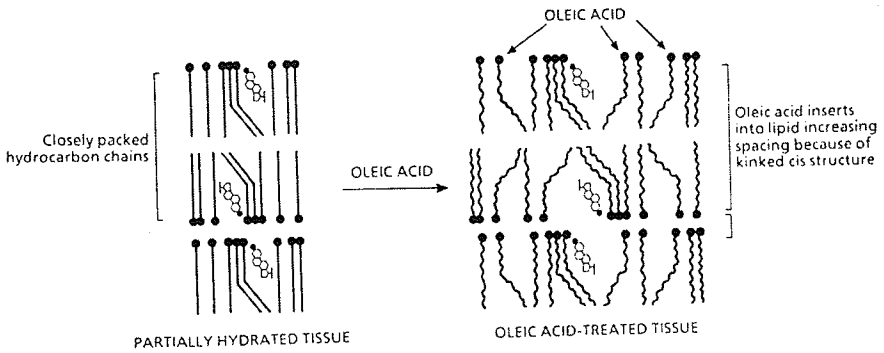


Figure 1.9. Oleic acid increases the fluidity of intercellular lipids of the stratum corneum (Barry 1987).

1.5.1.5. Esters

Several alkyl esters, such as ethyl acetate and isopropyl myristate (IPM), are effective skin penetration enhancers for a number of drugs. There is currently little information concerning how these chemicals act on the skin to alter permeability. There is some evidence to suggest that ethyl acetate extracts lipids from the stratum corneum, which would lower the diffusional resistance of the skin (Chattaraj and Walker 1995). IPM was suggested to have a direct action on the SC, permeating into the lipoidal bilayers of the membrane, hence increasing fluidity of the membranes and promoting permeation of biomolecules (Sato *et al.* 1988).

1.5.1.7. Azone

Azone or laucopram, chemically 1-dodecylazacycloheptа-2-one, has been investigated as a potential penetration enhancer for many drugs (Stoughton and McClure 1983); Hadgraft and Williams, 1993). Differential scanning calorimetry studies have shown that azone does not enter the corneocytes in any significant amount, therefore suggesting that it partitions directly in to the lipid bilayer structure and disrupting it (Barry 1987; Hadgraft and Williams 1993; Naik and Guy 1997). The mechanisn of action was studied using the dipalmitoyl phosphatidylcholine (DPPC) multilammelar vesicles as a model (Hadgraft *et al.* 1996). In the case of azone there was an expansion of the area per molecule compared to ideality, whereas for N-0915 there was an indication of compression, i.e. the molecules of N-0915 pull the DPPC molecules together by favorable molecular interaction.

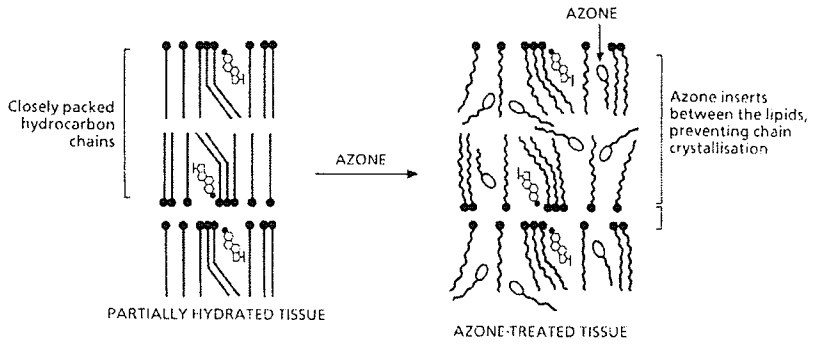


Figure 1.10. Azone modifies the permeability of human stratum corneum by interacting with intercellular lipids (Barry 1987).

Further evidence (Abraham *et al.* 1995; Potts and Guy 1995; Roberts *et al.* 1996) suggests that the H-bonding power of the penetrant is the major determinant of penetration. It is reasonable, therefore, to infer that modification of H-bonding within stratum corneum lipids is the possible mode of action of modifiers. Azone can bind firmly to a ceramide molecule, the displaced ceramide molecule now being unbound. Thus a region of fluidity appears in the lamella enabling penetration.

In addition to chemical penetration enhancement, there are other strategies through which the permeation of drugs through the skin can be increased (Figure 1.11.). Charged molecules do not readily penetrate stratum corneum. One technique is to ion

pair, by adding an oppositely charged species to create a more lipophilic character that diffuses across the stratum corneum. The complex partitions into the aqueous viable epidermis, where it dissociates into charged species (Megwa *et al.* 2000; Megwa *et al.* 2000; Sarveiya *et al.* 2001). Eutectic systems have been shown to promote drug permeation. This has been demonstrated by Kang et al (2000).

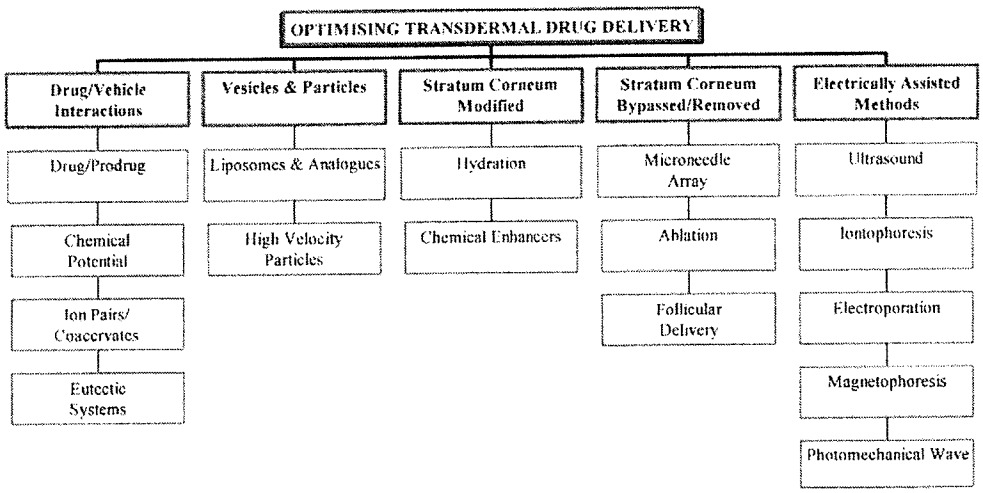


Figure 1.11. Some methods for optimising transdermal drug therapy (Barry 2001).

Liposomes and other vesicles are also being reported to modulate penetration. How well vesicles transport through skin is still debatable. Skin penetration can be enhanced by stratum corneum modification by hydration, chemical enhancers or partition promotion.

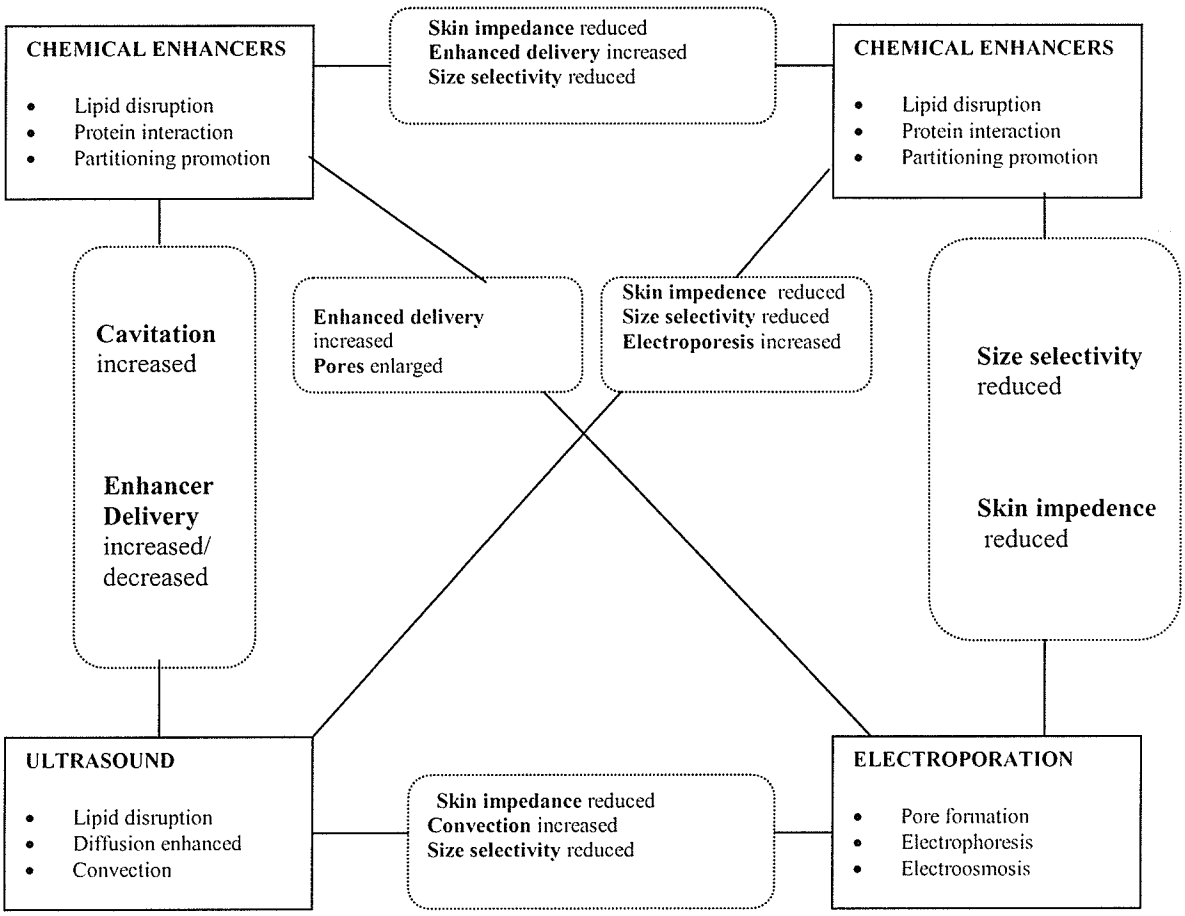


Figure 1.12. Suggested mechanisms for the actions of transdermal penetration enhancers (in main rectangular boxes) and possible synergistic actions between methods as illustrated in connecting boxes (rounded rectangles). Modified from (Mitragotri 2000)

1.5.3. Physical enhancement methods

Skin stripping using adhesive tape is commonly used to reduce the thickness of the stratum corneum, reducing the resistance and hence increasing the penetration of drugs. Stratum corneum can also be bypassed by microneedle array (Henry *et al.* 1998).

Ultrasound (sonophoresis or phonophoresis) has been used at both low (20 kHz) and high (5 to 10 MHz) levels. High-frequency ultrasound appears to disrupt

intercellular lipid lamellae (Menon *et al.* 1994), whereas low frequency induces cavitation to increase penetration. Iontophoresis is another popular technique for drug penetration enhancement. It is defined as the process of transferring ionized drugs across a membrane, such as skin, into a tissue by the use of an electrical potential difference across two electrodes. Iontophoretic delivery relies on the influence of an electromotive force repelling solute ions from an electrode of like charges and migrating to an electrode of opposite charges. Electroporation is another electrically assisted drug delivery technique and is the creation of transient, enhanced membrane permeability using short duration electrical pulse, by reversibly permeabilizing lipid bilayers and creating transient aqueous pores. Although both iontophoresis and electroporation involve the use of electrical fields, the main difference lies in their mechanism of action. Ionotophoresis acts primarily on the drug, involving skin structural changes as a secondary effect, while electroporation acts directly on the skin, making transient changes in tissue permeability (Prausnitz *et al.* 1993).

Synergism between various enhancement techniques has also been reported (Figure 1.12.). Mitragotri (2000) has published an excellent thought provoking review of synergistic interactions between chemical enhancers and ultrasound, iontophoresis or electroporation.

1.5.3. Penetration redardation

It may be desirable for a topically applied drug to stay on the surface of the skin rather than penetrate it. This is the case with applications such as sunscreening agents and insect repellants. To be effective, sunscreening agents should remain in a concentrated form on the skin surface, thereby providing a barrier between the potentially damaging UVR and living tissues. Retardation of absorption can be facilitated by the use of physical barriers or the azone analog N-0915.

Unlike Azone, N-0915 has an extra oxygen atom and thus has H-bonding groups potentially available to adjacent ceramide head groups on either side, raising the possibility of linking to both and thereby further stabilizing the structure of the lipid bilayer [Figure 1.13]. Condensation of lamella would be expected if the N-0915 ceramide bonds were stronger than the ceramide-ceramide bonds (0.18 units). It

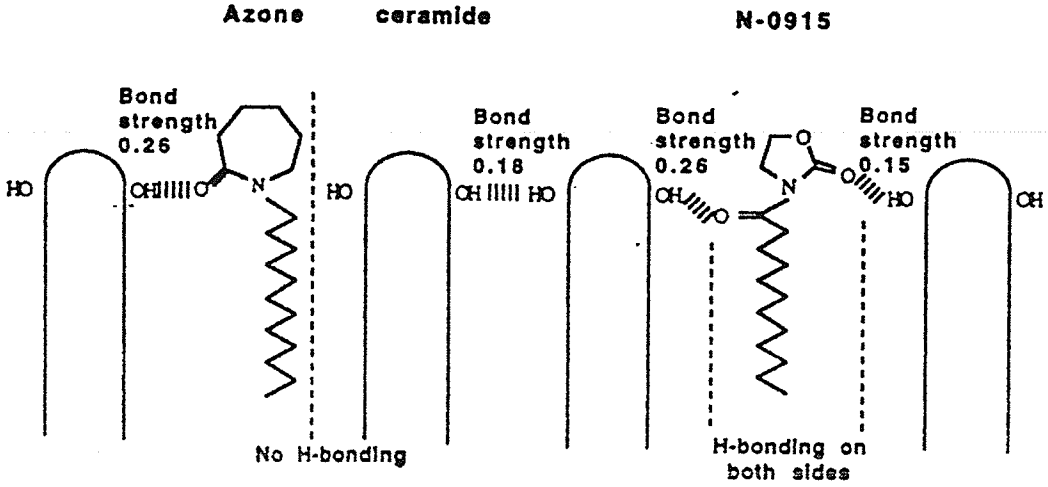


Figure 1.13. Proposed hydrogen bonding between ceramides and modified molecules (Hadgraft et al. 1996)

therefore seems possible that N-0915 acts as a retarder by producing condensation via more powerful H-bonding within the plane of polar head groups.

1.6. In vitro experimental models for assessing skin penetration

The most common method for evaluation of percutaneous absorption is the use of *in vitro* diffusion cells, and there is a plethora of literature on satisfactory performance of such experiments. Such experiments can be precisely controlled so that the only experimental variables are the skin and the test material.

1.6.1. Diffusion cell design

Diffusion cells basically consist of two compartments with a membrane clamped between the donor and receptor compartments. *In vitro* systems range in complexity from a simple two-compartment "static" diffusion cell to multijacketed flow-through cells [Figure 1.14.]. Construction materials must be inert with glass being the most common, although Teflon and stainless steel are also used. In all cases excised skin or synthetic membrane is mounted as a barrier between a donor chamber and a receptor chamber, and the amount of drug permeating from the donor to the receptor side is

determined as a function of time. Efficient mixing of the receptor phase (and where possible the donor phase) is essential, and the sample removal procedure should be simple. Neither of these processes should interfere with diffusion of the permeant. Comprehensive reviews on diffusion cell design are available (Flynn and Smith 1971; Nugent and Wood 1980). Continuous agitation of the receptor medium, sampling from the bulk liquid rather than the side arm, and accurate replenishment after sampling are important practical considerations. It is essential that air bubbles not be introduced below the membrane during sampling.

Static diffusion cells are usually of the upright ("Franz") or side-by-side type, with receptor chamber of varying volumes. Cell dimensions should be accurately measured and precise values used in subsequent calculations. Flow through cells can be useful when the permeant has a very low solubility in the receptor medium. Sink conditions are maximized as the fluid is continually replaced using a suitable pump (at

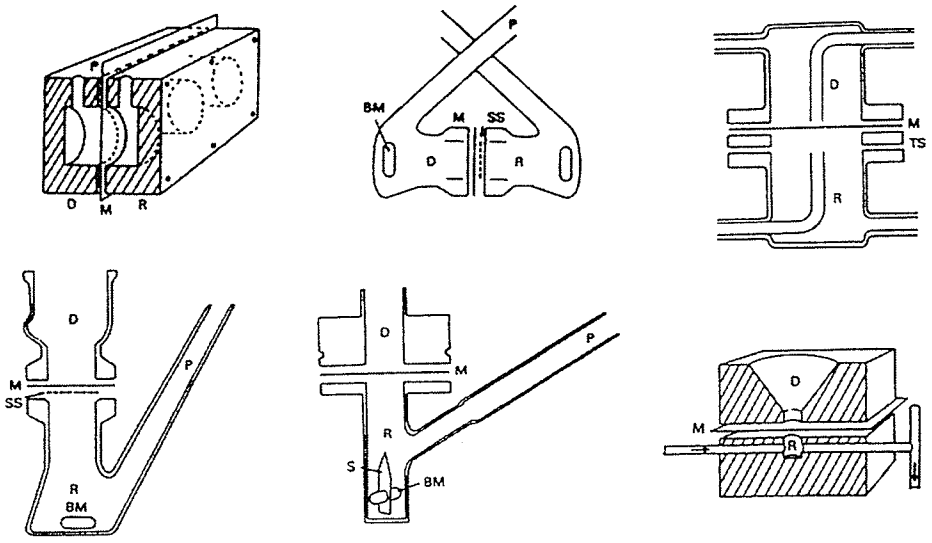


Figure 1.14. Examples of diffusion cells used to study percutaneous absorption. Key: D, donor compartment; R, receptor compartment; M, membrane; P, sampling port; BM, bar magnet; SS, Stainless steel support; TS, Teflon support; S, polyethylene sail.

a rate of ~1.5 ml/h) (Bronaugh 1996). However, the dilution produced by the continous flow can pose problems with analytical sensitivity, particularly if the permeation is low. Flow through and static systems have been shown to produce equivalent results. Automated systems can allow unattended sampling, and commercial systems are available.

1.6.2. Receptor chamber and medium

Receptor chamber dimensions are constrained by the conflicting requirements of guaranteeing that the receptor phase can act as a sink while ensuring that sample dilution does not preclude analysis. A large receptor volume may ensure sink conditions but will reduce analytical sensitivity unless large samples can be taken and subsequently concentrated.

The ideal receptor phase provides an accurate simulation of conditions pertaining to in vivo permeation of the test compound. As a general rule, the concentration of the permeant in the receptor fluid should not be allowed to exceed ~10% of saturation solubility (Skelly *et al.* 1987). Excessive receptor phase concentration can lead to a decrease in the rate of absorption, which may result in an underestimate of bioavailabilty. The most commonly used receptor fluid is pH 7.4 phosphate-buffered saline (PBS), although this is not always the most appropriate material, if the solute has limited aqueous solubility. It has been postulated that if a compound has a water solubility of <10 μg/ml, then addition of solubilizers such as such as ethanol and bovine serum albumin becomes necessary. Addition of solubilizers such as ethanol and bovine serum albumin is very common.

The problem of very lipophilic permeants has been addressed by the use of nonaqeous and non-liquid receptor media. Sheets of silicone rubber (0.02 in. thick) were used to collect pesticides with low water solubility, which were then desorbed from the rubber with an appropriate solvent and subsequently analyzed. Other solutions have included the use of flowing gaseous receptor phases for volatile permeants (Leinster *et al.* 1986).

1.6.3. Barrier membranes

A major potential variant in the design of *in vitro* skin diffusion experiments is the nature of the skin membrane. A membrane used in an *in vitro* study should simulate, as close as possible, the barrier layer in human skin. This is best achieved with human skin. Animal skin is widely used as substitutes for human skin (Wester 1980; Kadono *et al.* 1998) primarily due to difficulties in obtaining human skin. Human skin for *in vitro* is subject to variability due to age, race, anatomical site, and gender of the donor. Studies have also addressed the issues of intra- and intersubject variability in human skin permeability. Numerous authors have documented differences between the permeabilities of skin based on its racial origin. White skin is slightly more permeable than black skin (Wedig and Maibach 1981; Berardesca and Maibach 1990), which correlates with the observation that black skin has more cell layers within the stratum corneum (Weigand et al. 1974) and a higher lipid content (Reinertson and Wheatley 1959). Despite these variabilities, human skin is still the best choice for in vitro permeation studies.

Many studies use model synthetic membranes to evaluate the effect of changing vehicle and formulation variables on the observed overall drug availability. The membrane physically separates the donor formulation from the receptor phase, and the release rate is determined by the formulation. Solute transport through a membrane takes place either by partitioning into and diffusion through distinct membrane phase or by diffusion through a continuous donor-membrane-receptor phase via channels. Polydimethylsiloxane membrane is a commonly used non-polar membrane. It is highly permeable and characterized as a non-porous, homogenous hydrophobic barrier. Polyethylene and cellulose acetate are other membranes being used for diffusion. *In vitro* penetration studies using a model membrane system can provide useful information regarding vehicle effects, and can be particularly useful in the investigation of mechanism and the validation of theoretical concepts.

Chapter 2. Increased membrane diffusion of ibuprofen by ion pair formation

2.1. Introduction

The class of drugs known as "NSAID's" (non-steroidal anti-inflammatory drugs) are among the most commonly used medications (Garner 1992). They are prescribed largely for their anti-inflammatory, antipyretic, and analgesic properties but some are also very widely used in over-the counter preparations for the same indications. The extensive use of prescribed and over-the-counter non-steroidal anti-inflammatory drugs (NSAID's) is associated with significant adverse effect profiles which has prompted the search over recent years for solutions to this problem (Hansen *et al.* 1984; Figueras *et al.* 1994; Johnson *et al.* 1995; Lichtenstein *et al.* 1995). Strategies have included attempts to minimize NSAID use by education and legislation, co-administration of other (usually gastroprotective) agents, development of potentially better tolerated drugs such as cyclo-oxygenase-2 (COX-2) inhibitors or NSAID's incorporating nitric oxide together with modification of delivery systems (Wallace JL 1997; Vaile and Davis 1998). The possibility of delivering NSAID'S through the skin for either local or systemic effects is being increasingly investigated (Marty *et al.* 19889) (Hadgraft 1989; McNeill *et al.* 1992). There are conflicting reports in the literature concerning the actual depth and quantity of these drugs delivered to the local subcutaneous structures after topical application. Local direct deep tissue penetration of salicylate (Rabinowitz *et al.* 1982), ibuprofen (Giese 1990), and diclofenac (Riess *et al.* 1986) was demonstrated in dogs (Rabinowitz *et al.* 1982), pigs (Baldwin *et al.* 1984), guinea pigs (Giese 1990) and humans (Riess *et al.* 1986) respectively. Yano *et al.*(1986) studied the absorption of many drugs and showed that maximum absorption was observed with drugs that had a log P_{oct} of between 2 and 3. A similar parabolic relationship was observed between the log of the permeability coefficient for a group of NSAID's across human epidermal membrane *in vitro* and their respective log P_{oct} values with a optimal log P of around 3 (Singh and Roberts 1994).

Monteiro-Riviere et al. (1993) investigated the effect of the anatomy of the cutaneous vasculature of pigs on the penetration of piroxicam to the underlying masculature. The authors claimed that the pig has two different types of vasculature, cutaneous and musculo-cutaneous, at two different sites, the caudal and cranial,

respectively. They concluded that the musculo-cutaneous vasculature acts as a conduit for the piroxicam to the deeper tissues. However in an attempt to justify their choice of animal model, it remains to be seen whether the cutaneous vasculature in humans is similar to that of pigs. In a study to demonstrate the bioequivalence of oral ^{14}C-aspirin with topically applied triethanolamine ^{14}C-salicylate, it was shown that the local tissue levels were higher for the topically applied formulation than the orally administered formulation (Rabinowitz *et al.* 1982). It was also highlighted that the cutaneous vasculature of the skin does not act as an infinite sink and that penetration of the drug to the subcutaneous and other structures is a concept that ensures either bioequivalence to oral administration, or enhanced local tissue levels, but this has only been demonstrated to a limited depth with the drugs used.

By including vasoconstrictors or vasodilators in a topical formulation, it may be possible to control the degree of clearance of a permeant by the cutaneous vasculature. This theory was tested by Singh and Roberts (1994) in rats using the vasoconstrictor, phenyl epinephrine, to reduce the cutaneous blood flow and decrease the clearance of salicylic acid, lidocaine and tritiated water, with the result of elevating their levels in deeper tissues underlying the application site.

However, the transdermal use of NSAID's is limited because of their reduced penetration through the skin. Many types of penetration enhancement have been studied for topical and transdermal delivery of NSAID's. These range from physical methods of enhancement such as iontophoresis and phonophoresis (Bender 1995) (Okabe *et al.* 1989) to chemical methods, which incorporate enhancers in their formulation. The ability of many types of chemicals to enhance the penetration of NSAID's across skin has been recorded in the literature and some of the more widely studied enhancers are oleic acid (Francoeur *et al.* 1990), Azone (Ito *et al.* 1988; Tian *et al.* 1992), and the terpenes (Katayama *et al.* 1992). Furthermore, many topical formulations employ the use of vehicles that are also reported to give enhanced penetration enhancement.

One of the more attractive forms of penetration enhancement involves increasing the thermodynamic activity of a drug in a vehicle beyond that of a saturated solution to one in which it is supersaturated. (Iervolino *et al.* 2000) increased the penetration of ibuprofen using supersaturated solutions. Another method of penetration

enhancement involves chemical modification of the active form of a drug to a prodrug (Jona *et al.* 1995) (Davaran *et al.* 2003). Ion pairing is one of the very recent and novel techniques under investigation to improve the penetration of drugs across the skin.

Many of the clinically accepted drugs for delivery through the skin are of low molecular size, lipophilic and effective at low doses. Furthermore, the majorities of drugs are weak acids or bases, and are ionized under normal physiological conditions. The human stratum corneum acts as a significant barrier for the skin penetration of these hydrophilic ionizable drugs. Charged species are known to be poor penetrants across skin, other biological membranes and non-porous polymers. Their permeation coefficient has been estimated to be about 10^4 times smaller than for the respective uncharged species (Swarbrick *et al.* 1984). Many strategies, including the use of penetration enhancers, have been exploited to increase the penetration of drugs through the stratum corneum (Yu *et al.* 1988; Michnaik *et al.* 1995; Suh and Jun 1996; Wang *et al.* 1997). However, as many of the skin penetration enhancing chemicals have the potential to cause skin irritation (Okamoto *et al.* 1988; Wong *et al.* 1989; Okabe *et al.* 1990), more effective and safer penetration enhancement techniques need to be developed.

The formation of ion-pairs has been investigated for the enhancement of membrane permeability and hence bioavailability of hydrophilic ionized molecules. The theory is that when oppositely charged molecules interact, this association reduces or neutralizes the overall electrostatic charge of the ion-pair molecule that is formed. The consequent increase in lipophilicity of the ion-pair compared to the ion results in increased permeation of the molecule through a membrane (eg. intestinal, skin or synthetic).

Early studies on ion-pair transport focused on absorption from the gastrointestinal tract. Wilson and Wiseman (1954) were among the first to test the ion-pair hypothesis for the lipophilisation of the ionic drug tropsium. They reported an enhanced transfer rate across everted intestine using alkylsulphonates as counter ions. Irwin *et al.* (1969) reported an increased rate and efficiency of gastrointestinal absorption of isopropamide using trichloroacetate as the counter-ion. Gasco and colleagues (1984) reported an increase in the bioavailability of propranol in the

presence of taurodeoxycholate. Further, hexylsalicylate was found to be capable of enhancing the bioavailability of hydrophilic drugs such as pholedrine and bretylium after oral and rectal application, respectively.

The concept of forming ion-pairs to increase the skin permeability of hydrophilic drugs has also been reported (Hadgraft and Wotton 1984; Hadgraft *et al.* 1985; Hadgraft *et al.* 1986; Young *et al.* 1988; Pedersen. 1990). Kadono and colleagues (1998) reported increased penetration through shed snake skin of salicylate by ion-pair formation with alkylamines. Megwa and colleagues (2000) also found increased skin penetration and local tissue deposition of salicylate in the presence of alkylamines. In a further study, these researchers showed that secondary, tertiary and quaternary amines increased the permeation of salicylates through human epidermal membranes *in vitro* (McNeill *et al.* 1992). Permeability enhancement was greatest with tertiary amines and was found to increase with alkyl chain length. Increase in skin penetration of lignocaine (Valenta *et al.* 2000) and ondansetron (Takahashi and Rytting 2001) by ion-pair formation has also been reported. Although, most of these studies describe the ion-pair approach as the means to increase the permeation of drugs across biological and synthetic membranes few have provided direct evidence of ion-pair formation. This chapter and the next chapter deal with ion pair strategy as a technique to enhance the penetration of model NSAID's ibuprofen and benzydamine.

The objective of this study was to determine the significance of ion-pair formation on the permeation of ibuprofen (Figure 2.1.). The solubility of ibuprofen sodium was measured over a range of pH values. The saturated solutions obtained from the solubility determination were used to measure the diffusion at various pH values through a polydimethylsiloxane (PDMS) membrane. In further investigations, the effect on membrane permeability of a number of amine counter ions was examined. Nuclear

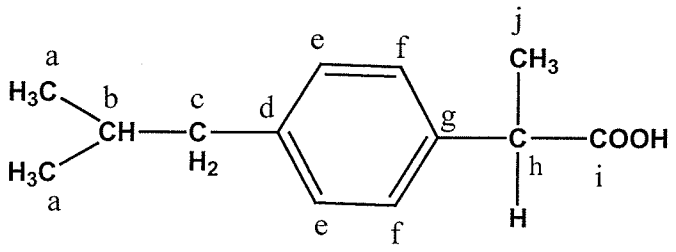


Figure 2.1. Structure of ibuprofen molecule

magnetic resonance (nmr) spectroscopy was utilized to establish the presence of ion-pair formation between ibuprofen and the respective amine counter ion. In addition, the partition characteristics of ibuprofen sodium and various organic ibuprofen salts were examined using an n-octanol-aqueous buffer system.

2.2. Materials and methods

2.2.1. Materials

Ibuprofen sodium, ethylamine hydrochloride, diethylamine hydrochloride, triethylamine hydrochloride and ethylene diamine dihydrochloride were from Sigma (St. Louis, MO, USA). PDMS membrane (thickness 0.005″) from Pillar Surgical (CA, USA). HPLC-grade acetonitrile and methanol (Fisher Scientific, USA) were used, and all other chemicals were of analytical grade.

2.2.2. High-performance liquid chromatography

A Waters liquid chromatographic system equipped with a model 717 plus auto sampler, model 600S controller and 996 photodiode array detector was used. Separation was achieved on a Symmetry C_{18} column (5μm, 3.9 x 150mm I.D., Waters Inc. MA, USA) at ambient temperature with an inline prefilter. Integration was undertaken using a PC with Millenium$^{3.2}$ version software.

The mobile phase consisted of dilute phosphoric acid adjusted to pH 2.2: acetonitrile [40:60], filtered through a 0.45 μm membrane filter (Durapore membrane filter, Millipore). The mobile phase was continuously degassed before and during use. The flow rate was 1.0 mL/min and the detection wavelength was 220 nm. The retention time for ibuprofen was ~ 4.8 min. Calibration curves were calculated on peak area measurements.

2.2.3. *NMR spectroscopy*

^{1}H and ^{13}C NMR spectra were recorded at 300 and 75 MHz respectively using a Bruker Avance 300 spectrometer (Karlsruhe, Germany). Samples were dissolved in deuteromethanol and chemical shifts (δ) for hydrogen and carbon resonance reported in ppm relative to TMS.

2.2.4. *Solubility-pH profile*

An excess of ibuprofen sodium was added to phosphate buffers with different pH values of 4.0, 5.0, 6.0, 7.0 and 8.0 in screw capped vials and stirred in the dark at 25 °C for 48 h. The pH of the resultant mixture was determined during this period and adjusted to the required value by adding phosphoric acid or KOH. The mixtures were then centrifuged at 10,000g for 10 min and the supernatants analyzed for ibuprofen content using HPLC. The pH of the solutions was confirmed after centrifugation. All experiments were repeated four times.

2.2.5. *Synthesis of ibuprofen salts*

Equimolar amounts of ibuprofen sodium and amine hydrochloride (ethylamine hydrochloride, diethylamine hydrochloride, triethylamine hydrochloride or ethylene diamine dihydrochloride) were dissolved in methanol and stirred for 24 h. The cloudy mixture was then filtered through a 0.45 μm membrane filter (Durapore). The precipitate was collected, weighed and was shown to be sodium chloride (NaCl) by reaction with silver nitrate. The molar yield of sodium chloride was more than 90% of the expected amount in all synthesis. The solvent from the clear filtrate solution obtained after filtration, was evaporated and the residue was dried in vacuo for 24 h with P_2O_5 as a drying agent. The salts were dissolved in deuteromethanol and ^{1}H-NMR and ^{13}C-NMR used to confirm the presence of ion-pairs in solution.

2.2.6. *Permeation Experiments*

In vitro permeation studies across a PDMS membrane were performed in Pyrex glass Franz-type diffusion cells. The membrane was immersed in de-ionized distilled water for 1 h before use. PDMS membrane (cross sectional area of 1.18 cm^2) was then mounted between the donor and receptor compartments of diffusion cells and the assembly held in place with a plastic clamp. The diffusion unit was immersed in a water bath at 37 °C. Phosphate buffer, pH 7.0 (approx. 3.5 mL) was the receptor fluid. For

permeation at different pH values the donor phase was 1 mL of the saturated solution at that particular pH. After equilibration with the buffer, 1.0 mL of the donor solution was added to the donor cell. A magnetic stirrer driven by an external magnet continuously stirred the receptor compartment at the same speed for all cells. Samples of the receptor phase were withdrawn and replaced by drug-free buffer at appropriate times throughout the 6 h period of the experiment. The ibuprofen content in the receptor phase was determined using HPLC. Experiments were repeated four times.

The cumulative amount of drug released through the PDMS membrane, Q(t), was determined from Q = (CV)/A, where C is the concentration of ibuprofen (sodium) in the receptor compartment in μg/mL for the corresponding sample time t, V is the volume of fluid in the receptor phase and A is the diffusional area of the membrane. The flux of ibuprofen through the membrane into the receptor from each of the formulations was determined from the slope of the plot of cumulative amount in the receptor phase versus time and expressed as μg cm^{-2} h^{-1}. Permeability coefficients were calculated for ibuprofen for each formulation.

2.2.7. Apparent partition coefficient

The apparent partition coefficients were investigated between n-octanol and phosphate buffers at various pH values. Each phase had been pre-saturated with the other by equilibration overnight before the experiments. A known amount of ibuprofen sodium was dissolved in buffers of different pH values to which n-octanol was added. The mixture was stirred continuously for 24 h at 25°C. After phase separation the ibuprofen content in the buffer was analyzed by HPLC. Since the initial amount of ibuprofen sodium was known, the amount in the organic phase was determined by difference.

2.2.8. Diffusion and partition of ibuprofen salts

The apparent partition coefficients and the diffusion studies were performed as described above. Phosphate buffer, pH=7.0, was used as an aqueous phase for determination of apparent partition coefficient. The diffusion studies were conducted containing 2% solutions of ibuprofen or its equivalent of the amine salt, using propylene glycol as the solvent. The ibuprofen content was analyzed using HPLC.

2.2.9. *Statistical analysis*

The difference between the flux of ibuprofen for the infinite dose application (saturated solutions) at different pH values, and for different ibuprofen salts was assessed using multiple regression with pair-wise comparison. One-way ANOVA, followed by Tukey's HSD post hoc test was used for assessing the difference in solubility due to pH or salts.

2.3. *Results and discussion*

2.3.1. *Solubility*

Ibuprofen is relatively non-polar, and accordingly its highest solubility is obtained in solvents of lower solubility parameter values such as acetone, ethyl acetate, and lipophilic alcohols. Solubility decreases in most polar solvents. By replacing the acidic proton by sodium, the region of maximum solubility is shifted to larger solubility parameter values as compared to the parent acid (Bustamante *et al.* 2000). As expected the solubility of ibuprofen sodium (pKa = 4.45; (Avdeef *et al.* 1998)) increases with increase in pH. The solubility profile is summarized in Table 1. The solubility at pH 7 and 8 is significantly greater than at pH 4, 5 and 6 ($p<0.001$). There is no significant difference in solubility from pH 7 to 8.

2.3.2. *Solvent-membrane interactions*

Percutaneous absorption involves partitioning of a solute from its vehicle into the skin and subsequent diffusion of solute through the skin. Identical solute flux would be expected from solutions in which the solute had equal thermodynamic activity. Non-ideal behavior is a result of solute and/or solvent interaction with the membrane (Twist and Zatz 1990; Jiang *et al.* 1998). In this situation the physicochemical properties of the barrier will change depending upon the interaction involved (Roberts and Anderson 1975). Therefore, it is often difficult to interpret results due to the highly complex nature of the stratum corneum. A synthetic membrane, such as PDMS membrane, offer advantages concerning the physicochemical properties of the diffusional barrier including perm selectivity, high diffusivity, thickness control, and less stringent storage and handling requirements. Moreover, solvents (e.g., water, glycerin, propylene glycol, and polyethylene glycol 400) are not sorbed to a significant extent by this material and behave as ideal vehicles; permeation from these vehicles is a function only of permeant

activity (Twist and Zatz 1988). Hence to determine the appropriate permeant activity and to eliminate membrane-solvent-solute interaction, PDMS membrane was chosen for the studies. Propylene glycol and water were used as solvents, since they are not significantly sorbed by this membrane. The flux through PDMS membranes is usually faster as compared to the human skin barrier, but the permeability relationships and trend are very similar (Valenta *et al.* 2000).

2.3.3. pH and penetration

Ibuprofen is a non-steroidal anti-inflammatory drug (NSAID) that has been formulated into a number of topical preparations. Its solubility and diffusion parameters as a function of pH, have been documented (Watkinson *et al.* 1993; Hadgraft and Valenta 2000). The low solubility of ibuprofen is one of the factors responsible for its reduced transfer across the skin. Sodium salts are more soluble than the parent drug, thereby increasing the amount of drug in solution in the aqueous vehicle. However, increasing the polar nature of the permeant will reduce its tendency to permeate the lipophilic stratum corneum. The overall effect on permeation will be a combination of these effects.

Donor depletion was observed at pH 4, 5 and 6, therefore flux was calculated using the cumulative amounts from the first 4 sampling times. The highest flux was determined at pH 7 (Table 2.1.). Flux increased as pH increased from pH 4 to pH 7 and then decreased as shown in Fig. 2.2. The total flux (J_{tot}) of a permeant through a membrane is a composite term, contributed by the diffusion of both the ionized and unionized moieties. The transport across the membrane can be described by the permeabilities of the ionized and unionized species and their respective concentrations $k_{p\ (ion)}$, $k_{p\ (union)}$, c_{ion}, and c_{union} respectively by the following equation (Hadgraft and Valenta 2000).

$$J_{tot} = k_{p\ (union)} * c_{union} + k_{p\ (ion)} * c_{ion}$$

Table 2.1. Solubility, log P values (n-octanol: buffer), permeability coefficient and steady state flux through PDMS membrane for ibuprofen-Na at different pH values.

pH	Solubility [mg/mL]	Log P	k_p (cm/h)	Fraction unionized	Flux $\mu g/cm^2/h$
4	0.028 ± 0.0007	-	2.297	73.81	64.13 ± 1.80
5	0.156 ± 0.008	3.28 ± 0.007	0.398	21.98	62.36 ± 0.91
6	1.0 ± 0.05	2.42 ± 0.02	0.187	2.74	187.10 ± 12.30*
7	340.51 ± 31.30	0.92 ± 0.04	0.00081	0.28	277.23 ± 4.23*
8	299.035 ± 21.4	0.63 ± 0.01	0.000126	0.03	37.83 ± 3.50*

Values represent the mean ± S.D. (n=4), *significantly different from value at pH = 4; $p<0.002$
LogP - between octanol and phosphate buffer pH=7.0

The ambient pH and the pK_a will give the relative amounts of ionized and unionized species. In the case of ibuprofen sodium, flux at lower pH is being dominated by the first term (unionized species), whereas at higher pH it is dominated by the second term (ionized species). Figure 2.2. shows the relationship between permeability coefficient as a function of percent of unionized ibuprofen present. The percent of drug that was ionized or unionized at a particular pH was calculated using the Henderson-Hasselbalch equation.

A linear relationship [$y = 31.984x + 1.322$; $r^2 = 0.978$] between the permeability coefficient and fraction ionized suggests that the diffusion was mostly as a result of partition and transfer of unionized ibuprofen present in the donor phase, and the insignificant intercept indicates the contribution of ionized species. A plot of permeability of ibuprofen as a function of pH (Figure 2.3.) also follows a reasonable trend expected of an acidic compound. The permeability coefficient of the ibuprofen

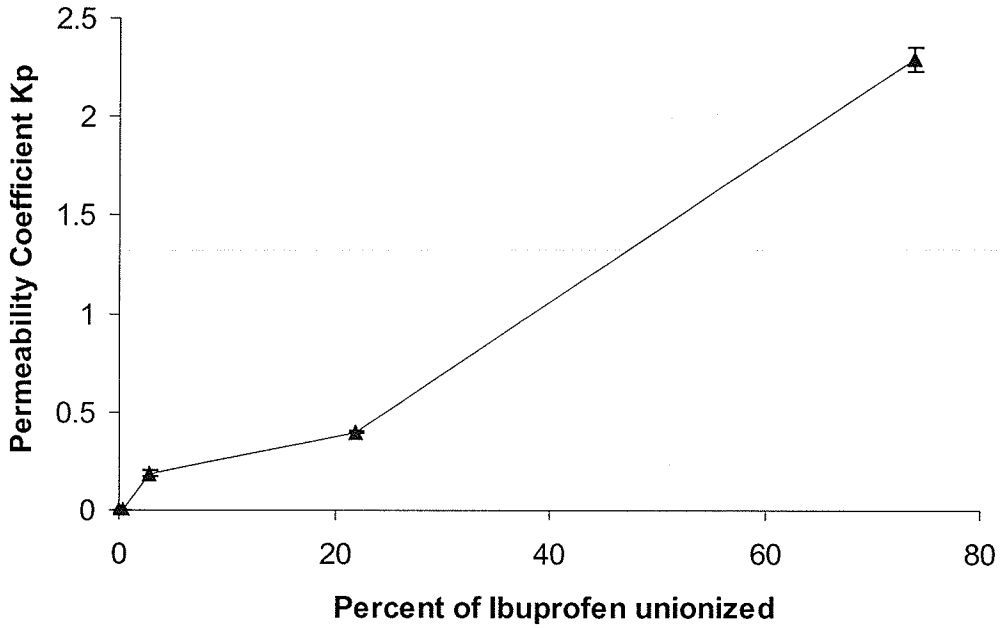


Figure 2.2. Relationship between percent of ibuprofen unionized and permeability coefficient (values : mean ± S.D).

sodium increases with decrease in pH. The highest permeability coefficient was determined at pH 4 (Figure 2.3.), when more than 50% of ibuprofen sodium is unionized (pK_a = 4.45, (Avdeef *et al.* 1998). It is interesting to note that the steady state flux of ibuprofen sodium is greater at higher pH, whereas its permeability coefficient is higher at lower pH when the fraction of unionized species is greater. This suggests that at higher pH the lower permeability of the ionized species is more than compensated for by the increased solubility, which is consistent with the findings of previous studies (Watkinson *et al.* 1993).

2.3.4. Partitioning experiments

The apparent partition coefficients were investigated between n-octanol (representative of skin lipids) and phosphate buffers at various pH values (see Table 2.1.). As expected, octanol:buffer partition coefficient increased with decrease in pH from pH 8 to 5. No drug was found in the aqueous phase at pH 4 indicating a very high partitioning into octanol for the unionized species.

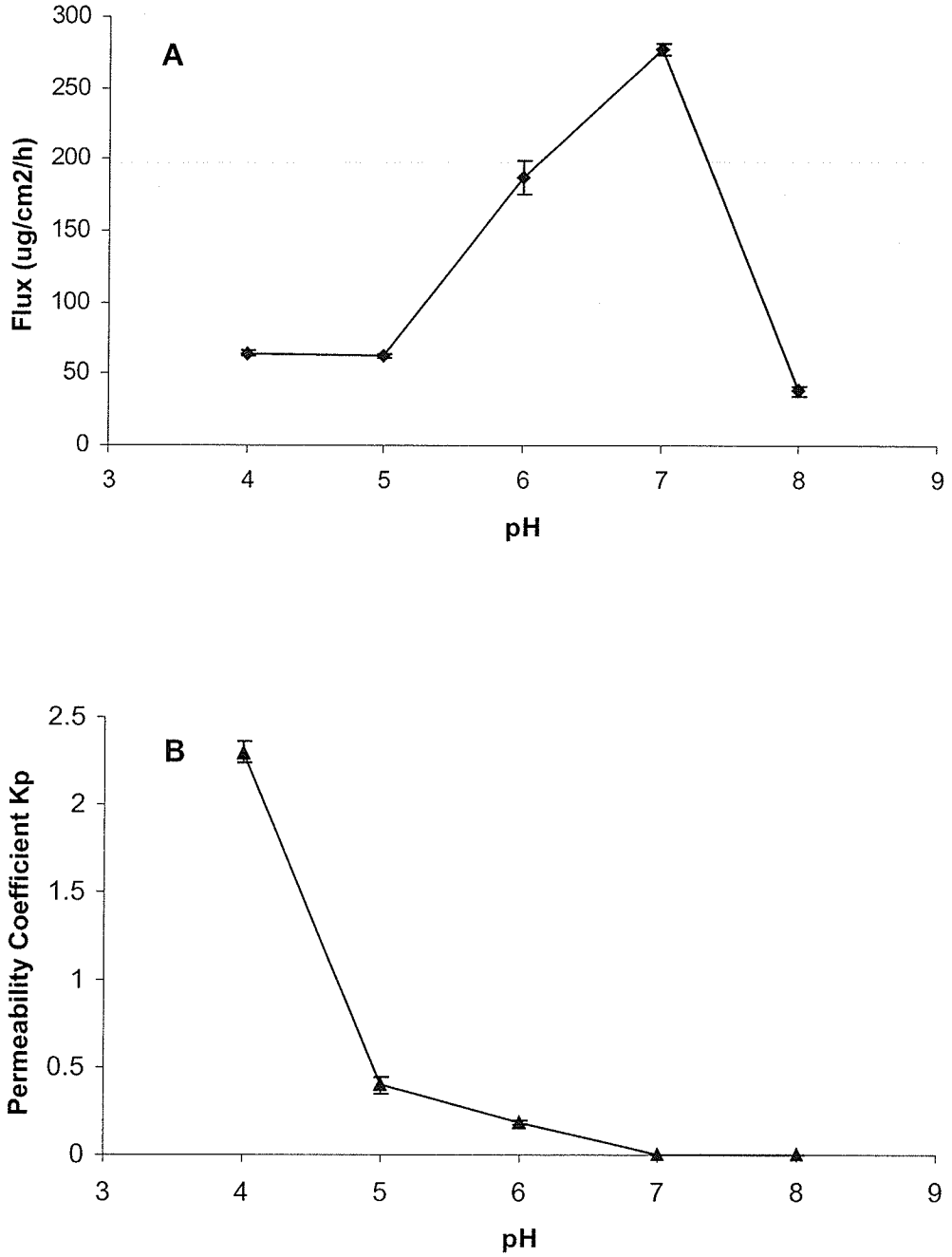


Figure 2.3. The relationship between steady state flux (A) and permeability coefficient k_p (B) with respect to change in pH. (values : mean ± S.D)

2.3.5 NMR Spectroscopy

The goal of both the ^{1}H and ^{13}C NMR measurements (see Tables 2.2., 2.3a and 2.3b) was to obtain evidence for the presence of ion-pair formation between ibuprofen (Figure 2.1.) and their respective amine salts, from the chemical shift changes to protons and carbons near the cationic and anionic charges. Ion-pair formation would be an indicator of increased permeability of the salt through skin.

Comparison of the proton spectra of ibuprofen and ibuprofen sodium, and the amine hydrochloride and ibuprofen salts in CD_3OD showed no significant changes in chemical shifts (Table 2.3a). Relative to the chemical shifts in the corresponding amine, the ^{1}H NMR spectra of the primary, secondary, tertiary and quaternary ethylamine hydrochloride and ibuprofen cations show small deshielding shifts in both the methylene and methyl protons adjacent to the nitrogen (Table 2.2.). The protons on the carbon adjacent to the nitrogen are recognized by their downfield position in the salt as compared to the free amine (Anderson and Silverstein 1965). The deshielding effect for primary, secondary and tertiary amine salts is smaller in the ibuprofen salts than in the corresponding hydrochlorides, indicative of greater charge neutralization as predicted from ion-pair formation. The largest downfield chemical shift is observed in the methylene group in the quaternary salts where charge separation is greatest and, therefore charge neutralization least, because of steric hindrance.

^{13}C NMR provided significant evidence for ion-pair formation between ibuprofen and the corresponding amine. The carboxylic acid group in ibuprofen (RCOOH) shows a chemical shift of carbon (i) at 178.55 ppm while the sodium salt ($RCOO^{-}Na^{+}$) shows the carboxylate anion at 186.67 ppm (Table 2.3b). Based on these values for complete protonation (or deuteration) in the acid and complete ionization in the sodium salt, intermediate chemical shift values would reflect the degree of charge neutralization resulting from interaction between the carboxylate anion and the nitrogen cation in the ibuprofen amine salts. The ammonium salt (181.96 ppm) indicates considerable charge neutralization which may be partly stabilized by solvation of an ion-pair including hydrogen bonding interactions as well as electrostatic attraction. Less charge neutralization occurs in the quaternary salt (183.39 ppm), where hydrogen

bonding interactions cannot occur and close contact between the charges is sterically hindered. The degree of neutralization in the quaternary salt is equivalent to that observed for the primary amine (183.56 ppm) but must result from a different mode of interaction in each case. As the lipophilic character resulting from increased ethyl substitution of the amine salts increases, ion-pair formation, as indicated by chemical shift changes as a measure of charge neutralization, is favored. This is shown by a decrease in the chemical shift values from the primary (183.56 ppm), to the secondary (182.79 ppm), and to the tertiary amine (178.74 ppm). The similarity of the chemical shifts for ibuprofen and the tertiary amine salt indicates a high degree of charge neutralization consistent with extensive close ion-pair formation. Tertiary amines have been previously reported to form more stable ion pairs when compared with primary and secondary amines (Megwa *et al.* 2000). Similar, but smaller chemical shift changes are observed in the methyl group (j) and the aromatic ring carbon (g). Correlation of the chemical shifts of the amine salts of ibuprofen with the carbon (h) adjacent to the carboxylate anion is not possible because this signal is obscured by the solvent protons. The similarity of the chemical shift for the carboxylate carbon in the primary amine salt with the mono ibuprofen salt of ethylenediamine is consistent with a similar degree of interaction between these primary amine salts and the carboxylate anion.

Relative to the corresponding amines, the ^{13}C NMR spectra of the cations in the primary, secondary, tertiary and quaternary hydrochloride and ibuprofen salts show significant deshielding in the cation of the methylene carbon and increased shielding of the methyl carbon (Table 2.2.). This reversal of shielding may be due to the conformational location of the methyl groups with respect to the C-C bonds. Nevertheless there is a consistent correlation between the chemical shift changes observed in all four series of salts. Similar, but smaller chemical shift differences are observed in the methylene carbon of the ibuprofen salts compared with the hydrochloride salts, again indicative of greater charge neutralization and ion-pair formation. No significant change is observed in the methyl carbons between the ibuprofen and hydrochloride salts.

Table 2.2. ^{1}H and ^{13}C chemical shifts of amines, amines hydrochloride and ibuprofen amine salt.

	^{1}H		^{13}C	
AMINE	CH2	CH3	CH2	CH3
1°	2.69	1.12	37.20	18.39
2°	2.63	1.13	44.64	14.95
3°	2.58	1.07	47.25	11.51
4°	-	-	-	-
AMINE HYDROCHLORIDE	CH2	CH3	CH2	CH3
1°	3.05 (+0.36)	1.35 (+0.23)	36.44 (-0.76)	13.31 (-5.08)
2°	3.09 (+0.46)	1.35 (+0.22)	43.77 (-0.87)	11.88 (-3.07)
3°	3.27 (+0.69)	1.38 (+0.31)	48.02 (-0.77)	9.63 (-1.88)
4°	3.34	1.32	53.50	7.87
IBUPROFEN AMINE SALT	CH2	CH3	CH2	CH3
1°	2.91 (+0.22)	1.24 (+0.12)	36.05 (0.39)	13.31 (-5.08)
2°	2.96 (+0.33)	1.25 (+0.12)	43.49 (-0.28)	11.77 (-3.18)
3°	3.21 (+0.63)	1.33 (+0.26)	-	9.39 (-2.12)
4°	3.30	1.30	53.44	7.79

The numbers in parenthesis () indicate the difference in chemical shifts of the salt with respect to the corresponding amine base.

Table 2.3a. ^{1}H NMR chemical shift, δ, of ibuprofen and its salts for proton on carbon

		Na^+	1°	2°	3°	4°	Diamine
	RCOOH	$RCOO^-Na^+$	$RCOO^-N^+H_3Et$	$RCOO^-N^+H_2Et_2$	$RCOO^-N^+HEt_3$	$RCOO^-N^+Et_4$	$RCOO^-N^+H_3CH_2CH_2NH_2$
a	0.92	0.92	0.89	0.89	0.91	0.89	0.90
b	1.87	1.84	1.84	1.84	1.85	1.83	1.84
c	2.47	2.45	2.44	2.44	2.46	2.44	2.44
e	7.11	7.07	7.06	7.07	7.1	7.05	7.06
f	7.24	7.32	7.27	7.27	7.23	7.28	7.27
h	3.61	3.61	3.57	3.58	3.68	3.56	3.58
j	1.46	1.46	1.42	1.43	1.44	1.41	1.42

Table 2.3b. ^{13}C NMR spectra of ibuprofen and its salts

		Na^+	N^+H_4	1°	2°	3°	4°	Diamine
	RCOOH	$RCOO^-Na^+$	$RCOO^-N^+H_4$	$RCOO^-N^+H_3Et$	$RCOO^-N^+H_2Et_2$	$RCOO^-N^+HEt_3$	$RCOO^-N^+Et_4$	$RCOO^-N^+H_3CH_2CH_2N\ H_2$
a	22.90	22.91	22.90	22.94	22.96	22.87	22.89	22.93
b	46.15	46.21	46.24	46.26	46.26	46.19	46.25	46.25
c	31.48	31.54	31.61	31.64	31.64	31.58	31.65	31.63
d	139.74	140.25	140.92	140.57	140.74	139.96	140.42	140.74
e	128.33	128.39	128.44	128.47	128.49	128.40	128.52	128.46
f	130.34	129.87	130.16	130.07	130.13	130.41	129.97	130.13
g	141.54	142.85	141.81	142.83	142.45	141.67	143.08	142.39
h	46.34	49.97	-	-	-	-	-	-
i	178.55	186.67	181.96	183.56	182.79	178.74	183.39	183.126
j	19.23	20.21	19.86	20.21	20.08	19.26	20.27	20.06

Therefore, these measurements provide evidence for the existence of an ion-pair between ibuprofen and amines in solution. The conclusion based on NMR measurements that the tertiary amine has the largest degree of ion-pair formation is in agreement with the changes observed in the diffusion rates for primary, secondary and tertiary salts.

An ion-pair is a pair of oppositely charged ions held together by Coulomb attraction and not by formation of a covalent bond. Experimentally, an ion-pair behaves as one unit in determining conductivity, kinetic behavior, osmotic properties, etc. Following Bjerrum, oppositely charged ions with their centers closer together than a distance

$$q = 8.36 \times 10^6 z^+ z^- / (\varepsilon_r T) \text{ pm}$$

are considered to constitute an ion pair ('Bjerrum ion pair'). z^+ and z^- are the charge numbers of the ions, and ε_r is the relative permitivity (or dielectric constant) of the medium. Solvents with lower dielectric constant favors formation of ion pair (Marcus 1985; Hwang *et al.* 2003). The distance q increases with temperature and solvents at high temperature behave like solvents of lower dielectric constants, permitting long range ion-pairing (Marcus 1985). Because the dielectric constant for propylene glycol is lower than methanol and there is evidence for ion-pair formation in methanol, therefore we assume ion-pair formation of ibuprofen with various amine counter-ions in propylene glycol. Propylene glycol has been used as the solvent in the penetration experiments as specified in the methods section.

2.3.6. Ibuprofen salts and diffusion through PDMS membrane

Propylene glycol was used as a solvent because it is not significantly sorbed by the PDMS membrane (Twist and Zatz 1990). The measured apparent partition coefficients of the ibuprofen salts into n-octanol seems to be in agreement with its lipophilicity (Table 2.4.). Ibuprofen showed the highest partition coefficient with triethylamine as a counter ion, followed by diethylamine and ethylamine. The partition coefficient of ibuprofen ethylene diamine was similar to that of diethylamine.

Table 2.4. Steady state flux and log P values of ibuprofen salts

Salt type	Log P	Flux
Ibuprofen Na	0.92 ± 0.04	3.09 ± 0.091
Ibuprofen ethylamine	0.967 ± 0.02	5.42 ± 0.092*
Ibuprofen diethylamine	1.12 ± 0.03	7.91 ± 0.14*
Ibuprofen triethylamine	1.18 ± 0.03	48.14 ± 1.34*
Ibuprofen ethylene diamine	1.11 ± 0.02	15.31 ± 0.35*

Each value represent the mean ± S.D. (n=4), *significantly different from Ibuprofen Na, a) $p<0.0005$.
Log P determined at pH 7.0.

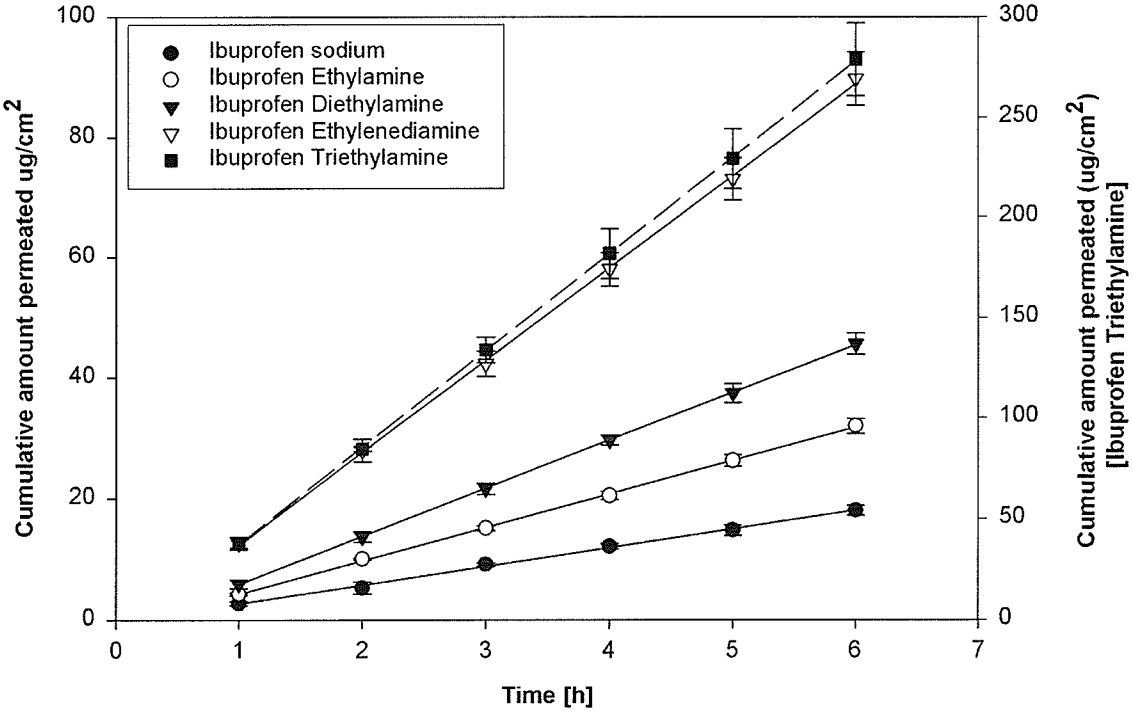


Figure 2.4. Comparison of diffusion profiles of different ibuprofen salts from propylene glycol through PDMS membrane. (values : mean ± S.D)

All the salts showed higher steady state flux for ibuprofen through the PDMS membrane when compared to the sodium salt (Figure 2.4.). Ibuprofen ethylenediamine is plotted on the right hand y axis, whereas the other salts are plotted on the left hand y axis. The highest steady state flux was measured from ibuprofen triethylamine, followed by ibuprofen ethylene diamine, ibuprofen diethylamine, and ibuprofen ethylamine. Although the partition coefficient of ibuprofen ethylene diamine was similar to ibuprofen diethylamine, its steady state flux is almost 2-fold more than ibuprofen diethylamine.

2.4. Conclusions

The results of this study suggest that it is possible to enhance the flux of salts across lipophilic membranes using an ion-pair approach. The degree of enhancement is associated with the lipophilicity, extent of ion-pairing, and reduction in the charge over the drug molecule.

Chapter 3. Influence of ion pairing on topical delivery of benzydamine

3.1. Introduction

The concept of ion pairing was discussed in detail in the previous chapter is a technique to facilitate the transdermal delivery of ionic drugs across the skin and other membranes. Ibuprofen was observed to have increased diffusion across the membrane using the ion-pair technique with various counter-amines (Sarveiya *et al.* 2001). This chapter extends the ion-pair concept from the acidic ionic drug ibuprofen to a basic ionic drug benzydamine. This study highlights the effect of various acidic counter-ions on the permeation of benzydamine. In fact the study also presents the most interesting pairing of benzydamine with ibuprofen.

Benzydamine or N,N-dimethyl-3-{[1-(phenylmethyl)-1H-indazol-3-yl]oxy}-propanamine is a non-steroidal anti-inflammatory drug that acts through an inhibitory effect on the phospholipase A2 by diminishing the liberation of arachidonic acid from phospholipids and partly as a weak inhibitor of PGHS 1 and/or 2 by reducing the production of PGs (Blackwell *et al.* 1975; Modeer and Yucel-Lindberg 1999; Nettis *et al.* 2002). Nettis *et al.* (2002) recently suggested that benzydamine may be a tolerable NSAID and could be a valid alternative in NSAID-sensitive subjects. Benzydamine has been shown to be active after topical (Catanese *et al.* 1966; Andersson and Larsson 1974) and systemic absorption (Schlag *et al.* 1970). It has been reported that local tissue concentrations of benzydamine are higher after topical application of the drug than are those obtained after oral administration (Chasseaud and Catanese 1985; Maamer *et al.* 1987; Schoenwald *et al.* 1987; Baldock *et al.* 1991); plasma concentrations are correspondingly lower. Despite of its unique properties (Silvestrini 1987) the use of benzydamine is mainly limited to topical treatment of oro-pharyngeal and gynaecological conditions. Although, benzydamine is widely used in the form of Difflam® cream for the relief of symptoms associated with painful inflammatory conditions of musculoskeleton system, there is not much literature regarding the skin penetration of benzydamine. Moore *et al.* (1998) published an excellent quantitative analysis of topically applied NSAID's. Ketoprofen, felbinac, ibuprofen, and piroxicam were all significantly superior to placebo, in contrast to indomethacin and benzydamine which were not. In a few studies the authors identified compared topical with oral NSAID's; three in acute conditions (Akermark and Forsskahl 1990; Vanderstraeten and

Schuermans 1990; Hosier 1993) and two in chronic conditions (Golden 1978; Browning and Johson 1994). None showed a significant benefit of oral over topical preparations. The authors (Moore *et al.* 1998) concluded that topical non-steroidal anti-inflammatory drugs are effective and safe.

The lower efficacy of benzydamine may be attributed to its limited penetration across the skin. In an attempt to increase the penetration of benzydamine across the skin, Benson *et al.* (1989) investigated the use of ultrasound to enhance the percutaneous absorption of benzydamine. It was observed that ultrasound was not able to enhance the percutaneous absorption of benzydamine. We therefore propose to investigate the dermal absorption and the effect of ion pairing on the percutaneous absorption of benzydamine. The concept of ion pairing is not new and has already been discussed in detail in the previous chapter.

3.2. Materials and Methods

3.2.1. Materials

Benzydamine HCl, ibuprofen sodium, sodium benzoate, octane sulphonic acid sodium were obtained from Sigma (St. Louis, MO, USA). PDMS (thickness 0.005") from Pillar surgical (CA, USA). Sodium dodecyl sulphate was from Fisher Scientific, USA and was of analytical grade. HPLC-grade acetonitrile (Fisher Scientific, USA) was used. All other chemicals were of analytical grade.

3.2.2. High performance liquid chromatography

An Alliance liquid chromatographic system (Waters Inc., USA) equipped with a 2690 Separations Module and 996 Photodiode Array detector was used. Separation was achieved on a Symmetry C_{18} column (5μm, 3.9 x 150mm I.D., Waters Inc., USA) at ambient temperature, with an inline pre-filter. Integration was undertaken using a personal computer equipped with Millenium 4.0 version software.

Benzydamine was analyzed as reported by Benson and McElnay (1987) using the mobile phase of acetonitrile-water-acetic acid (62:37.5:0.5, v/v) and contained 5 mM sodium dodecyl sulphate. Incorporation of acetic acid maintained the mobile phase at pH 4.0. The mobile phase was filtered through a 0.45 μm membrane filter, Millipore. The mobile phase was continuously degassed before and during use. The flow rate was

0.9 mL/min and detection wavelength was 218 nm. The retention time for benzydamine was 3.2 min.

There was no interference with sodium octanesulphonic acid. The method was slightly modified for benzydamine with ibuprofen and benzydamine with sodium benzoate. For analysis of benzydamine in the presence of ibuprofen, the mobile phase was acetonitrile-water-acetic acid (50:49.5:0.5, v/v) and contained 5 mM sodium dodecyl sulphate. The pH was set at 4.5. The flow rate was 1 mL/min and detection wavelength was 218 nm. The retention times for benzydamine and ibuprofen were 2.8 min and 3.8 min respectively. In the case of benzydamine and sodium benzoate the retention times were 2.8 min and 3.9 min respectively.

3.2.3. NMR spectroscopy

^{1}H and ^{13}C NMR spectra were recorded at 300 and 75 MHz respectively using a Bruker Avance 300 spectrometer (Karlsruhe, Germany). Samples were dissolved in deuteromethanol and chemical shifts (δ) for hydrogen and carbon resonance reported in ppm relative to TMS.

3.2.4. Diffusion through an artificial membrane

In vitro permeation studies across PDMS membrane were performed in Pyrex glass Franz-type diffusion cells. The membrane was immersed in de-ionized distilled water for 1 h before use. PDMS membrane (cross sectional area of 1.18 cm^2) was then mounted between the donor and receptor compartments of diffusion cells and the assembly held in place with a plastic clamp. The diffusion unit was immersed in a water bath maintained at 37 ± 0.5 °C. Phosphate buffer, pH 3.5 (approx. 3.5 mL) was the receptor fluid. For permeation at different pH values the donor phase was 1 mL of 2% benzydamine HCl in buffer solution. After equilibration with the buffer, 1.0 mL of the donor solution was added to the donor cell. A magnetic stirrer driven by an external magnet continuously stirred the receptor compartment at the same speed for all cells. Samples of the receptor phase were withdrawn and replaced by drug-free buffer at appropriate times throughout the 6 h period of the experiment. The benzydamine

content in the receptor phase was determined using HPLC. Experiments were repeated four times.

The cumulative amount of drug released through the PDMS membrane, Q(t), was determined from Q = (CV)/A, where C is the concentration of benzydamine in the receptor compartment in μg/mL for the corresponding sample time t, V is the volume of fluid in the receptor phase and A is the diffusional area of the membrane. The flux of benzydamine through the membrane into the receptor from each of the formulations was determined from the slope of the plot of cumulative amount in the receptor phase vs time and expressed as μg cm^{-2} h^{-1}. Permeability coefficients were calculated for ibuprofen for each formulation.

3.2.5. Diffusion through human epidermis

Ethical approval for using human skin was obtained from the Health Research Ethics Board at the University of Manitoba and St. Boniface Hospital Ethics Committee. Skin tissue from the breast region of female human aged 24 to 47 was stored at –20°C. Epidermal membrane was obtained by a heat separation method (Kligman and Chistophers 1963). Briefly, after thawing, the subcutaneous tissue was removed by dissection. The resultant full-thickness skin was then, immersed in water at 60°C for 45 seconds. The epidermis was removed by blunt dissection of the full-thickness skin and floated onto the water surface. The epidermal membrane was placed onto pieces of aluminum foil and air-dried before storage at –20°C. To simulate acceptable in vivo physiological conditions, experiments were conducted using buffer donor solutions at pH 5.0, 6.0 and 7.0. Each permeation study was run for 6 hours. To ensure sink conditions, the receptor solution consisted of buffer pH 3.5. The receptor solutions were analyzed for benzydamine by HPLC. The integrity of the skin epidermal membranes was ascertained by measuring the permeability of tritiated water (^3H). Trace, but known amount of tritium was added to the donor phase of the Franz diffusion cell. The samples drawn at various time intervals were analyzed for benzydamine as well as for tritium. If the permeability of water was greater than 1.5 x 10^{-3} cm/hr, the skin was considered damaged.

3.2.6. Partition coefficient

The partition coefficients were investigated between n-octanol and phosphate buffers at various pH values. Each phase had been pre-saturated with the other by equilibration overnight before the experiments. A known amount of benzydamine hydrochloride was dissolved in buffers of different pH values to which n-octanol was added. The mixture was stirred continuously for 24 h at 25°C. After phase separation the benzydamine content in the buffer was analyzed by HPLC. Since the initial amount of benzydamine was known, the amount in the organic phase was determined by difference.

3.2.7. Effect of ion pairing on diffusion and partition of benzydamine

The apparent partition coefficients and diffusion studies were performed as described above. Phosphate buffer, pH = 7.0, was used as an aqueous phase for determination of partition coefficient of benzydamine in the presence of ibuprofen sodium, sodium octane sulphonate and sodium benzoate. To study the effect of ion pairing on the permeation of benzydamine, 1.5 mL of 3% solution of benzydamine in propylene glycol. Equimolar amounts of the counter ions were used. For calculations of molar quantity, only the molecular weights of the ions were considered, ignoring chloride and sodium.

3.2.8. Statistical analysis

The difference between the flux of benzydamine at different pH values, and in the presence of different counter ions was assessed using multiple regression with pair-wise comparison.

3.3. Results and discussion

3.3.1. pH and penetration

Percutaneous absorption involves partitioning of a solute from its vehicle into the skin and subsequent diffusion of solute through the skin. Identical solute flux would be expected from solutions in which the solute had equal thermodynamic activity. Non-

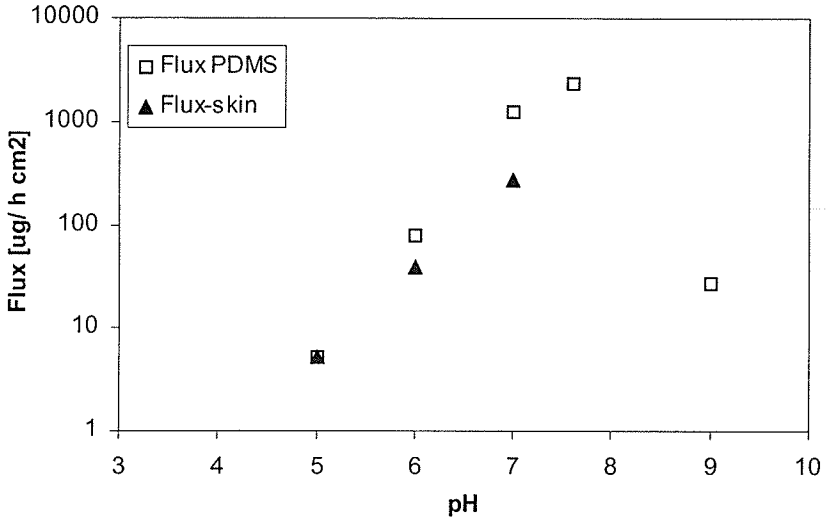


Figure 3.1. The relationship between steady state flux and pH of benzydamine hydrochloride through PDMS and human skin.

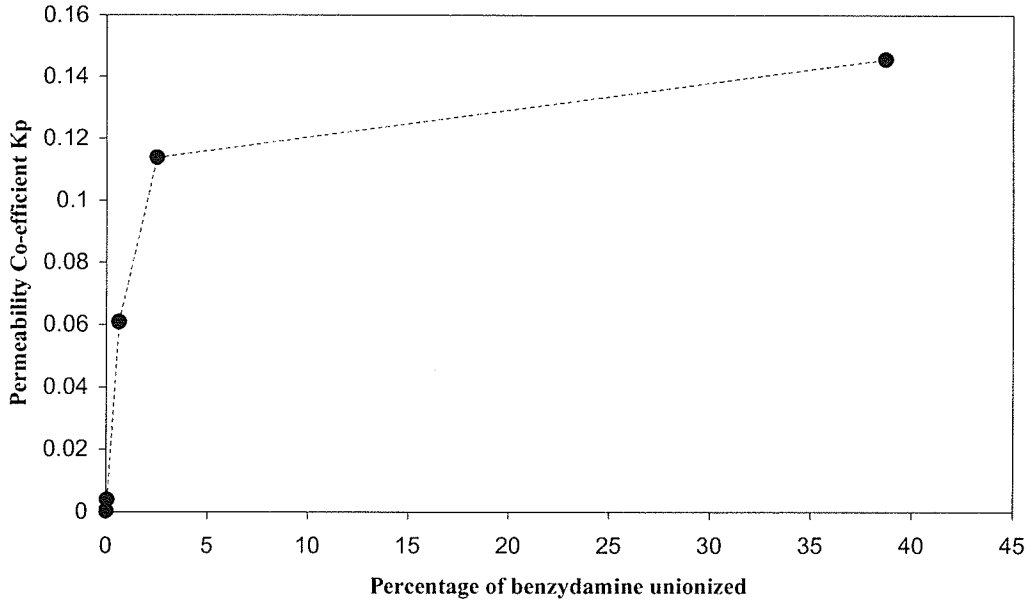


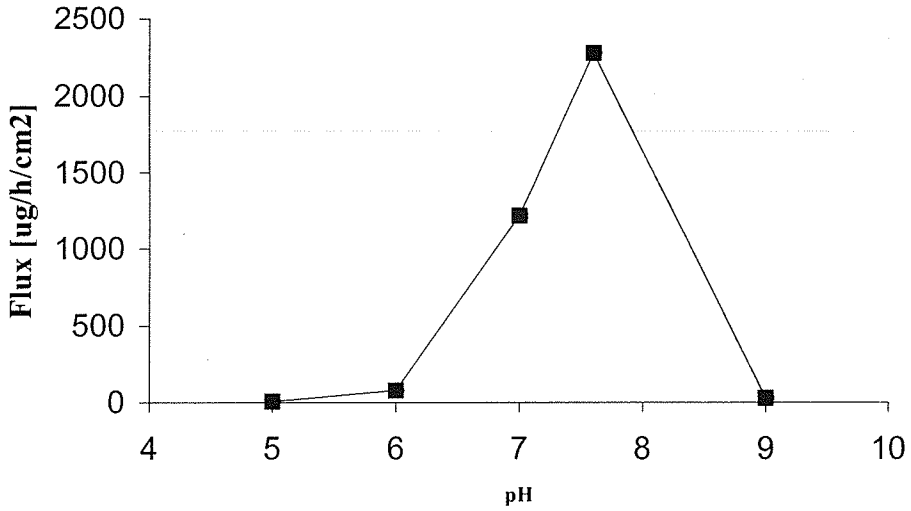
Figure 3.2. The relationship between permeability coefficient Kp and percentage of benzydamine unionized.

-ideal behavior is a result of solute and/or solvent interaction with the membrane (Twist and Zatz 1990; Jiang *et al.* 1998). A synthetic membrane, such as PDMS membrane, offers advantages concerning the physicochemical properties of the diffusional barrier including perm selectivity, high diffusivity, thickness control, and less stringent storage and handling requirements. Moreover, solvents (e.g., water, glycerin, propylene glycol, and polyethylene glycol 400) are not sorbed to a significant extent by this material and behave as ideal vehicles; permeation from these vehicles is a function only of permeant activity (Twist and Zatz 1988). Hence to determine the appropriate permeant activity and to eliminate membrane-solvent-solute interaction, PDMS membrane was chosen for the studies. Propylene glycol and water were used as solvents, since this membrane does not significantly sorb them.

Increase in solubility of drugs in the vehicle has been shown to improve the transdermal penetration of drugs. Salts are known to be more soluble in water than the parent drug, thereby increasing the amount of drug in the solution in the aqueous vehicle. However, increasing the polar nature of the permeant will reduce its tendency to permeate the lipophilic stratum corneum. The overall effect on permeation will be a combination of these effects. The highest flux was determined at pH 7.6. Flux increased as pH increased from pH 5 to pH 7.6 and then decreased as shown in Figure 3.1. The total flux (J_{tot}) of a permeant through a membrane is a composite term, contributed by the diffusion of both the ionized and unionized moieties. The transport across the membrane can be described by the permeabilities of the ionized and unionized species and their respective concentrations $k_{p\,(ion)}$, $k_{p\,(union)}$, c_{ion}, and c_{union} respectively (Hadgraft and Valenta 2000).

$$J_{tot} = k_{p\,(union)} * c_{union} + k_{p\,(ion)} * c_{ion}$$

The ambient pH and the pK_a will give the relative amounts of ionized and unionized species. In the case of benzydamine hydrochloride, flux at higher pH is being dominated by the first term (unionized species), whereas at lower pH it is dominated by the second term (ionized species) (Figure 3.2.). It is interesting to note that the flux of benzydamine decreases after pH 7.6, which is primarily due to limited solubility of



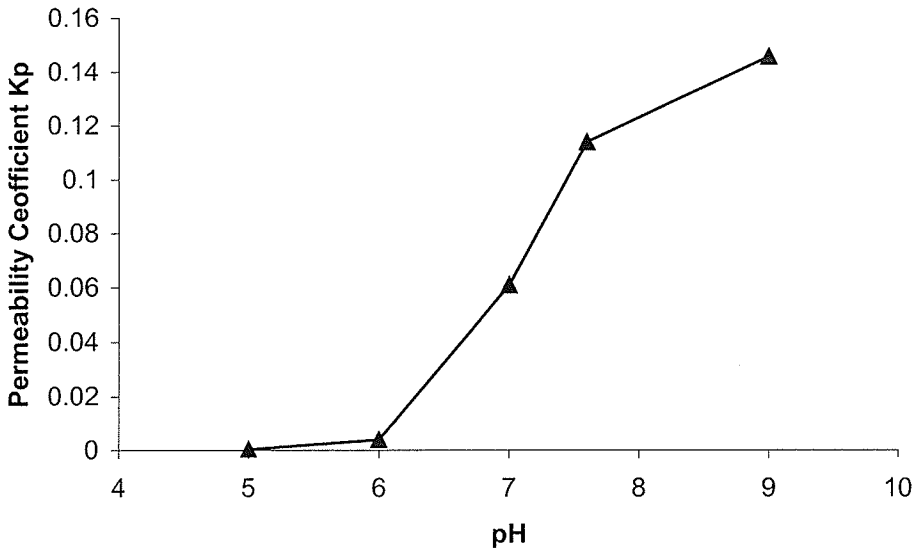


Figure 3.3. The relationship between steady state flux and permeability coefficient k_p with respect to change in pH

benzydamine. It is clear from Figure 3.3., that if the donor concentration (or solubility) at pH 9.0 was higher, than the flux would have been much greater. This is supported by the permeability co-efficient value of benzydamine, which is higher than the values at all the pH studied.

In order to see if similar results could be obtained using human skin, permeation experiments were performed. Epidermal membranes were used and the donor phases at pH 5.0, 6.0 and 7.0 were chosen to simulate conditions, which could be used in topical formulations (Katz and Poulsen 1971; Valenta *et al.* 2000). Although, the flux across the skin is much slower as compared to the PDMS, it follows a similar trend in these experiments (Figure 3.1.).

3.3.2. Partitioning experiments

The apparent partition coefficients were investigated between n-octanol (representative of skin lipids) and phosphate buffers at various pH values (see Table 3.1.). As expected, octanol: buffer partition coefficient increased with increase in pH from pH 5 to 8. Drug was below the level of detection in the aqueous phase at pH 9 indicating a very high partitioning into octanol for the unionized species.

Table 3.1. Concentration, log P values (n-octanol: buffer), permeability coefficient and steady state flux through PDMS membrane for benzydamine hydrochloride at different pH values.

PH	Concentration [mg/mL]	Log P	k_p (cm/h)	Fraction unionized	Flux (PDMS) $\mu g/cm^2/h$	Flux (Skin) $\mu g/cm^2/h$
5	20	0.21 ± 0.008	0.00026	6.3×10^{-3}	5.304 ± 1.8	5.12 ± 2.42
6	20	0.76 ± 0.041	0.0038	0.0631	77.12 ± 0.9	39.07 ± 10.50
7	20	1.45 ± 0.028	0.061	0.627	1215.6 ± 12.4	269.09 ± 58.50
7.6	20	2.08 ± 0.01	0.114	2.51	2275.4 ± 4.2	-
9	0.186	-	0.145	38.7	27.17 ± 3.5	-

Values represent the mean ± S.D. (n=6).

3.3.3. NMR Spectroscopy

The goal of ^{1}H and ^{13}C NMR measurements was to determine if ion pair formation occurs in mixtures containing a carboxylic acid and the tertiary amine benzydamine hydrochloride by chemical shift changes to protons and carbons near the cation and anion.

A great deal of energy is necessary to overcome the powerful electrostatic forces holding together an ionic lattice. Only water or other highly polar solvents are able to dissolve ionic compounds appreciably. In dilute aqueous solutions an ion is strongly solvated and effectively insulated from the charge of its counter-ion. But in a less polar solvent – in methanol for example or one of the aprotic solvents- it feels its charge, and is attracted by it. There is a measure of ionic bonding, and the pair of oppositely charged ions is called an ion pair.

Various modes of ion pair types classified as loose, tight, solvated as contrasted with "free" ions. Ibuprofen, ibuprofen sodium, benzydamine hydrochloride, sodium benzoate, and sodium octane sulfonate were employed for measurements (Figure 3.4.). Chemical shift changes between ibuprofen, ibuprofen sodium, and equimolar mixture of benzydamine hydrochloride and the counter acids in a suitable solvent. For example, equimolar mixtures of CD_3OD solutions would be expected to precipitate NaCl leaving a solution containing a cation and an anion. Association between the cation and anion could induce chemical shift changes in the nearby atoms.

3.3.3.1. Benzydamine hydrochloride and ibuprofen sodium

Comparison of the proton NMR spectra of benzydamine hydrochloride with a mixture of benzydamine hydrochloride with ibuprofen (sodium) in deuteromethanol showed significant shift differences (Table 3.2.a). As expected, despite chemical shift differences, coupling constants were unaltered. Some aromatic protons from each molecule were overlapping and unresolved. The largest changes observed were in the methylene protons ($-CH_2-N^+$) and the two methyl groups (Me_2N^+) attached to nitrogen, which showed downfield shifts of -0.27 to –0.23 ppm, respectively. Smaller shift changes occurred in the remaining methylene groups as the inductive effect of the

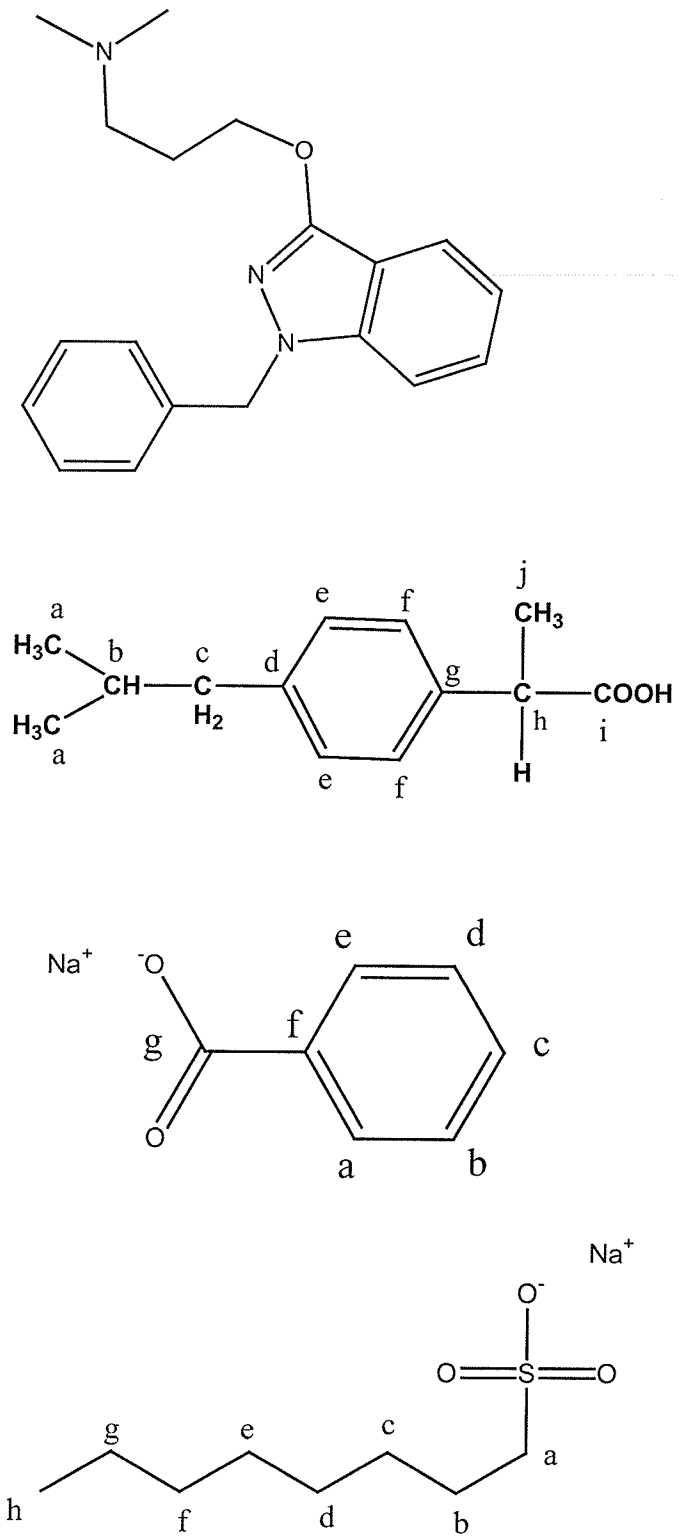


Figure 3.4. Structures of benzydamine and ibuprofen, benzoate and octane sulphonate molecules

Table 3.2.a :- ^1H NMR of benzydamine hydrochloride, and shift differences in the presence of ibuprofen sodium, sodium benzoate and octane sulphonate molecules.

Benzydamine hydrochloride		Ibuprofen sodium **Δ**	Sodium benzoate **Δ**	Octane sulphonate **Δ**
$-CH_2-$	2.31	-0.16	-0.08	-0.09
Me x 2	2.87	-0.23	-0.19	-0.12
$-CH_2-O-C=N$	4.47	-0.07	-0.04	-0.03
$-CH_2-N^+$	3.33	-0.27	-0.04	-0.06
$Ph-CH_2$	5.36	-0.01	-0.01	-0.10
ArH	7.06	overlap	overlap	overlap
ArH	7.21	overlap	overlap	overlap
ArH	7.33	overlap	overlap	overlap
ArH	7.69	-0.02	-0.01	-0.07

Table 3.2.b :- ^1H NMR shift differences (Δ) in ibuprofen sodium, sodium benzoate and octane sulphonate molecules in the presence of benzydamine hydrochloride.

Ibuprofen sodium		Sodium benzoate		Octane sulphonate	
a	-0.09	a	-0.05	a	-0.07
j	-0.01	b	-0.01	b	-0.03
b	-0.13	c	0.0	c	-0.06
c	-0.11	d	-0.01	d	-0.06
h	+0.01	e	-0.05	e	-0.06
ArH	overlap			f	-0.06
ArH	overlap			g	-0.09
				h	-0.09

cationic nitrogen decrease due to their increasing distance from the charged groups. Ion-pair formation would be expected to also lower the charge on the nitrogen leading to similar chemical shift changes. Therefore, these observed chemical shift differences do not clearly distinguish between either "free" cation and anion or ion-pair formation. Smaller changes observed in the ibuprofen portion of the spectrum surprisingly occur in the isobutyl chain furthest from the carboxylate anion (Table 3.2.b). While inductive effects can be transmitted through an aromatic ring, it is unlikely that no change would be observed in the much nearer C-H(h) and Me(j) protons. However, these more remote changes are consistent with a hydrophobic interaction between the isobutyl group and the benzydamine group.

Table 3.3.a :- ^{13}C NMR of benzydamine hydrochloride, and shift differences (Δ) in the presence of ibuprofen sodium, sodium benzoate and octane sulphonate molecules.

Benzydamine hydrochloride		Ibuprofen sodium Δ	Sodium benzoate Δ	Octane sulphonate Δ
$-CH_2-$	25.71	+ 0.09	+ 0.07	-0.09
-N(Me)2	43.55	-0.13	-0.15	-1.6
$-CH_2-$	52.95	0	-0.01	-0.02
$-CH_2-$	56.36	-0.28	-0.18	-0.07
$PhCH_2N$	67.00	+0.21	+0.05	+0.02
ArH	110.39	-0.09	-0.08	-0.09
ArH	113.73	+0.07	+0.07	-0.01
ArH	120.68	-0.09	-0.03	-0.02
ArH	128.27	-0.07	-0.07	-0.08
ArH	128.60	-0.05	-0.05	-0.01
ArH	128.88	-0.05	-0.06	-0.01
ArH	129.63	-0.03	-0.03	-0.02
ArH	139.01	-0.05	-0.03	-0.05
-O-C=N	156.66	+1.06	+ 0.06	+0.17

Table 3.3.b :- ^{13}C NMR shift differences (Δ) in ibuprofen sodium, sodium benzoate and octane sulphonate molecules in the presence of benzydamine hydrochloride.

Ibuprofen sodium		Sodium benzoate		Octane sulphonate	
j	-0.14	a	-0.02	a	-1.4
a	-0.02	b	-0.2	b	-0.8
c	-0.23	c	-0.08	c	-0.4
b	-0.24	d	-0.2	d	-0.5
h	-0.70	e	0.4	e	-0.5
e	-0.05	f	-3.8	f	-0.2
f	+0.14	g	-7.9	g	-0.5
d	+0.27			h	-0.7
g	-0.61				
i	-3.92				

Comparison of the ^{13}C-NMR spectra of benzydamine hydrochloride with that obtained from the solution prepared by mixing equimolar amounts of ibuprofen sodium and benzydamine hydrochloride showed significant changes. For example, the changes observed in the methyl carbons attached to nitrogen and neighboring methylene protons indicating an interaction with ibuprofen molecule (Table 3.3.a). Similarly, the ibuprofen molecule showed some significant shift changes due to the presence of the benzydamine moiety (Table 3.3.b). Large chemical shifts were observed in the carbonyl group (-3.92 ppm), C(h)(0.70), and C(g)(0.61) with moderate changes to other carbon atoms. The most striking change was the large downfield shift (-3.92 ppm) in the carbonyl group, giving a chemical shift value between that of the acid (178.55 ppm) and the sodium salt (186.96 ppm). This value suggests that the charge on the carbonyl group has been partially neutralized which would be consistent with ion-pair formation. These measurements therefore provide evidence for the existence of an ion pair in the solution. Corresponding changes were not observed in the proton attached to these carbon atoms demonstrating the greater sensitivity of the carbon atoms to these electronic effects.

3.3.3.2. Benzydamine hydrochloride and sodium benzoate

Comparison of the proton NMR spectra for each molecule with the mixture showed a significant change in the protons on the two methyl groups on nitrogen in the benzydamine molecule (Table 3.2.a, Figure 3.4). This may be due to the interaction of these protons with the carboxylic group of the sodium benzoate as a result of ion-pair formation.

The ^{13}C-NMR spectra of benzydamine hydrochloride and sodium benzoate molecule seperately were compared with the spectrum of an equimolar mixture of the two. Benzoate showed significant upfield chemical shifts in the carbonyl group and adjacent carbon, similar to that observed in ibuprofen (Table 3.3.b). Large chemical shifts were observed in the carboxyl carbon (-7.9), carbon "f" (-3.8) and moderate changes in other carbon atoms. Benzydamine also showed large changes in the methyl carbon atoms attached to nitrogen and neighbouring carbons atoms due to inductive effects. These measurements thus provide strong evidence for ion-pair formation between the benzoate and benzydamine molecules.

3.3.3.3. Benzydamine hydrochloride and sodium octane sulphonate

Comparison of the proton NMR spectra of sodium octane sulphonate and in a equimolar mixture with benzydamine hydrochloride, showed changes in the protons on carbons g, h, and a (Table 3.2.b; Figure 3.4.). When the proton NMR spectra of benzydamine hydrochloride was compared with the mixture, changes were also observed in the methyl group protons of the benzydamine molecule. These changes provide evidence of interaction between the molecules (Table 3.2a).

Comparison of the carbon NMR spectra of the molecules alone and as a mixture showed major downfield shift changes in the carbons "a" (-1.4) and "b" (-0.8) next to sulphur, and carbons "g" and "h" of the octane sulphonate (Table 3.3b, Figure 3.4). Major significant changes in the benzydamine molecule occurred in the methyl carbons (-1.6). There were other significant changes in the benzydamine molecule. These measurements indicate interaction between the two molecules.

In summary, we can conclude that there is ion-pair formation between the benzydamine molecule and the three counter-ions used in this study. The chemical shift changes in benzydamine and octane sulphonate when compared with their mixtures is smaller than those with ibuprofen and sodium benzoate, which shows that ibuprofen and benzoic acid are able to pair with benzydamine more effectively than does octane sulphonate.

An ion pair is a pair of oppositely charged ions held together by Coulomb attraction and not by formation of a covalent bond. Experimentally, an ion pair behaves as one unit in determining conductivity, kinetic behavior, osmotic properties, etc. Following Bjerrum, oppositely charged ions with their centers closer together than a distance

$$q = 8.36 \times 10^6 z^+ z^- / (\varepsilon_r T) \text{ pm}$$

are considered to constitute an ion pair ('Bjerrum ion pair'). z^+ and z^- are the charge numbers of the ions, and ε_r is the relative permitivity (or dielectric constant) of the medium. Solvents with lower dielectric constant favor formation of ion pair

Table 3.4. Steady state flux and log P values of benzydamine and in the presence of other counter-ions.

Salt type	Log P	Flux
Benzydamine HCl	0.21 ± 0.008	0.087 ± 0.016
Benzydamine HCl + Ibuprofen	1.54 ± 0.23	12.54 ± 0.94*
Benzydamine HCl + Sodium benzoate	1.32 ± 0.09	11.31 ± 0.83*
Benzydamine HCl + Sodium octane sulphonate	0.96 ± 0.05	0.121 ± 0.08

Each value represent the mean ± S.D. (n=6), *significantly different from Ibuprofen Na, a) p<0.0005

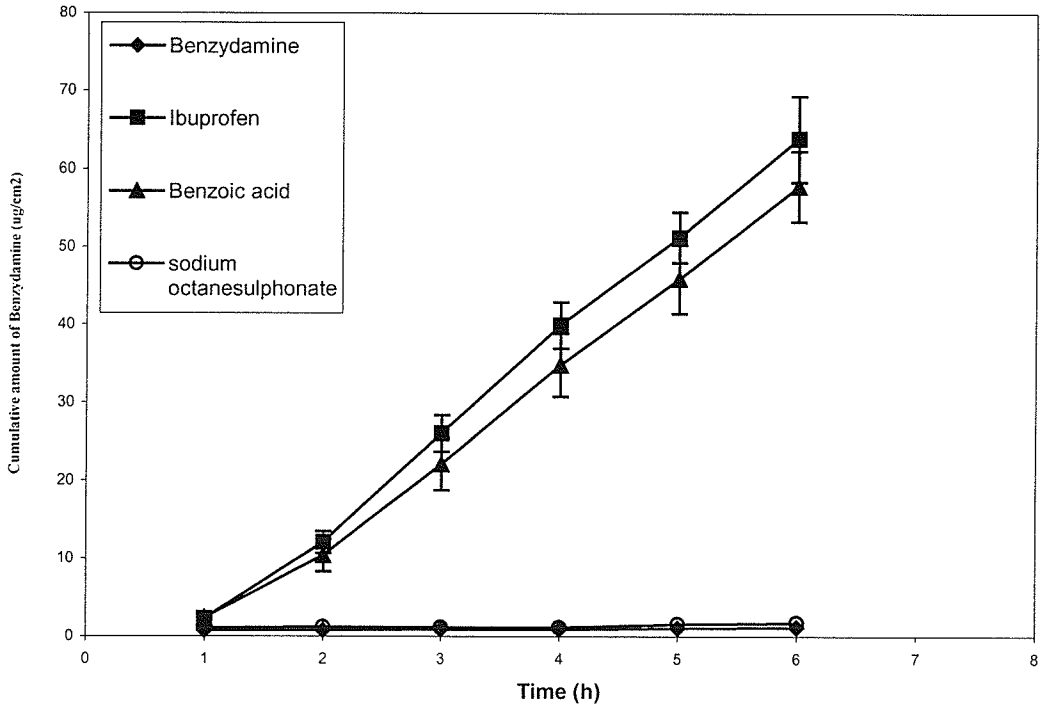


Figure 3.5. . Comparison of diffusion profiles of different ibuprofen salts from propylene glycol through PDMS membrane (values : mean ± S.D).

(Marcus 1985; Hwang *et al.* 2003). The distance q increases with temperature and solvents at high temperature behave like solvents of lower dielectric constants, permitting long range ion-pairing (Marcus 1985). Because the dielectric constant for propylene glycol is lower than methanol and there is evidence for ion pair formation in methanol, therefore we assume ion pair formation of ibuprofen with various amine counter-ions in propylene glycol. Propylene glycol has been used as the solvent in the penetration experiments as specified in the methods section.

3.3.4. Effect of ion pairing on the permeation and partition characteristics of benzydamine

The measured partition coefficients of the salts into n-octanol seems to be in agreement with their lipophilicity (Table 3.4.). Benzydamine showed the highest partition coefficient with ibuprofen as a counter ion, followed by benzoate and octane sulphonate ions.

The permeation of benzydamine in the presence of ibuprofen or sodium benzoate was significantly greater than permeation of benzydamine alone (Figure 3.5.). These results are quite consistent with the ability of benzydamine to form ion pairs with ibuprofen and sodium benzoate. Although, the partitioning of benzydamine in the presence of sodium octane sulphonate was significantly greater (> 4.5 times), there wasn't a significant effect on permeation. This may be due to the reason that the benzydamine-octane sulphonate ion pair or the octane sulphonate ion interact differently with n-octanol than with the PDMS membrane. The NMR data suggest that although there is interaction between the benzydamine and octane sulphonate ion, the charge on benzydamine ion is not neutralized to an extent (compared with ibuprofen and benzoate), which may be required to produce a significant permeation enhancement.

3.5. Conclusions

The results of this study suggest that it is possible to enhance the flux of salts across lipophilic membranes using an ion pair approach. This study shows that for both PDMS and human skin there are similarities in the behavior, the larger the unionized fraction, higher the flux.

The partition coefficients of ion pairs of benzydamine seem to be in agreement with their lipophilicity. However, a correlation with steady state flux could not be established, especially in the case of benzydamine-octane sulphonate ion pair. In addition to the reasons specified above, it could be a possibility of hydrophobic interaction between the open chain hydrocarbon (C-8) of the octanesulphonate ion pair and the PDMS memebrane. Similar observations have previously been reported with lignocaine-octanesulphate salt (Valenta et al. 2000).

Overall we conclude that the degree of enhancement is associated with the lipophilicity, extent of ion pairing, and reduction in the charge over the drug molecule.

Chapter 4. High performance liquid chromatographic assay for common sunscreen agents

4.1. Introduction

The use of sunscreens has increased with the awareness of the detrimental effects of sun exposure on human skin such as erythema, skin ageing and cancers. Sunscreen products are formulated to provide a specific sun protection factor (SPF) and to absorb a broad spectrum of ultraviolet radiation (UVR). In addition to traditional sunscreen products, sunscreen chemicals are also incorporated into a wide range of everyday hair and skin products and may therefore be used without the wearer making a conscious decision to apply a sunscreen.

The actives used in topical formulations are generally classed as either chemical or physical sunscreens. Physical sunscreens comprise of particles that act by scattering, reflecting, or absorbing the passage of radiation. Chemical sunscreens act by absorbing incident UVR and then dissipating it as longer wavelength energy, thereby protecting the skin from potentially damaging UVR. The efficiency of sunscreens is estimated by the sun protection factor (SPF), which depends on the content of UV filters in the formulation. The necessity to provide a high SPF and screening efficiency against both UV-A (320-400 nm) and UV-B (290-320 nm) wavelengths has led to the development of sunscreen preparations containing many different sunscreen chemical combinations. Benzophenones, dibenzoylmethanes and anthranilates are the most common UV-A filters, whereas the UV-B filters include p-aminobenzoic acid (PABA) derivatives, salicylates, cinnamates, digalloyl triolate, lawsone, acrylates and benzimidazole derivatives. Of the approved sunscreen chemicals oxybenzone (benzophenone-3), octylmethoxycinnamate, butylmethoxydibenzoylmethane, octylsalicylate and homosalate are some of the most common active ingredients used in sunscreen formulations.

Recent studies have provided evidence that some sunscreens are absorbed systemically following topical application to the skin (Treffel and Gabard 1996; Hayden et al. 1997; Benson 2000). These studies involved determination in skin layers only or measurement of urinary excretion of absorbed sunscreens and their metabolites. Neither provided a full pharmacokinetic analysis as only a single measure of absorption or excretion was assessed. It would be advantageous to quantify penetration within the skin tissue and systemic distribution of sunscreen

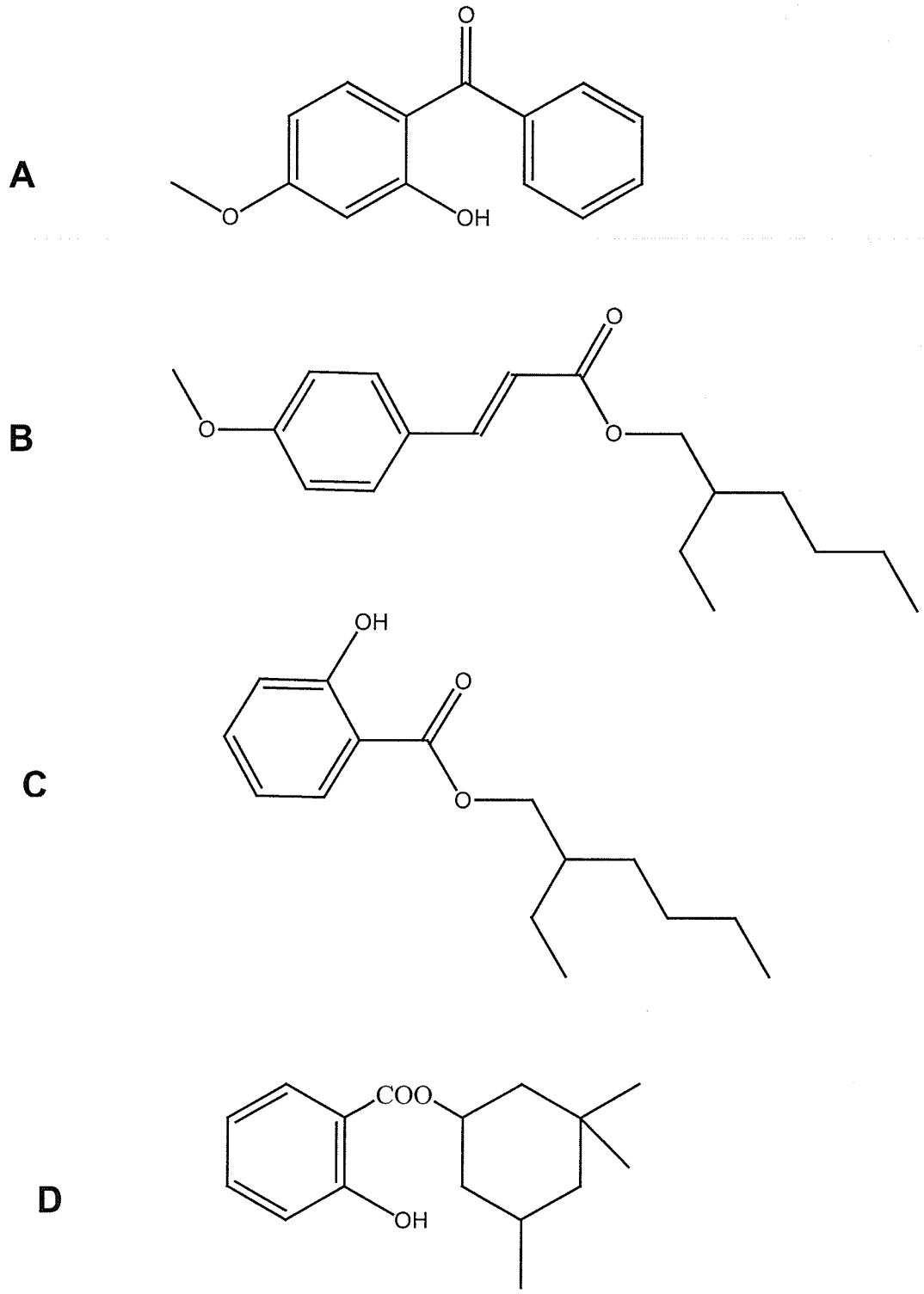


Figure 4.1. Structures of the sunscreen agents. (A) Oxybenzone; (B) Octylmethoxycinnamate; (C) Octylsalicylate; (D) Homosalate

agents following topical application. This would aid in determination of the exposure of viable tissues to sunscreen chemicals, provide a better understanding of the potential for toxicity both locally and systemically, and facilitate design of novel formulations to target the outer skin layers.

In addition to *in vivo* studies, skin penetration of chemicals and drugs is frequently investigated using in vitro techniques. The *in vitro* technique utilizes diffusion cells, which consist of a receptor and donor phase separated by a synthetic or skin membrane. Where lipophilic solutes are investigated, as is the case for many sunscreens, bovine serum albumin or other solubility modifiers are used as receptor fluids to provide adequate solubility and ensure sink conditions (Bronaugh 1996; Jiang et al. 1998). A suitable extraction procedure and HPLC assay is required to facilitate these studies.

Many of the HPLC assays published for sunscreen agents are designed for product evaluation and determination of concentrations in cosmetic formulations (Gagliardi *et al.* 1989; Chisvert *et al.* 2001; Chisvert *et al.* 2001; Chisvert *et al.* 2001aa; Chisvert *et al.* 2001bb). Few assays for evaluation of sunscreens in biological samples have been reported (Jiang et al. 1996). A reliable analytical method for the quantitative determination of the common sunscreen chemicals in biological fluids will facilitate the evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetic data.

The aim of this study was to develop simple, rapid and reliable operating procedures for quantification of sunscreen chemicals in a range of biological matrices. Butyl methoxybenozoylmethane (BDM), octyl methoxycinnamate (OMC), octyldimethyl PABA, octylsalicylate (OS), oxybenzone (OX) and homosalate (HS) are the most commom sunscreen agents used in commercial sunscreen products. Most of these sunscreen agents present similar retention times on previously published assays and are therefore diffcult to resolve. HS is especially problematic because it presents two peaks corresponding to two isomeric forms (Chisvert *et al.* 2001). This study also provides a reproducible and accurate assay, by which four of the most common sunscreen agents (Figure 4.1.), including HS, can be resolved simultaneously. Using this assay procedure, a clinical investigation of the penetration into skin tissues, plasma

and excretion in the urine of four common sunscreens, as active ingredients in a commercially available sunscreen product, was conducted. Extraction procedures for the extraction of sunscreens from tape strips, skin tissue and biological matrices including plasma, urine and bovine serum albumin are also provided. The clinical study utilizing this assay is described in Chapter 5.

4.2. Experimental

4.2.1. Materials and Methods

OX, OS and BSA were purchased from Sigma-Aldrich (USA). OMC and HS were gifts from BASF Corporation (NJ, USA) and EM Industries (Germany) respectively. Coppertone sunblock lotion (Schering-Plough Health Care Products Inc) was the commercially available sunscreen product used for the study. HPLC grade methanol was from Fisher Scientific, USA. De-ionized water (Milli-Q, Waters Inc) was used and all other chemicals used were analytical reagent grade.

4.2.2. HPLC instrumentation and conditions

An Alliance liquid chromatographic system (Waters Inc., USA) equipped with a 2690 Separations Module and 996 Photodiode Array detector was used. Separation was achieved at ambient temperature on a Symmetry C_{18} column (5μm, 3.9 x 150mm I.D., Waters Inc., USA) with an inline pre-filter. Integration was undertaken using a personal computer equipped with Millenium 4.0 version software.

4.2.2.1. Analysis of sunscreen

The mobile phase consisted of methanol: water, filtered through a 0.45 μm membrane filter (Durapore, Millipore, USA). Gradient flow from 75:25 methanol: water to 92:8 methanol: water was used from 0 to 4 min; thereafter the flow was isocratic with 92:8 methanol: water. The solvent composition was returned to initial conditions after 11 minutes. The mobile phase was continuously degassed before and during use. The flow rate was 1.0 mL/min. To obtain a satisfactory UV response for all the analytes, each chemical was measured at its wavelength of maximum absorbance: oxybenzone at 289 nm, octylmethoxycinnamate at 310 nm; homosalate and octylsalicylate at 237 nm. Injection volumes of 10 μL were used for the assay.

Stock solutions were prepared by accurately weighing the sunscreen agents (OX, OS, OMC, HS) and dissolving in methanol. Three working solutions of the four sunscreens were freshly prepared from their stock solutions by 1:10 dilution. Appropriate dilution of these working solutions gave concentrations of 0.1 to 0.5 ug/mL. The entire procedure was repeated on six different days to test inter-day variation and repeated six times at low and high concentrations to test intra-day variation.

The minimum detectable limits were measured by diluting the sunscreen agents with methanol to give an appropriate range from 0.01 to 20 μg/mL. Aliquots of 10 μL of the samples were injected onto the HPLC column.

4.2.2.2. Analysis of oxybenzone and its metabolites in urine

The mobile phase consisted of acetonitrile: water at pH =3, filtered through a 0.45 μm membrane filter (Durapore, Millipore, USA). The water was acidified using acetic acid. The flow was 0.8 mL/min from 0 to 6 minutes and thereafter 1.5 mL/min until 11 minutes. The flow conditions were then returned to normal conditions.

4.2.3. Sample treatment and preparation

4.2.3.1. Plasma and Bovine Serum Albumin (BSA):

The four sunscreen standards were spiked into human plasma and 4% w/v BSA in phosphate buffer (pH 7.4) at low and high concentrations (0.5 and 5.0 μg/mL). Sample solutions were stirred for 30 min following spiking to ensure complete dissolution of the sunsceen agents. After protein precipitation with two sample volumes of acetonitrile (200 μL acetonitrile to 100 μL of sample), the samples were centrifuged at 10 000 g for 10 min. The supernatant was injected onto the HPLC system. Six replicates were performed at each concentration. Blank plasma and BSA in phosphate buffer were treated identically and injected onto the HPLC system to ensure that there were no peaks interfering with the sunscreen active substances.

4.2.3.2. Urine

The four sunscreen standards were spiked (5 μg/mL) into fresh human urine. To 1 mL of this solution was added an equal volume of phosphate buffer (pH 6.8). The sample was then treated with beta-glucuronidase enzyme (600 u) and heated at 37°C for

24 h. The reaction was stopped with an equal volume of acetonitrile resulting in protein precipitation. It was vortex mixed and centrifuged at 5000 g for 10 minutes. The sample was evaporated to dryness then resuspended in methanol and 20 μL of the supernatant was then injected onto the HPLC column.

4.2.3.3. Skin tissue

Human epidermal membranes were immersed in 5 mL of standard sunscreen solution for 24 hours in dark conditions at 25°C. The formulation residue was removed from the epidermal tissue by rinsing with 5 mL distilled water three times and drying. Retained sunscreen was then extracted with 2 mL of absolute methanol for 30 min. The extraction procedure was repeated three times with methanol. After centrifuging each extract at 10,000 g for 10 minutes, the resultant supernatants were diluted appropriately and quantified by HPLC.

Sunscreen in the stratum corneum tape strip samples was extracted by a two-step procedure adapted from Potard and co-workers (Potard *et al.* 1999). This involved overnight contact of the tape with isopropanol to destructure the polymeric glue, followed by dissolution of the polymeric glue and the hard polymeric tape support by acetonitrile. The solvent was then evaporated and the residue resuspended in 1 mL methanol for analysis of sunscreen content by HPLC.

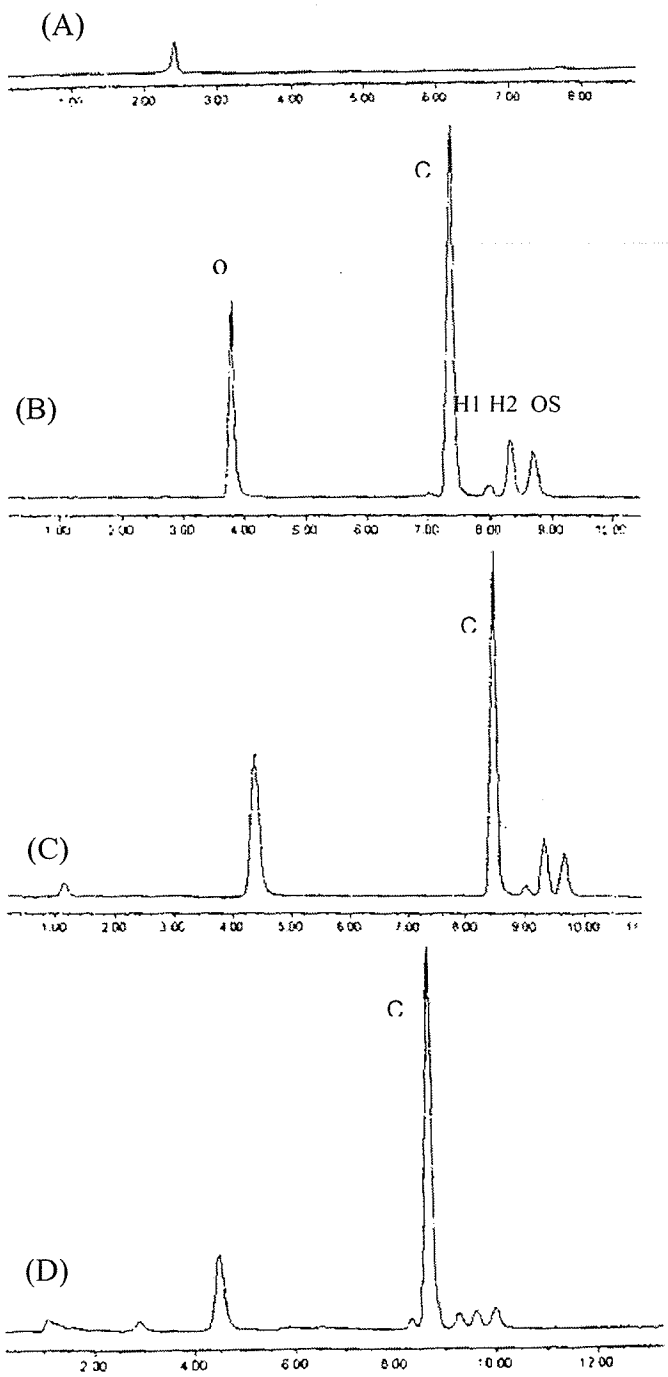


Figure 4.2. Chromatograms of a blank of 4% BSA (A); an extract from 4% BSA in phosphate buffer (B); plasma (C) and skin strips (D).
Peaks: O = oxybenzone; C = octylmethoxycinnamate; H1 and H2 = homosalate and OS = octylsalicylate.

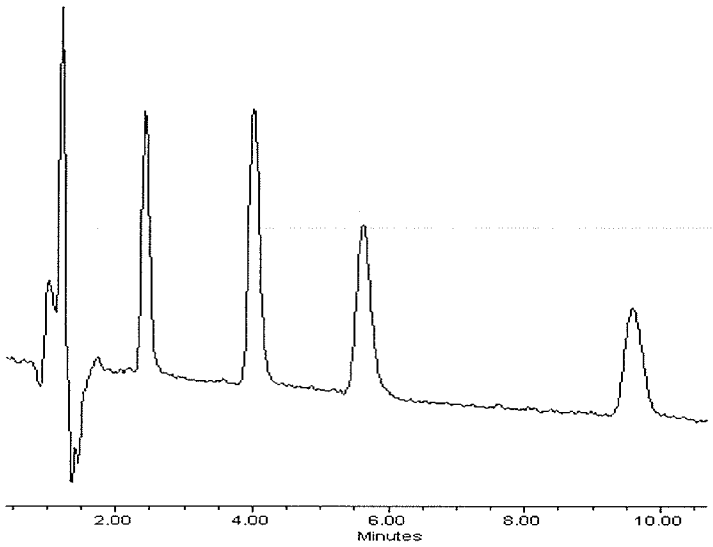


Figure 4.3. Chromatogram of four benzophenones standard solution peaks
(1) trihydroxy-benzophenone 2.5 min, (2) dihydroxy-benzophenone 4min, (3) dioxybenzone 5.5 min, (4) oxybenzone 9.5 min.

4.3. Results and Discussion

4.3.1. Chromatography and resolution

HPLC chromatograms of the four sunscreen agents after sample preparation from an extract of 4% BSA, human plasma and tape strips are shown in Figure 4.2. Most of the HPLC assays published are designed for product evaluation and determination of concentrations in cosmetic formulation (Gagliardi *et al.* 1989; Rastogi and Jensen 1998; Chisvert *et al.* 2001a; Chisvert *et al.* 2001b). The assay method previously published for evaluation of sunscreens in biological samples is useful but offers certain limitations in terms of sensitivity, especially with octylsalicylate (Jiang et al. 1996). Increased sensitivity is particularly important while measuring biological samples. Moreover, the method does not include the UV-B filter homosalate, which is present in many of the commercially available sunscreen formulations. Homosalate is particularly difficult to measure due to the low extinction coefficient, and the presence of two peaks corresponding to two isomeric forms designated H1 and H2 (Figure 4.2.). H2 was used for calibration and quantitation. The present method, which includes homosalate, also provides increased sensitivity and resolution for all the sunscreens measured compared to previous published methods. The procedure is relatively rapid with a run time of only approximately 10 minutes.

4.3.2. Linearity

Table 4.1. reports the results for calibration plot linearity. Excellent linearity was obtained over the range 0.1 – 5.0 μg/mL for the four sunscreen agents.

4.3.3. Assay precision

Calibration graphs were constructed by plotting the peak area versus concentration of standards injected. The best straight lines were determined using the method of least squares. To obtain a satisfactory UV response for all analytes, each chemical was measured at its wavelength of maximum absorbance: oxybenzone at 289 nm, octylmethoxycinnamate at 310 nm, homosalate and octylsalicylate at 237 nm. The intra- and inter-day variation for the four sunscreens was less than 3% at the upper end of the linear range and less than 6% at the lower end (as summarized in Table 4.1.). There was no significant difference between day-to-day analyses (slopes evaluation, $p<0.001$).

4.3.4. Minimum detectable limits

The lower limits of quantitation calculated as greater than ten times the baseline noise in the assay were 2 ng (0.2 μg/mL) for oxybenzone, 1 ng (0.1 μg/mL) for octylmethoxycinnamate and 4 ng (0.4 μg/mL) for homosalate and octylsalicylate. The minimum detectable limits, calculated as greater than three times the baseline noise level in the assay, were 0.8 ng (0.08 μg/mL) for oxybenzone, 0.3 ng (0.03 μg/mL) for octylmethoxycinnamate, and 2 ng (0.2 μg/mL) for homosalate and octylsalicylate.

The limits of detection in previously published assay by Jiang et al. (1996) is 0.1 ng (0.01 μg/mL) for oxybenzone, 1 ng (0.1 μg/mL) for octylmethoxycinnamate and 5 ng (0.5 μg/mL) for octylsalicylate (Jiang et al. 1996). Chisvert et al (2001a,b) reported detection limits for oxybenzone of 1.7 μg/mL, 2.2 μg/mL for octylmethoxycinnamate, 2.3 μg/mL for homosalate and 1.5 μg/mL for octylsalicylate. These are not directly comparable with the current data as the method for determination of minimum detection limits is different. Jiang et al. (1996) argued that lower sensitivity, particularly for octylsalicylate was due to using a compromise wavelength. A similar wavelength was

Table 4.1. Quantitative results for HPLC assay of sunscreens

	OX	OMC	OS	HS
Wavelength (nm)	289	310	237	237
Linear range (μg/mL)	0.1 – 5.0	0.1 – 5.0	0.1 – 5.0	0.1 – 5.0
Slope (10^6)	3.53 ± 0.037	4.42 ± 0.20	2.01 ± 0.026	1.81 ± 0.026
Intercept (10^4)	-(5.76 ± 2.1)	(2.15 ± 0.31)	-(3.78 ± 1.3)	-(3.47 ± 1.95)
Correlation coefficient	0.9998	0.9998	0.9998	0.9998
Minimum detection limit (ng)	0.8	0.3	2	2
Inter-day variation: mean ± s.d. & %CV @ 0.1 μg/mL	0.092 ± 0.002 2.72	0.1002 ± 0.004 4.139	0.101 ± 0.005 5.439	0.983 ± 0.005 5.594
Inter-day variation: mean ± s.d. & %CV @ 5 μg/mL	4.976 ± 0.0304 0.612	5.01 ± 0.0357 0.713	4.99 ± 0.027 0.557	5.02 ± 0.037 0.745
Intra-day variation: mean ± s.d. & %CV @ 0.1 μg/mL	0.095 ± 0.002 2.47	0.099 ± 0.005 5.55	0.105 ± 0.004 4.15	0.107 ± 0.005 4.94
Intra-day variation: mean ± s.d. & %CV @ 5 μg/mL	4.985 ± 0.058 1.16	4.965 ± 0.146 2.95	4.947 ± 0.058 1.176	4.953 ± 0.048 0.998

*RSD of peak area (n=6)

Table 4.2. Recovery of sunscreens from human plasma, 4% BSA in phosphate buffer and urine spiked with 0.5 μg/mL or 5 μg/mL of each sunscreen

	OX	OMC	OS	HS
Plasma (0.5 μg/mL):				
% recovery	99.29	103.83	97.33	95.92
% CV	2.75	3.78	2.22	4.10
Plasma (5 μg/mL):				
% recovery	98.4	96.38	92.61	90.76
% CV	3.47	2.53	2.13	4.41
4% BSA (0.5 μg/mL):				
% recovery	102.1	99.47	102.31	97.95
% CV	2.56	1.90	2.22	3.28
4% BSA (5 μg/mL):				
% recovery	100.91	101.25	100.88	98.30
% CV	1.45	2.38	1.18	2.70
Urine (5 μg/mL):				
% recovery	90.03	89.22	86.82	92.25
% CV	3.05	4.45	4.32	3.22
Epidermal membranes:				
% recovery	98.84	99.20	98.49	99.55
% CV	2.53	1.82	3.07	3.20

Mean of six extractions

Table 4.3. Recovery of oxybenzone and its metabolites from urine spiked with 5 μg/mL of each chemical

	OX	DHMB	DHB	THB
% recovery	89.88	94.88	92.76	93.21
% CV	2.84	2.39	2.49	4.13

DHMB = 2,2' dihydroxy 4- methoxy-benzophenone; DHB = 2,4 dihydroxybenzophenone ; THB = 2,3,4 trihydroxybenzophenone
Mean of six extractions

used by Chisvert and colleagues (2001b). This has been resolved in our method, leading to increased sensitivity. In addition to the use of multiple wavelengths, gradient flow in our method favored better peak shape and separation. Therefore, this method would be more useful for measuring sunscreen agents, especially in biological samples.

4.3.5. Recovery study in human skin, plasma, urine and 4% BSA in phosphate buffer

The recovery of the four sunscreen agents from plasma and 4% (w/v) BSA solution is summarized in Table 4.2. Recoveries were within the range of 90-104%. The coefficients of variation calculated from the six replicates were all less than 5%. Extraction of the sunscreens from the epidermal membranes and urine is also summarized in Table 4.2. Recoveries were within the range of 98-100 % and 86-92% respectively. Data for oxybenzone and its metabolites are summarized in Table 4.2. and Table 4.3.

4.4. Conclusions

This assay provides an efficient means of quantifying the most common sunscreens in a range of biological matrices relevant to both *in vitro* and *in vivo* assessment of skin penetration. As such it will facilitate the development of novel sunscreen products with high SPF and substantivity (skin retention) but also minimal absorption to deep tissues or the systemic circulation.

Chapter 5. Systemic absorption of sunscreens after topical application

5.1. Introduction

There is limited literature about how people protected themselves against the sun in ancient times. Evidence from paintings suggest that clothing covering the body, veils and large brim hats were used by the ancient Greeks, and that umbrellas existed in ancient Egypt, Mesopotamia, China and India (Urbach 2001). In 1889, acidified quinine sulfate was used to absorb UVB, apparently because, since quinine fluoresces when irradiated with UVR. Further in 1891 quinine prepared in lotion or ointment as the first human sunscreen (Urbach 2001). Over the next 40 years a number of different chemicals were introduced for sunscreening purposes. The first reported use of commercial sunscreen occurred in the United States in 1928, with the introduction of an emulsion containing benzyl salicylate and benzyl cinnamate (Patini 1988). In the early 1930's, a product containing 10% Salol (phenyl salicylate) appeared on the Australian market (Groves 1997). Many products including tannic acid (1925), para-amino benzoic acid derivatives and 2-phenylimidazole derivatives (1942), anthranilic acid (1950), various cinnamates (1954) and benzophenones (1965) have been introduced since then (Urbach 2001).

A sunscreen active ingredient is defined by the Food and Drug Administration (FDA) as: 'an active ingredient that absorbs at least 85% of radiation in the UV range at wavelengths from 290-320 nm, but may not transmit radiation at wavelengths longer than 320 nm. This definition was subsequently modified to include sunscreen active ingredients whose absorption maxima is within the UV range of 320-400 nm (Federal Register 1993 May 12; Federal Register 1996 Sep 16) (Benson 2000).

The choice of sunscreen agent or combination of agents is crucial for providing the formulation with a suitable SPF value, broad UV absorption spectrum and waterproof capacity. The level of protection offered by a sunscreen product is generally assessed often determined by the measurement of a sun protection factor (SPF). The SPF is assessed by measuring the dose of UVR that produces erythema in unprotected skin or in other words namely the Minimal Erythemal Dose (MED). Then the MED for sunscreen protected skin in measured. The ratio of MED of protected skin to MED of unprotected skin is the SPF. It should be noted that although the SPF provides information as to how long it is possible to exposed and avoid a sunburn, this value

offers only limited information as to the extent of UVA protection provided. To appreciate fully the nature of a SPF for a given product it should be remembered that doubling the SPF does not equate to double protection. The increase in SPF is correctly related to the % of UVR that is transmitted, not that which is absorbed. For example, if SPF 15 transmits approximately 6.7% of UVR, SPF 30 would allow 3.3% (Hayden et al. 1998). Desired SPF values can be obtained for a combination of sunscreen agents based on the knowledge of their synergistic activity. Ideally, an efficient sunscreen combination will minimize the concentration of sunscreen agents required. This is particularly important because it will reduce possible irritation and cost (Klein 1990). Modern sunscreening agents are classified as either chemical sunscreens or physical blockers.

Sunscreens are regularly applied to large areas of the body and it is essential to have an understanding of their safety. In particular, it is necessary to quantify the skin penetration and distribution of sunscreens agents. Even if the degree of skin penetration is small, the total amount absorbed may be significant and therefore potential consequences should be considered. The limited data on skin absorption currently available suggests that sunscreens penetrate into the upper layers of skin to varying degrees (Hayden *et al.* 1998). Appreciable absorption and urinary excretion of oxybenzone and its metabolites following topical application to the skin of healthy volunteers have been reported (Hayden et al. 1997). There is also report of sunscreen excretion in the breast milk of humans (Hany and Nagel 1995; Hayden *et al.* 1998).

It has been shown that anatomical site of application is a major determinant of drug absorption. In a letter to the Lancet commenting on the previously published results (Hayden et al. 1997). Ong (1998) noted that skin penetration depends on the site of application and that further in vivo studies should measure plasma concentration. The following study examines sunscreen absorption following topical application to the face and back. The face is the most common site for sunscreen application, as it is most frequently exposed to sunlight without the protection of clothing. The back is less frequently exposed on a routine basis, but is the site generally used for determination of SPF in the development of sunscreen products.

5.2. Experimental design

Ethical approval was obtained from the Health Research Ethics Board at the University of Maniotba and St. Boniface Hospital Ethics Committee. The study involved application of a commercially available sunscreen product, Coppertone Colorblok® for kids (SPF 30), to two anatomical sites on the face and back in a crossover fashion. Permeation of sunscreen into the skin, systemic absorption and elimination were monitored following application. A preliminary study was conducted on three healthy volunteers to determine absorption kinetics. This permitted identification of the time of steady-state plasma concentration of sunscreen so that only a single time point blood sample was required in the experimental study.

5.2.1. Preliminary study protocol

A commercially available sunscreen product, Coppertone Colorblok for kids (SPF 30) was applied at a dose of 2 mg/cm^2 to the arms and back of three female human volunteers, aged 22-42 years. This lotion contains homosalte (HS) 8%, octylmethoxycinnamate (OMC) 7.5%, oxybenzone (OX) 6%, and octylsalicylate (OS) 5% as active ingredients. Baseline blood and urine samples were collected prior to sunscreen application. Permeation of sunscreen into the skin, systemic absorption and urinary elimination were monitored for up to 48 hr following application. At 30 minutes after application, a small area of the skin was wiped with Kleenex tissue and skin strip samples taken by application and removal of Scotch® crystal clear tape (3 cm x 1.9 cm). Tape stripping is a relatively non-invasive technique, which permits samples of stratum corneum (0.5-1 μm thickness) to be collected from the treated area. The tapes were applied to the treated areas by application of a consistent pressure generated by stroking the thumb 10 times along the tape. The stratum corneum was sequentially stripped up to 16 times and the 16 strips taken from each site were grouped into 4 groups for subsequent analysis of sunscreen content (Group 1: strip 1; Group 2: strips 2-6; Group 3: strips 7-11; Group 4: strips 12-16). This procedure was repeated on a separate site at 4 hours and 8 hours. The stratum corneum was removed by 16 sequential strips, focusing on the upper layers of the stratum corneum. It has been found that on the flexor surface of the forearm about 30 tape strips are needed to strip off most of the horny layer (Pinkus 1951). Complete removal of the stratum corneum was not possible

even after 30-40 strippings (Hojyo-Tomoka and Kligman 1972) and a certain barrier function in the tissue so treated remains (Malkinson 1958; Feldmann and Maibach 1965). Ohman and Vahlquist (1994) showed that even after 100 tape strippings the entire stratum corneum could be removed. The stripping procedure was not normalized, since the inconsistent cohesion of the corneocyte layers means that reproducible amounts of SC (within and between subjects) cannot be removed (King et al. 1979). The product was washed off the skin at 8 hours post application. Blood samples were taken from all subjects at pre-application baseline and at 1, 2, 4, 6, 8, and 24 hours post-application. Urine output of all subjects over the 48-hour post-application period was collected. All plasma and tape strip samples were analyzed as described in chapter 4.

Sunscreen in the stratum corneum tape strip samples was extracted by a two-step procedure adapted from Potard and co-workers (Potard *et al.* 1999). This involved overnight contact of the tape with isopropanol to destructure the polymeric glue, followed by dissolution of the polymeric glue and the hard polymeric tape support by acetonitrile. The solvent was then evaporated and the residue resuspended in 1 mL methanol for analysis of sunscreen content by HPLC.

5.2.2. Experimental protocol

A single application of the test product was made to the face of one group of subjects and an equal area on the back of the second group. The application sites were randomly assigned. The product was washed off the skin at 8 hours post-application. After washing, skin strip samples were taken from each site by application and removal of Scotch® tape. A total of 16 strips were taken at the site on the back and 6 from the face. The strips were grouped into 4 and 2 groups respectively (as above) for subsequent analysis of sunscreen content.

Blood samples were taken from all subjects at pre-application baseline and at a suitable steady state time (as determined in the preliminary study, 7.5 hrs) post-application. Urine output of all subjects over the 48-hour post-application period was collected. All plasma, urine and skin strip samples were assayed by high performance liquid chromatography (HPLC).

Following a 7-day washout period, the application and sample collection procedure was repeated. The sunscreen product was applied to the back of those subjects whose faces were previously treated, and to the face of those who were previously treated on the back. The sampling procedure described above was repeated.

5.2.3. Subject Selection

5.2.3.1. Inclusion Criteria

a. Males and Females, 18 years of age or older
b. Signed informed consent
c. Willing to avoid exercise or strenuous activity, sunlight, cigarettes, alcohol, caffeine and other drugs for the duration of the study.
d. Willing to suspend use of cosmetics, moisturizers, or other skin care products at the test site 24 hours prior to and for the duration of the study.

5.2.3.2. Exclusion Criteria

a. Insulin-dependent diabetes
b. Clinically significant skin diseases which may contraindicate participation, including psoriasis, eczema, atopic dermatitis, and active cancer
c. Asthma that requires medication
d. Immunological disorders such as HIV positive, AIDS and systemic lupus erythematosus
e. Treatment for any type of cancer within the last six months
f. Use of any prescribed or over-the-counter medication
g. Use of topical drugs at test site
h. Pregnancy, lactation, or planning a pregnancy
i. Medical conditions which, in the Investigator's judgement, make the subject ineligible or place the subject at undue risk

j. Concurrent participation in any clinical testing or participation in any clinical testing within the last four weeks, including other studies being conducted at Hill Top Research, Inc
k. Damaged skin in or around test sites, which includes sunburn, extremely deep tans, uneven skin tones, tattoos, scars, excessive hair, numerous freckles or other disfiguration of the test site
l. Known allergy or sensitivity to sunscreen or sunscreen ingredients, cosmetics, or cosmetic ingredients

5.3. Results and Discussion

This clinical study demonstrates systemic absorption of sunscreens after topical application. Data from the preliminary study showed a significant amount of sunscreen penetrated the epidermal barrier (Figure 5.1. and Table 5.1.), a finding which is consistent with previously published *in vitro* and *in vivo* research (Treffel and Gabard 1996). Higher amounts of sunscreens were recovered from the upper layers of the stratum corneum at 30 minutes post application. At 4 and 8 hours post application, similar depth of penetration profiles were obtained but with overall lower sunscreen concentration (Table 5.1.). There was no significant difference in the absorption of sunscreens into the skin after application to the arms and back, though slight differences were observed (as shown in bold in Table 5.1.). This is consistent with previous reports of similar absorption kinetics through the arms and back after topical application of other drugs (Rougier et al. 1986; 1987). In addition, the amount quantified in the first strip (Group 1) seems to predict which components will have better substantivity for the stratum corneum. From Figure 5.1., it appears that oxybenzone, octylmethoxycinnamate and octylsalicylate have more affinity towards the stratum corneum when compared to homosalate.

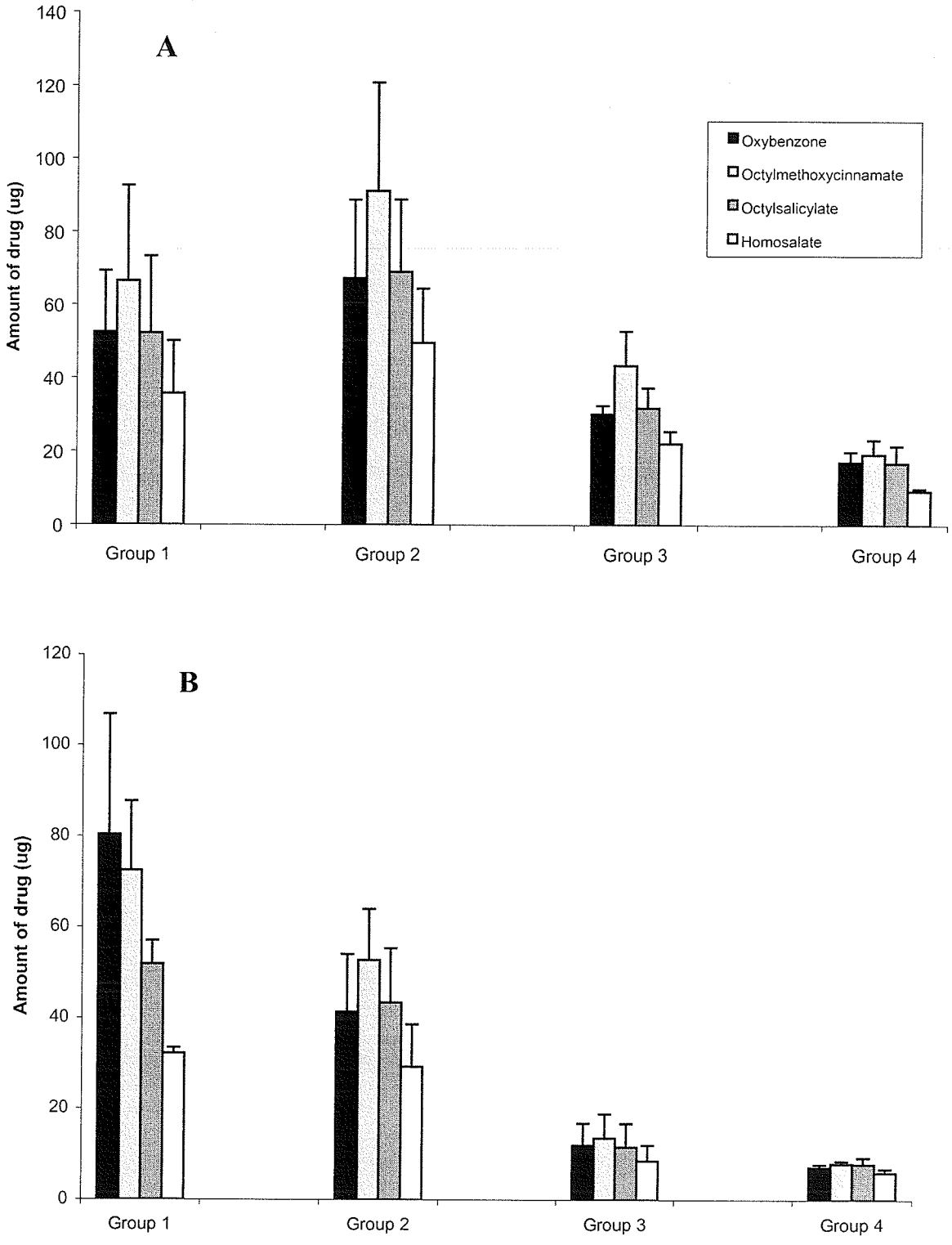


Figure 5.1. Amount of sunscreen in the tape strips from the arms (A) and back (B) of volunteers after 30 minutes of application of the sunscreen formulation.

Table 5.1. Amount (μg per pool of strippings) of UV filters recovered from the tapes (n=3)

	After 30 minutes		After 4 hours		After 8 hours	
Stripping group	**Arm**	**Back**	**Arm**	**Back**	**Arm**	**Back**
Group 1						
Oxy	52.39 ± 16.87	80.33 ± 26.44	**8.69 ± 3.61**	31.76 ± 6.49	19.78 ± 14.44	21.56 ± 4.10
OMC	66.59 ± 25.88	72.42 ± 15.22	**10.91 ± 2.76**	40.07 ± 5.18	25.67 ± 13.20	27.95 ± 4.62
OS	52.21 ± 21.12	51.925 ± 5.10	**9.88 ± 1.10**	32.20 ± 4.34	10.89 ± 3.39	24.44 ± 7.43
HS	35.71 ± 14.32	32.21 ± 1.24	**6.36 ± 1.82**	21.32 ± 7.25	13.28 ± 8.12	14.49 ± 4.06
Group 2						
Oxy	67.29 ± 21.37	41.37 ± 12.71	50.78 ± 32.60	29.42 ± 7.61	25.53 ± 16.59	16.09 ± 2.39
OMC	91.07 ± 29.75	52.74 ± 11.17	67.66 ± 41.45	35.93 ± 8.26	38.51 ± 24.53	21.12 ± 5.45
OS	69.01 ± 19.70	43.41 ± 11.95	54.21 ± 31.21	29.24 ± 4.69	26.93 ± 17.94	19.79 ± 9.15
HS	49.55 ± 14.86	29.24 ± 9.36	36.84 ± 21.36	19.6 ± 4.68	16.27 ± 12.53	11.9 ± 2.68
Group 3						
Oxy	**30.14 ± 2.32**	12.03 ± 4.81	16.49 ± 8.53	8.25 ± 3.60	23.24 ± 8.62	7.42 ± 1.22
OMC	**43.46 ± 9.30**	13.63 ± 5.20	22.59 ± 11.07	11.17 ± 3.35	12.30 ± 6.36	10.05 ± 2.33
OS	**31.89 ± 5.49**	11.67 ± 5.10	18.905 ± 9.71	10.12 ± 3.92	10.41 ± 4.61	9.23 ± 3.09
HS	22.12 ± 3.39	8.66 ± 3.39	13.67 ± 7.71	6.09 ± 2.02	6.35 ± 4.26	5.43 ± 0.64
Group 4						
Oxy	**17.12 ± 2.88**	7.18 ± 0.67	11.98 ± 6.05	4.86 ± 0.64	6.29 ± 1.65	4.58 ± 2.10
OMC	**19.29 ± 3.94**	8.02 ± 0.54	12.61 ± 2.54	7.27 ± 3.53	10 ± 1.91	6.77 ± 4.95
OS	16.86 ± 4.71	7.9 ± 1.42	10.17 ± 1.68	7.51 ± 5.7	7.5 ± 1.56	6.65 ± 5.63
HS	9.27 ± 0.63	6.00 ± 0.84	7.43 ± 2.04	4.14 ± 1.94	4.23 ± 1.01	3.63 ± 2.56

Group 1 = Strip 1; Group 2 = Strip 2-6; Group 3 = Strips 7-11; Group 4 = Strips 12-16
Bold numbers are significantly different ($P<0.05$). Values (mean ± SD) for arms are compared with back at a particular time.

Systemic absorption of oxybenzone was confirmed through detection of oxybenzone in the plasma and in the urine. The plasma was only measured for the four sunscreens, excluding their metabolites. Minute amounts of oxybenzone were observed in the plasma, whereas other sunscreens were below the limits of detection. Up to approximately 1% of oxybenzone and its metabolites were detected in the urine (Figure 5.2.). The major metabolite was 2,4-dihydroxybenzophenone (DHB), whereas 2,3,4-trihydroxy-benzophenone (THB) was detected only in trace amounts. The low levels of oxybenzone in the blood may be due to rapid metabolism and distribution, as has been demonstrated previously in rats (Okereke et al. 1993). Okereke et al (1993) further reported that oxybenzone and its metabolites were found in liver, kidney, spleen,

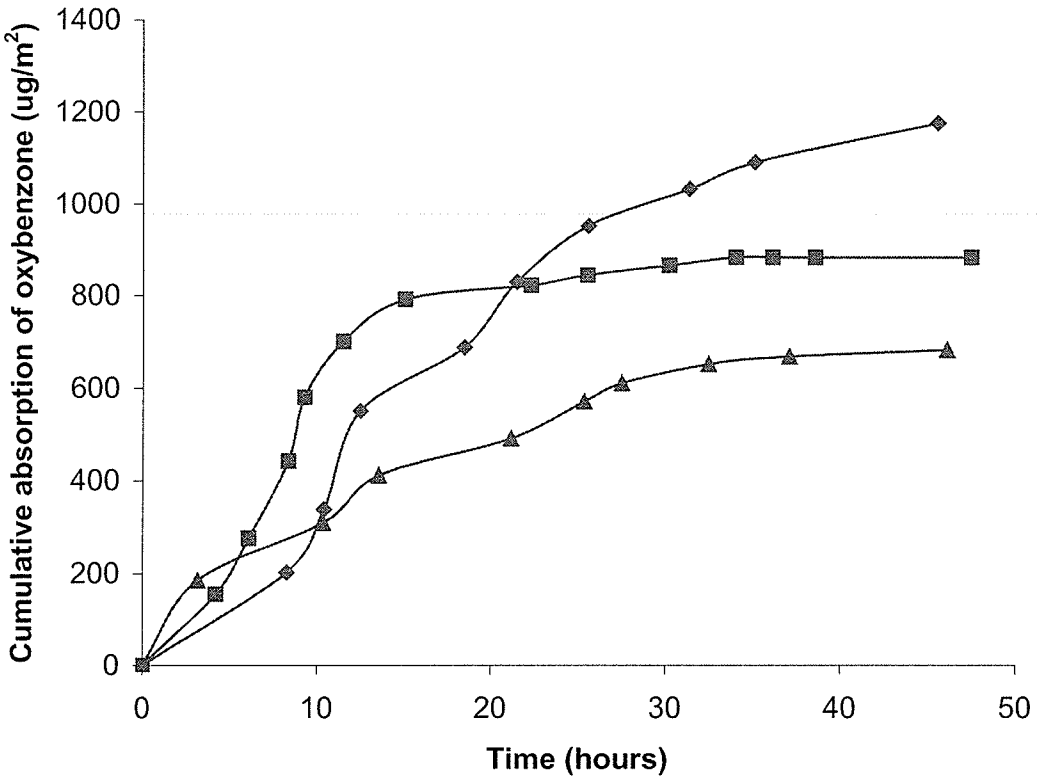


Figure 5.2. Systemic absorption of oxybenzone following topical application of a commercial sunscreen product to 3 healthy human volunteers; absorption was determined from urinary excretion of oxybenzone and its metabolite

heart and even testes. Since the area of application of sunscreen in our study was approximately 864 cm^2, which is almost half the area that one would apply in practice, the total amount systemic absorption of oxybenzone could be higher in practice.

The results from the preliminary study clearly demonstrate that oxybenzone penetrates across the human skin and enters the systemic circulation in significant amounts. Others sunscreens were below the limits of detection and appear to be retained in the stratum corneum rather than penetrating across the skin.

The main study primarily focussed on comparing the penetration of sunscreens after topical application to face and back. On the basis of the preliminary study, it was decided to have only one time point blood sample at 7.5 hours. Since the area and amount of sunscreen formulation applied was much lower than in the preliminary study,

sunscreen in the blood or urine samples could not be detected. The main study was therefore focused on a comparison on skin penetration from the face and the back. The amount specified in the protocol was applied (2 mg/cm^2). This is consistent with the amount specified in the FDA guidance for SPF testing and general use (Administration 1993). Facial application was to 3 areas: forehead and both cheeks. The forehead template was 10 cm x 4 cm. Because of the difference in facial shape from one subject to another, either of two templates was used for the cheeks: 8 cm x 4.5 cm or 6 cm x 6 cm.

10x4=40 plus 2(6x6) = 112 cm^2

OR

10x4=40 plus 2(8x4.5)=112 cm^2

0.224 g (224 mg) of product was applied in total, so 224/112=2 mg/cm^2.

Figure 5.3. shows a substantial amount of sunscreen in the stratum corneum of the back after 8 hours. This figure clearly shows higher amounts of sunscreen in the superficial layers than in the deeper layers. Since there were only 2 groups of tape strips from the facial area, first two groups of tape strips from the back and two groups from the facial area were compared.

Figure 5.4. shows a comparison of the distribution of sunscreens in the stratum corneum of the face and back of the volunteers. The facial tape stripping was conducted on the cheeks with the 6 strips taken divided in two groups for analysis parallel to the first two groups from the back. The figure clearly demonstrates almost twice the amount of sunscreen in the facial stratum corneum as compared to that in the stratum corneum of the back. It is evident that more sunscreen is retained in the facial stratum corneum as compared to the back. This may result in greater sunscreen efficacy on the face and providing sun protection for a longer period of time. Because the amount of sunscreen formulation was applied over a smaller surface area in the study compared with the preliminary study, the blood and urine levels of the sunscreens were below the limits of detection. Hence, we were unable to appropriately correlate absorption of sunscreen into the skin with systemic absorption. The high levels of sunscreen in the facial stratum

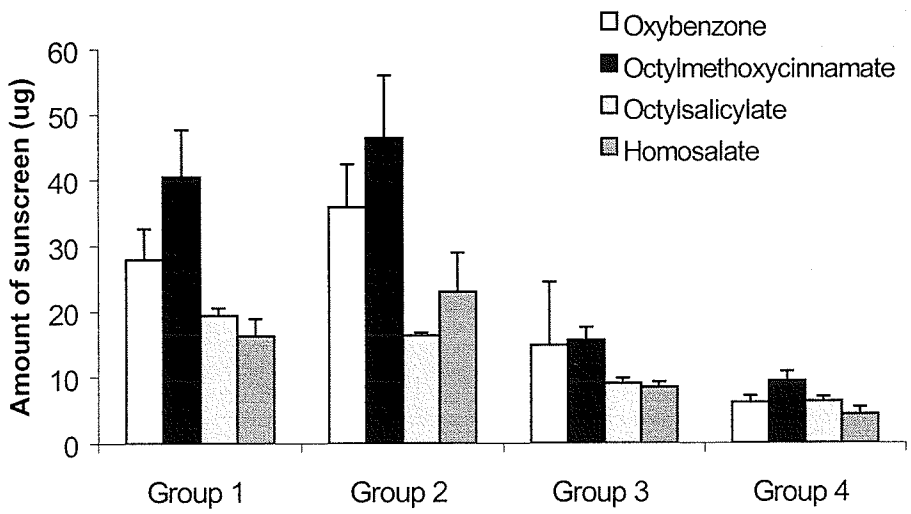


Figure 5.3. Amount of sunscreen in the stratum corneum after an application time of 8 h (mean ±s.e., n = 12).

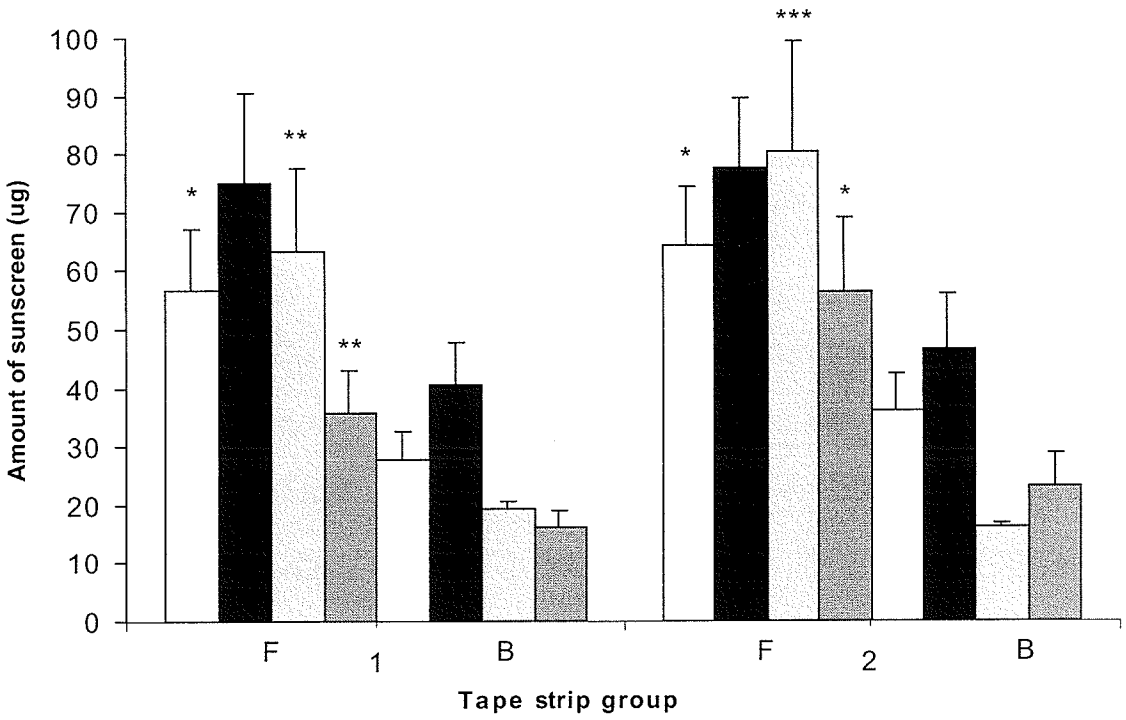


Figure 5.4. Amount of sunscreen in the stratum corneum after an application time of 8 hours (mean ±s.e.). F = facial area and B = back. 1 and 2 denotes the tape strip group from the respective areas. Facial group 1 compared to back group 1 tape strips, and Facial group 2 compared to back group 2 tape strips (= $p<0.05$; ** = $p<0.02$; *** = $p<0.01$).*

corneum as compared to the back may be due to higher partitioning of the sunscreen from the formulation vehicle to the stratum corneum, higher permeability of facial stratum corneum and possible back partitioning from the viable epidermis to the stratum corneum. The viable epidermis in the facial area is expected to be more hydrophilic as compared to the back due to the presence of more vascular tissues. The facial area, particularly the forehead is reported to be more permeable for many drugs relative to the other areas (Rougier et al. 1986). Each site has anatomical differences which result in the differences observed during experiments. *In vivo,* results from the stratum corneum clearly confirm that as much as twice the amount of sunscreen is retained in the facial stratum corneum than the back stratum corneum. The higher sunscreen concentration in the facial area for a longer period of time suggests that less sunscreen is required in the facial area to produce the similar sun protection as in the back. Our studies strongly indicate that anatomic site could influence the distribution of sunscreen in the human stratum corneum and thereby resulting in different sun protection efficiency at different anatomical sites. Further studies are required to assess the sun protection efficacy of sunscreen formulation with respect to different anatomic sites.

Oxybenzone was the only sunscreen found to be absorbed across the skin There have been suggestions that certain sunscreen agents may have mutagenic effects if absorbed into the skin to the viable cells of the epidermis and beyond (Knowland et al. 1993). In this study it was shown that while the sunscreen agent padimate O (PdO) is not mutagenic in normal Ames tests, under illumination with UV light it induces mutations and attacks DNA. It is interesting that the mechanism of mutagenecity is suggested to be due to the generation of free radicals, the same as implicated in UV induced skin damage and cancer (Nishi et al. 1991). Studies by the National Toxicology Program suggested a possible decrease in sperm motility, an increase in abnormal sperm formed, and alteration in sperm production with oxybenzone (National Toxicology Program 1991; National Toxicology Program 1991). It is also reported that sunscreens caused mitochondria stress and inhibited cell growth in cultured human cells (Xu and Parsons 1999). Oxybenzone has also been identified to induce liver and kidney toxicity in rats and mice. Benzophenone is listed among 'chemicals suspected of having

endocrine disrupting effects' by the World Wildlife Fund, the National Institute of Environmental Health Sciences in the USA and the Japanese Environment Agency. Infact, oxybenzone and its metabolite DHB have been recently suggested as endocrine modulators (Schlumpf et al. 2001; Nakagawa and Suzuki 2002). The oxybenzone metabolite, DHB is reported to be more toxic to rat hepatocytes and be more prone to free radical formation than the parent compound (Nakagawa and Moldeus 1992). DHB has been detected in urine after topical application of a sunscreen formulation containing oxybenzone to human volunteers (Hayden et al. 1997; Sarveiya et al. 2003). Moreover, oxybenzone has been reported to be rapidly metabolized to DHB and other metabolites, further increasing the concern (Okereke et al. 1993). It is clear through *in vitro* (Jiang et al. 1999) and *in vivo* studies (Hayden et al. 1997; Sarveiya et al. 2003) that oxybenzone penetrates into and across the skin in appreciable amounts. In addition, recent studies from our laboratory suggest increased penetration of oxybenzone in the presence of the insect repellant DEET and *vice versa* (Kasichayanula et al. 2002). This further raises the concern. There may be additional concern for young children who have less well-developed processes of elimination, and a large surface area per body weight than adults, with systemic availability of a topically applied dose. Further, the absorption is likely to exceed after repeated application.

5.4. Conclusions

It is clearly evident from the current study that oxbenzone is absorbed across the skin in appreciable quantities. Penetration of other more lipophilic sunscreens to the systemic circulation is minimal. Appreciable quantities of oxybenzone were detected in the plasma and the urine. A comparison of the skin penetration of sunscreen at different anatomical sites (face and back) demonstrates that as much as twice amount is present or retained in the facial stratum corneum. This suggests that less amount of sunscreen would be required on the face to produce the same sun protection as would be required on the back. Further studies are required to assess the sun protection efficacy of sunscreen formulation with respect to different anatomical sites.

Chapter 6. Inclusion complexation of the sunscreen 2-hydroxy-4-methoxy benzophenone (oxybenzone) with hydroxypropyl-β-cyclodextrin: effect on membrane diffusion

6.1. Introduction

Increased evidence linking sun exposure with skin aging, damage and development of skin cancers has led to more extensive use of sunscreens in beach products and everyday cosmetics. Sunscreen products are formulated to provide a specific sun protection factor (SPF) and to absorb a broad spectrum of ultraviolet radiation (UVR). Since sunscreens are regularly applied to large areas of the body, it is essential to have an understanding of their safety and minimize absorption of actives to viable tissues.

Acute toxic adverse effects of specific sunscreen agents include contact irritation, allergic contact dermatitis, phototoxicity, photoallergy, and staining of the skin. However the incidence is relatively small considering their widespread use. Relatively little information is available on the mutagenic and carcinogenic potential of sunscreen agents. It has been suggested that under UVR some sunscreens and related chemicals may have mutagenic effects in the viable tissues of the epidermis and dermis (Knowland *et al.* 1993). A recent report that sunscreens caused mitochondrial stress and inhibited growth in cultured human cells has added to safety concerns (Xu and Parsons 1999). Although the reports are not conclusive, the best means to ensure sunscreen safety is to minimize penetration to viable tissues.

Ideally a topically applied sunscreen would be localized in the stratum corneum without penetration to deeper viable tissues. This is likely the case for many lipophilic UVB sunscreens, but the UVA sunscreen 2-hydroxy-4-methoxy benzophenone (commonly known as benzophenone-3 or oxybenzone), which has an absorption spectrum of 270-350 nm, is known to be systemically absorbed following administration to the skin (Benson 2000). For example, topical absorption leading to excretion of oxybenzone and its metabolites in the urine (Hayden *et al.* 1997; Sarveiya *et al.* 2003) and breast milk (Hany and Nagel 1995) has been reported.

Various strategies to reduce the systemic uptake of drugs by targeting the molecules to the upper layers of the skin have been investigated. Jiang et al studied the influence of a range of vehicles on the diffusion of oxybenzone into and across polyethylene membrane and human epidermis (Jiang *et al.* 1998). They reported that the flux and permeability coefficient of oxybenzone is related to vehicle solubility

parameter (Jiang *et al.* 1998). Increased solubility of oxybenzone in the vehicle reduced the thermodynamic activity of the solute in the vehicle, thereby decreasing solute availability from the vehicle, leading to reduced permeability. Walters *et al.* (1997) also demonstrated the influence of vehicle on skin penetration of the highly lipophilic sunscreen octyl salicylate. Due to the poor water solubility of many sunscreen chemicals, vehicles for sunscreen products tend to include oils or alcohols to facilitate formulation. In some cases these vehicles may interact with the stratum corneum barrier to act as skin penetration enhancers (Williams and Barry 1992). Enhanced sunscreen solubility with minimal use of organic solvent would be advantageous.

The current study investigates the use of cyclodextrins to facilitate aqueous solubility of sunscreens and minimize release from the vehicle. Cyclodextrins of pharmaceutical relevance contain 6, 7 or 8 dextrose molecules (α-, β-, γ-cyclodextrin) bound in a 1,4-configuration to form rings of various diameters. The ring has a hydrophilic exterior and lipophilic core in which appropriately sized organic molecules can form non-covalent inclusion complexes (Loftsson and Brewster 1996). This can result in improved stability and/or increased aqueous solubility and dissolution of poorly water-soluble drugs (Rajewski and Stella 1996). β-cyclodextrin (βCD) has low aqueous solubility (18.5 mg/mL at 25 °C); therefore derivatives with enhanced solubility such as 2-hydroxypropyl-β-cyclodextrin (HPβCD) and dimethyl-β-cyclodextrin (dimethylβCD) are more commonly used in pharmaceutical formulation.

Cyclodextrins may be useful in topical formulation of lipophilic solutes. First, they could enhance aqueous solubility thereby facilitating formulation without the need for organic and mixed solvents that may have penetration enhancement properties. Second, the molecular size of the solute-cyclodextrin complex may act to retard skin penetration. A limited number of studies have been published on the use of cyclodextrins in topical formulation. Szente and co-workers (1990) reported improved stability, wettability and dissolution of the lipophilic insect repellent N,N-diethyl-m-toluamide (DEET) by formulation of inclusion complexes with βCD. The solubility and photostability of sunscreen chemicals was increased by complexation with HPβCD (Scalia *et al.* 1998; Scalia *et al.* 1999).

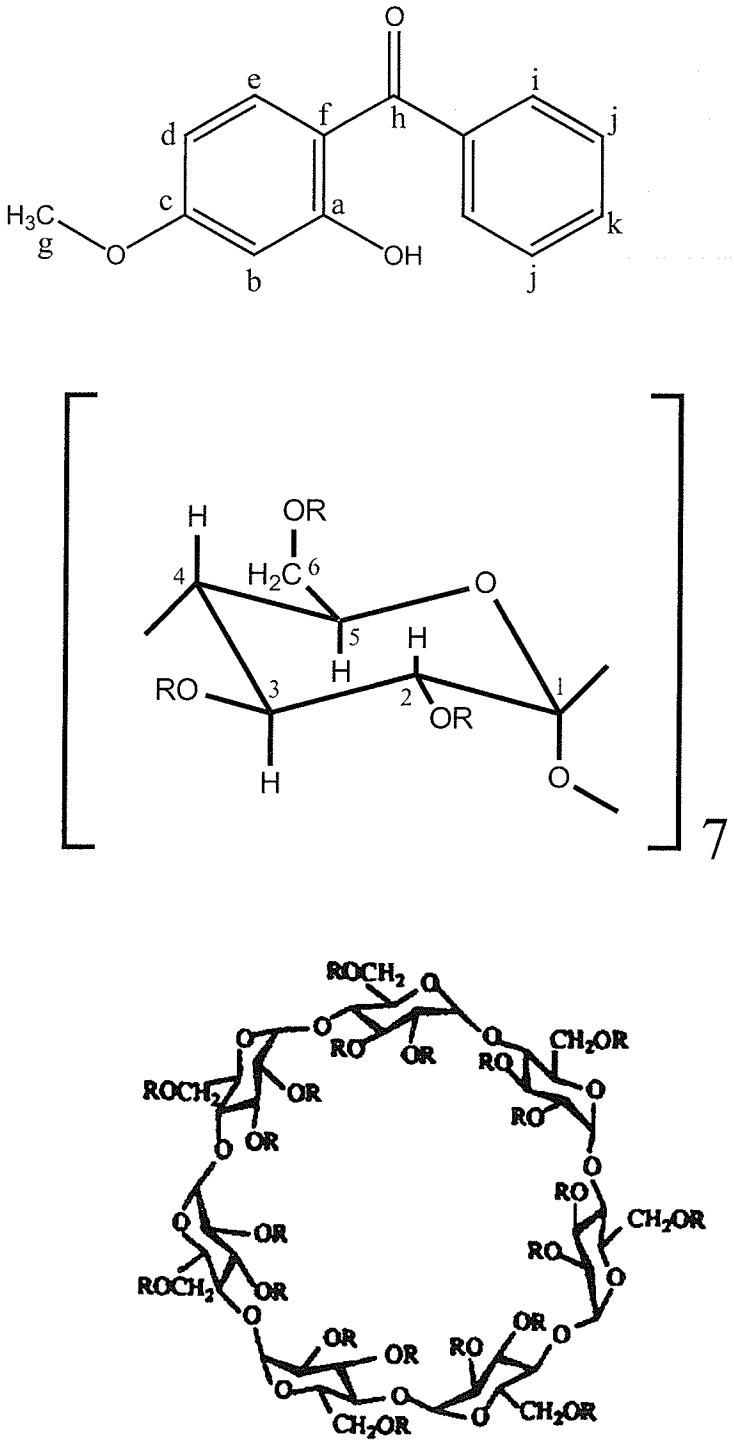


Figure 6.1. The chemical structures of 2-hydroxy-4-methoxy-benzophenone or oxybenzone (upper) and HPβCD. R = -$CH_2CH(OH)CH_3$ or –H, with average molar substitution of 0.75.

There are reports of both increased and decreased skin penetration of drugs due to cyclodextrin complexation. For example, penetration of lirazole across rat skin was increased by complexation with HPβCD and dimethylβCD (Vollmer *et al.* 1994). Based on thermal analysis data, the authors suggested that dimethylβCD interacts with proteins and disorders the stratum corneum bilayer lipid structure. Similar changes were not observed with HPβCD. Recently, the ability of HPβCD and γCD to reduce the release of deet from vehicles was demonstrated (Proniuk *et al.* 2002). Williams and co-workers demonstrated the use of cyclodextrins to reduce skin penetration of estradiol and toluene (Williams *et al.* 1998). The effect on skin penetration may be related to CD concentration. In a recent review of the literature, Loftsson and Masson (2001) concluded that reduced permeability is generally observed at relatively high concentrations of cyclodextrins.

The aim of the present study was to prepare and assess a complex between oxybenzone and HPβCD (Figure 6.1). This complex was characterized by phase solubility studies, nuclear magnetic resonance (NMR) spectroscopy and thermal analysis. The effect of complexation on the UV transmittance spectra of oxybenzone (as a measure of sunscreen efficacy) and its diffusion across a synthetic membrane was assessed.

6.2. Experimental

6.2.1. Materials

Oxybenzone was purchased from Aldrich (USA). β-cyclodextrin (βCD) and hydroxypropyl-β-cyclodextrin (HPβCD) (molar substitution 0.6 – 0.9) were generously provided as gifts by Wacker Biochem Corporation (USA). Bovine serum albumin was from Sigma, USA. Methanol and acetonitrile were high-performance liquid chromatography (HPLC)-grade from Fisher Scientific, USA. De-ionized water was used and all other chemicals were analytical-reagent grade.

6.2.2. Determination of oxybenzone solubility

A moderate excess of oxybenzone was placed in 10 mL of each solvent (30%, 45% and 60% aqueous ethanol) and stirred in the dark at 25 ± 0.1 °C for 72 h. Each mixture was then centrifuged at 10,000 g for 10 min. This step was repeated with the resulting supernatant, before analysis of oxybenzone concentration by HPLC.

6.2.3. Phase Solubility Studies

Solubility measurements were performed according to the method of Higuchi and Connors (Higuchi and Connors 1965). An excess amount of oxybenzone was added to aqueous solutions containing different concentrations of the cyclodextrins (0-15 mM for βCD and 0-60 mM for HPβCD). The suspensions were stirred in 10 mL screw-capped vials at 25 ± 0.1 °C and shielded from light. After equilibration for 72 hr (previously validated), the contents of each vial were filtered through a 0.45 μm membrane filter (Millipore, USA) and analyzed by HPLC to determine the concentration of oxybenzone.

6.2.4. Preparation of the inclusion complex

The inclusion complex was prepared at an equimolar ratio of oxybenzone to HPβCD as reported below. Oxybenzone (114.1 mg, 0.5 mmol) dissolved at room temperature in 95% ethanol (10 mL) was added to purified water (10 mL) containing HPβCD (700 mg, 0.5 mmol). The resulting solution was stirred for 24 hr at room temperature and shielded from light. The solvent was evaporated under vacuum at 40 °C by rotary evaporation. The solid complex was dried under vacuum in a desiccator for 3 days with phosphorus pentoxide as a drying agent. The oxybenzone content of the complex was determined by HPLC.

6.2.5. Thermal analysis

Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) were performed on a Metler TGA/SDTA 851 and Metler DSC/821 respectively. Samples of oxybenzone, HPβCD and the complex were scanned at a heating rate of 20 °C per min under a flow of nitrogen (50 mL/min).

6.2.6. *NMR Spectroscopy*

^{1}H and ^{13}C NMR spectra were recorded at 300 and 75 MHz respectively using a Bruker Avance 300 spectrometer (Karlsruhe, Germany). Samples were dissolved in deuteromethanol and chemical shifts (δ) for hydrogen and carbon resonance reported in ppm relative to TMS.

6.2.7. *High-performance liquid chromatography*

An Alliance liquid chromatographic system (Waters Inc., USA) equipped with a 2690 Separations Module and 996 Photodiode Array detector was used. Separation was achieved on a Symmetry C_{18} column (5μm, 3.9 x 150mm I.D., Waters Inc., USA) at ambient temperature, with an inline pre-filter. Integration was undertaken using a personal computer equipped with Millenium 4.0 version software.

The mobile phase consisted of methanol : water [85:15], filtered through a 0.45 μm membrane filter (Durapore membrane filter, Millipore, USA) The mobile phase was continuously degassed before and during use. The flow rate was 1.0 mL/min and the detection wavelength set at 289 nm. The retention time for oxybenzone was ~ 2.4 min. Calibration curves were calculated on peak area measurements and the assay was fully validated. Extraction of oxybenzone from 4% BSA solutions was achieved using solvent precipitation of proteins. Briefly, 100 μL sample of oxybenzone in BSA solution was mixed with 200 μL acetonitrile : methanol (95:5), refrigerated for 15 minutes, then centrifuged at 10,000 g for 10 minutes to precipitate proteins prior to HPLC analysis. This extraction was validated with a recovery of 99.6% ± 1.08% from BSA solutions.

6.2.8. *UV Transmittance spectral analysis*

Solutions for UV spectral analysis were prepared in ethanol using oxybenzone alone and in combination with cyclodextrin. The method to determine transmittance has been reported previously (Agrapidis-Paloympis *et al.* 1987; Rosenstein *et al.* 1999). Four combinations were prepared in 100 mL of ethanol containing (i) 1 mg oxybenzone (4.38×10^{-3} mmol), (ii) 1 mg oxybenzone and 12.6 mg HPβCD (8.75×10^{-3} mmol), (iii) 1 mg oxybenzone and 6.3 mg HPβCD (4.38×10^{-3} mmol), (iv) 1 mg oxybenzone and 3.15 mg HPβCD (2.19×10^{-3} mmol). UV transmittance curves of each solution were obtained with the aid of a UV/VIS spectrophotometer with scanning wavelengths

between 290 to 400 nm (Shimadzu, Japan). A background correction was performed using 1cm quartz cells filled with blank solvent. The UVA transmittance was determined from the area under the transmittance curve in the 320-400 nm region for each solution divided by the area under the transmittance spectrum for these wavelengths in the absence of sunscreen and/or HPβCD, multiplied by 100.

6.2.9. In vitro release and membrane diffusion

Oxybenzone release and membrane permeation was determined across a synthetic model membrane (polydimethyl siloxane, PDMS). In vitro permeation studies across PDMS were performed in Franz-type diffusion cells (cross sectional surface area 1.18 cm^2). The membrane was immersed in de-ionized water for 1 hr then mounted between the donor and receptor compartments of diffusion cells and the assembly held in place with a plastic clamp. The receptor chamber (approx. 3.5 mL) was filled with 4% BSA in phosphate buffer (pH 7.4), stirred continuously in a water bath at 37±0.1 °C. The donor phase consisted of 1 mL of each of the test formulations of oxybenzone i.e., saturated solutions of oxybenzone in 30, 45 and 60% aqueous ethanol; saturated solutions of oxybenzone in 45 and 60% ethanol containing equimolar or 2 times molar concentrations of HPβCD; and saturated solutions in 30% ethanol with 1, 2 and 4% HPβCD. Aliquots of 100 µL of the receptor fluid were withdrawn for HPLC analysis and replaced periodically for 6 hr. Experiments were conducted in triplicate.

The flux of oxybenzone through the membranes into the receptor fluid from each of the formulations was determined from slopes of plots of cumulative concentration in the receptor phase vs time and expressed as µg $cm^{-2}h^{-1}$. Permeability coefficients were calculated for oxybenzone from each formulation. The difference between the cumulative amount of oxybenzone in the receptor versus time plots was assessed using multiple regression with pair wise comparison. One-way ANOVA followed by Bonferroni's comparision test was used for assessing the decrease in permeability coefficient of oxybenzone with increased concentration of ethanol and on addition of HPβCD. The level of significance was set at $P<0.05$.

6.3. *Results and Discussion*

6.3.1. *Characterization of the complex*

The phase solubility studies of oxybenzone in aqueous solutions of different concentrations of βCD and HPβCD examined in this study are shown in Figure 6.2A. Of the two cyclodextrins used only HPβCD caused a substantial increase in aqueous solubility of oxybenzone and consequently only HPβCD was selected for further experiments. The slope of the solubility curve (Figure 6.2B) is less than unity and the linear A_L-type relationship suggest a 1:1 complex stoichiometry of oxybenzone with HPβCD (Higuchi and Connors 1965). The apparent stability constant (K) for the oxybenzone-HPβCD complex was calculated from the slope and intercept values of the initial straight-line portion of the solubility diagram, according to the following equation:

$$K_{1:1} = \text{slope}/(\text{intercept}\ (1 - \text{slope}))$$

The apparent stability constant (K) for the oxybenzone-HPβCD complex was calculated to be 5839 M^{-1}.

The complex was characterized in the solid state by thermal analysis using DSC and TGA. DSC curves for HPβCD showed a shallow endotherm at 110°C. A sharp melting point endotherm was observed for oxybenzone at 67°C. A slight deviation in this sharp melting point endotherm for oxybenzone was observed in the presence of HPβCD (65°C) and the shallow endotherm for HPβCD was shifted from 110 °C to 117 °C in the presence of oxybenzone. These deviations indicate an interaction between oxybenzone and HPβCD. Confirmation was provided by the TGA curve of the oxybenzone-HPβCD complex which shows that the interaction of oxybenzone with HPβCD leads to a dramatic reduction in weight loss of oxybenzone, compared to oxybenzone alone (Figure 6.3). The weight loss at the inflect point for oxybenzone

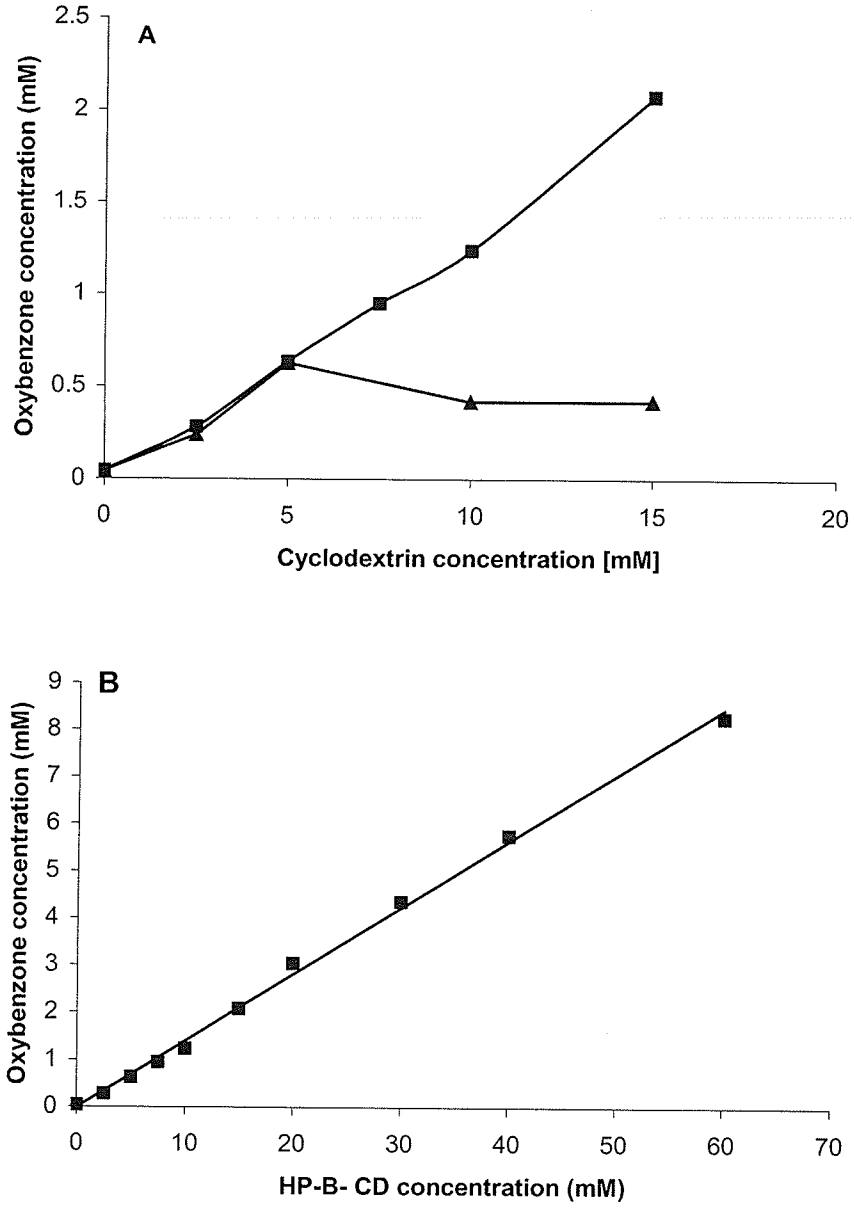


Figure 6.2. A. Phase solubility diagrams in water at 25 °C for oxybenzone with (0-15 mM) (▲ βCD and ■ HPβCD) B. Phase solubility diagram in water at 25 °C for oxybenzone in the presence of HPβCD (0-60 mM).

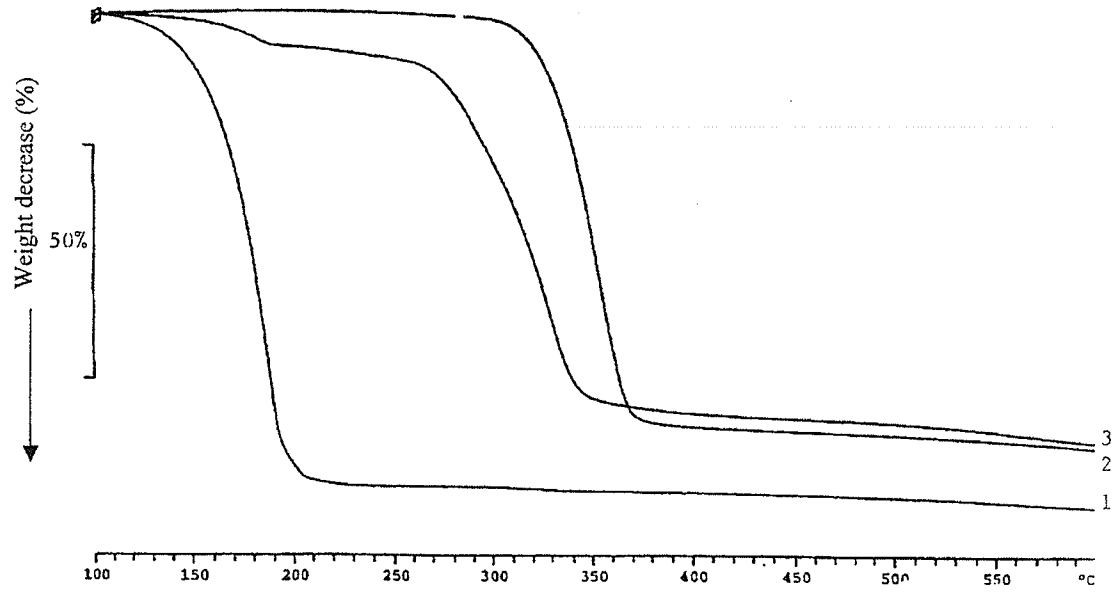


Figure 6.3. TGA thermograms for (1) oxybenzone, (2) HPβCD, (3) oxybenzone - HPβCD complex.

(185.11°C) and HPβCD (352.12°C) alone were 99.18 % and 86.79 % respectively. The complex has two inflect points with a weight loss of only 5.44 % at 179.62 °C and a major weight loss of 74.21 % at 328.23 °C. These changes can be ascribed to interaction of oxybenzone with HPβCD. Hence, the results of thermal analysis indicate an interaction between the two moieties.

NMR studies were performed to obtain further evidence to determine the interaction between the sunscreen molecule and the cyclodextrin moiety in deuteromethanol. Comparison of the ^{13}C NMR spectrum of cyclodextrin alone and in the presence of oxybenzone shows significant upfield chemical shifts in carbons 3 and 5 in the ring (Table 6.1; see Figure 6.1). These shifts are indicative of an interaction between the oxybenzone and cyclodextrin molecule. No significant changes were observed in the cyclodextrin protons in the ^{1}H NMR spectra.

Table 6.1. ^{1}H and ^{13}C NMR chemical shifts (δ, ppm) for HPβCD in the absence and presence of oxybenzone.

	^{1}H NMR chemical shift, δ, for the proton on carbon no.:					
	1	2	3	4	5	methyl
HPβCD	5.003	3.733	3.973	3.525	3.849	1.15
Oxybenzone-HPβCD complex	4.995	3.731	3.972	3.515	3.838	1.145
Δδ	-0.008	-0.002	-0.001	-0.01	-0.011	-0.005

	^{13}C NMR chemical shift, δ, for carbon no.:					
	1	2	3	4	5	methyl
HPβCD	102.051	74.944	83.472	74.088	62.294	19.525
Oxybenzone-HPβCD complex	102.012	74.933	83.344	74.071	62.062	19.594
Δδ	-0.039	-0.011	-0.128	-0.017	-0.232	0.069

Table 6.2. ^{1}H and ^{13}C NMR chemical shifts (δ, ppm) for oxybenzone in the absence and presence of HPβCD

	^{1}H NMR chemical shift, δ, for the proton on carbon:						
	b	d	e	g	i	j	k
Oxybenzone	6.449	6.478	6.528	3.858	7.58	7.63	7.606
Oxybenzone-HPβCD complex	6.505	6.535	6.561	3.893	7.61	7.665	7.641
Δδ	0.056	0.057	0.033	0.035	0.03	0.035	0.035

	^{13}C NMR chemical shift, δ, for carbon:									
	b	c	d	e	f	g	h	i	J	k
Oxybenzone	102.339	168.061	108.313	132.796	114.446	56.365	201.68	129.987	129.552	136.605
Oxybenzone-HPβCD complex	102.449	168.086	108.451	132.911	114.433	56.52	201.708	130.012	129.682	136.54
Δδ	0.11	0.025	0.138	0.115	-0.013	0.155	0.028	0.025	0.13	-0.065

Alternatively, comparison of the ^{13}C NMR spectrum of oxybenzone in the absence and presence of cyclodextrin shows significant downfield chemical shifts in the carbons b, d, e, and the methoxy carbon (g) of oxybenzone (Table 2). This is in agreement with the deshielding phenomenon previously reported (Inoue *et al.* 1985), in which the carbons interacting with the external part of the cavity are shifted downfield. The aromatic carbon k is shifted upfield, in contrast to downfield shift in carbon i and j. A similar comparison of the ^{1}H NMR spectra shows small downfield chemical shifts (-0.03 to –0.057 ppm) for the protons of oxybenzone (Table 6.2), which is also suggestive of an intermolecular interaction. Although, the NMR results do not clarify the exact inclusion mode, it strongly indicates complexation of oxybenzone with HPβCD.

6.3.2. Ultra-violet transmittance studies

Measured sun protection factors (SPF) provide a measure of the time of skin exposure to sunlight required to induce erythema in the presence of a sunscreen compared to no protection. Theoretical SPFs calculated from UV transmittance spectra have been reported to show correlation with the actual measured SPF values (Schallreuter *et al.* 1996). This study measures the UV transmittance spectra of oxybenzone, as a preliminary measure for SPF and the effect of complexation with HPβCD (Figure 6.4). Since, oxybenzone is primarily considered as a UVA sunscreen agent, percent UVA transmittance was also calculated. The UVA transmittance was determined from the area under the transmittance curve in the 320-400 nm region for each solution (A, B, C, and D) divided by the area under the transmittance spectrum for these wavelengths in the absence of sunscreen and/or HPβCD (Figure 6.4). The percent transmittance decreased with the increase in the concentration of HPβCD, with percent UVA transmittance of 74.76, 74.05, 68.94, and 66.11 for solution A, B, C, and D respectively. The results suggest that HPβCD complexation does not decrease the UVA absorbing properties of oxybenzone, and that a sunscreen formulation incorporating the HPβCD complex would be as effective as oxybenzone alone.

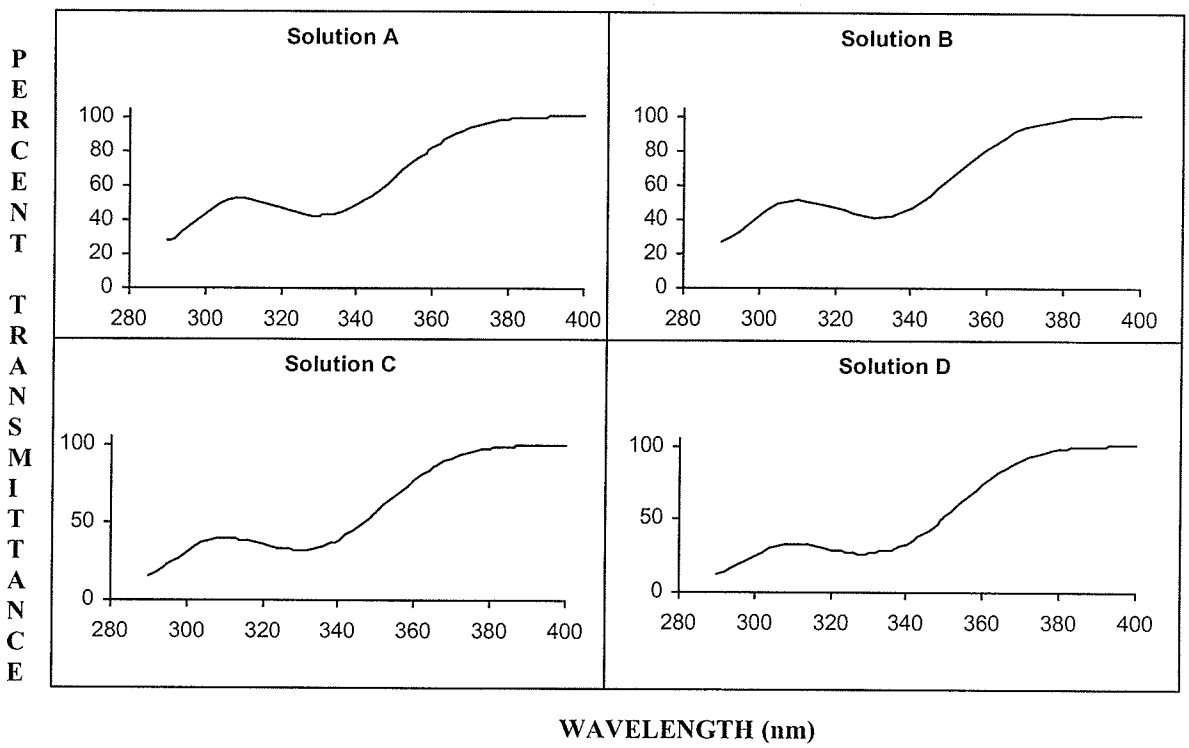


Figure 6.4. Transmittance Spectra of (A) 1 mg oxybenzone (4.38×10^{-3} mmol); (B) 1 mg oxybenzone and 12.6 mg HPβCD (8.75×10^{-3} mmol); (C) 1 mg oxybenzone and 6.3 mg HPβCD (4.38×10^{-3} mmol); (D) 1 mg oxybenzone and 3.15 mg HPβCD (2.19×10^{-3} mmol) in ethanol.

6.3.3. In vitro release and membrane diffusion

The release study was designed to determine if increased affinity of oxybenzone towards the vehicle would result in a decrease in release rate and thereby decrease penetration. Since the solubility of oxybenzone increases with increase in percentage of ethanol (0.134 ± 0.005 mg/mL, 1.43 ± 0.06 mg/mL, and 6.39 ± 0.17 mg/mL in 30, 45, and 60% ethanol respectively), the thermodynamic activity of the drug is expected to decrease in the same order and thereby result in decreased release from the vehicle. Permeability coefficient, K_p values are often used to compare penetration profiles of solutes examined under different conditions and relate to the rate of diffusion of a solute within a membrane adjusted for differences in concentration. As expected, the

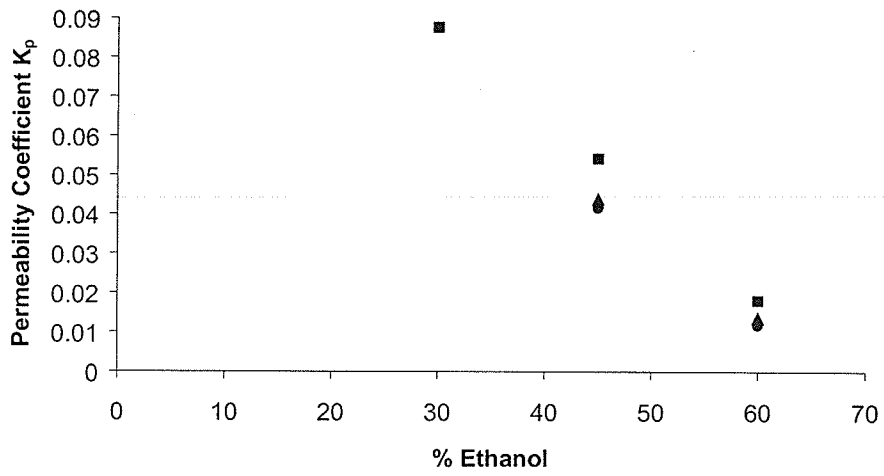


Figure 6.5. Effect of ethanol (■) with HPβCD (▲ equimolar; ● 2 times molar) on permeability of oxybenzone through PDMS membrane.

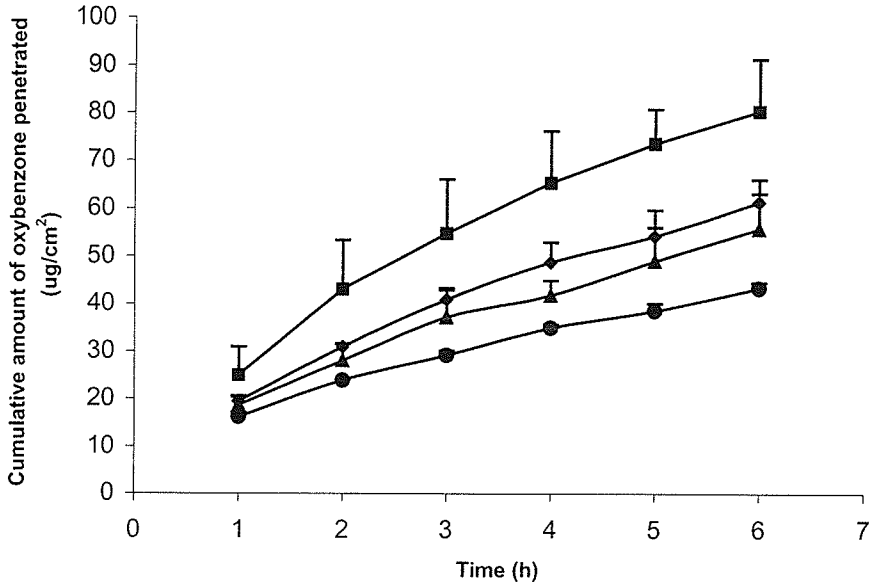


Figure 6.6. Penetration profiles of oxybenzone across PDMS membrane. Data represents mean ± sd of 3 replicates. The donor phase was saturated solution of oxybenzone in 30% ethanol (■■), with 1% (♦); 2% (▲); 4% HPβCD (●·).

permeability coefficient of oxybenzone decreased significantly with increase in the percentage of ethanol from 30 to 60% (Figure 6.5).

Further studies were designed to evaluate whether HPβCD is capable of increasing the affinity of oxybenzone towards the vehicle, which would result in decreased thermodynamic activity of the solute in the vehicle. This would decrease the release of solute from the vehicle, and thereby reduce permeability. Equimolar and 2 times molar concentration of HPβCD decreased the release of oxybenzone from the vehicle containing 45 and 60% ethanol when compared to oxybenzone alone. This is reflected by lower values of permeability coefficient ($p < 0.01$) as shown in Figure 6.5. Oxybenzone release was also reduced in the 30% ethanol vehicle when an excess of HPβCD (1%) was added (Figure 6.6.) ($p < 0.01$). Further, decrease in release was seen with increase in the concentration of HPβCD to 2% and 4% ($p < 0.01$).

6.4. Conclusions

Some studies have reported the influence of vehicle on the skin penetration and efficacy of sunscreen agents. Agrapidis-Paloympis *et al.* (1987) observed that absorbance of many sunscreen agents is profoundly influenced by the polarity and chemical structure of both the sunscreen and the solvent. (Gupta *et al.* 1999) reported that penetration of oxybenzone and octylmethoxycinnamate into and across micro-Yucatan pig skin was formulation dependent. (Agin *et al.* 1998) also commented that sunscreen penetration could be formulation dependent. Treffel and Gabard (1996) demonstrated significantly greater concentrations of three sunscreen agents in the stratum corneum of human volunteers when the sunscreens were applied in an oil in water (O/W) emulsion-gel compared with petroleum jelly. In addition, the sunscreen efficacy as determined was significantly higher for the emulsion (SPF 14) than the petroleum jelly (SPF 5). In a parallel *in vitro* study, they reported greater sunscreen retention in the stratum corneum and less skin penetration to the receptor phase for the emulsion-gel, compared to the petroleum jelly. Similarly, greater skin retention and higher SPF were recently also reported from o/w emulsion gel as compared to petroleum jelly (Chatelain *et al.* 2003). The authors concluded that sunscreen retention,

penetration and SPF could be optimised by appropriate choice of vehicle. In fact, recent studies by (Schulz *et al.* 2002) demonstrated that influence of cosmetic formulation on the SPF or efficacy. The w/o formulation led to an increase of SPF of approximately 40% compared to the o/w emulsion. In case of emulsifier free hydrodispersion, a loss of efficacy was observed. The authors further concluded that efficacy or SPF of a sunscreen formulation is remarkably influenced by the distribution of sunscreen agents in the skin. Previously reported studies confirm that sunscreen retention, penetration and SPF could be optimized by appropriate choice of vehicle and sunscreen distribution in the skin.

Complexation of oxybenzone with HPβCD was demonstrated in the solid state by thermal analysis (DSC and TGA) and in solution by phase solubility and NMR spectroscopic studies. It is concluded that HPβCD can reduce the release and membrane diffusion of oxybenzone whilst retaining its efficacy as a sunscreen agent. This formulation strategy may be useful in controlling skin penetration of sunscreens and other topically applied chemicals. It is possible that inclusion of oxybenzone in HPβCD may also be beneficial in limiting interaction of UV filter with the skin and thus reducing irritation and allergic side effects. Further studies on human epidermis are currently being undertaken. In addition, the influence of cyclodextrin complexation on oxybenzone oxidation (Schallreuter *et al.* 1996) and formation of toxic photoproducts following UVR exposure could be minimized and should be investigated, as this may be a further advantage.

Chapter 7. Summary and Conclusions

In recent years there has been a great deal of interest worldwide in the dermal and transdermal delivery of drugs. Many drugs have been formulated in transdermal systems, and others are being examined for the feasibility of their delivery in this manner (e.g., nicotine, antihistamines, beta-blockers, calcium channel blockers, non-steroidal anti-inflammatory drugs, contraceptives, anti-arrhythmic drugs, insulin, antivirals, hormones, alpha-interferon, and cancer chemotherapeutic agents). Transdermal drug delivery offers the advantages of avoiding local gastrointestinal irritation and hepatic first-pass metabolism providing controlled plasma levels of potent drugs and improved patient compliance (Ranade 1991).

The first step in optimization of drug delivery through the skin is to assess the absorption of drug into and across the skin. Also important is to know where the drug is being targeted into the layers of the skin (upper or deeper layers) or intended for systemic absorption. This work focuses on the aspect of optmization of transdermal permeation of drugs/chemicals into and across the skin. Figure 7.1. outlines the techniques and drugs investigated to optimize transdermal drug delivery.

The transdermal use of NSAIDs is limited due to their relatively poor penetration through the skin. The majority of clinically useful NSAIDs are ionized under normal physiological conditions and therefore have poor physicochemical characterization for penetration across the skin. The formation of ion pairs has been investigated for the enhancement of membrane permeability and hence bioavailability of hydrophilic ionized molecules. Several researchers have investigated the ion pair technique to improve the permeation of drugs through the skin and other biological membranes (Megwa *et al.* 2000; Megwa *et al.* 2000; Valenta *et al.* 2000). Although, most of the studies describe ion pair approach as a means to increase the permeation of drugs across biological and synthetic membranes, few have provided direct evidence of ion pair formation. Chapters 2 and 3 demonstrate the technique of ion pair formation for increasing the percutaneous absorption of the NSAIDs - ibuprofen and benzydamine. Nuclear magnetic resonance (NMR) spectroscopy was used to demonstrate the existence of ion pairs and to rationalize the permeation behavior in terms of molecular interactions.

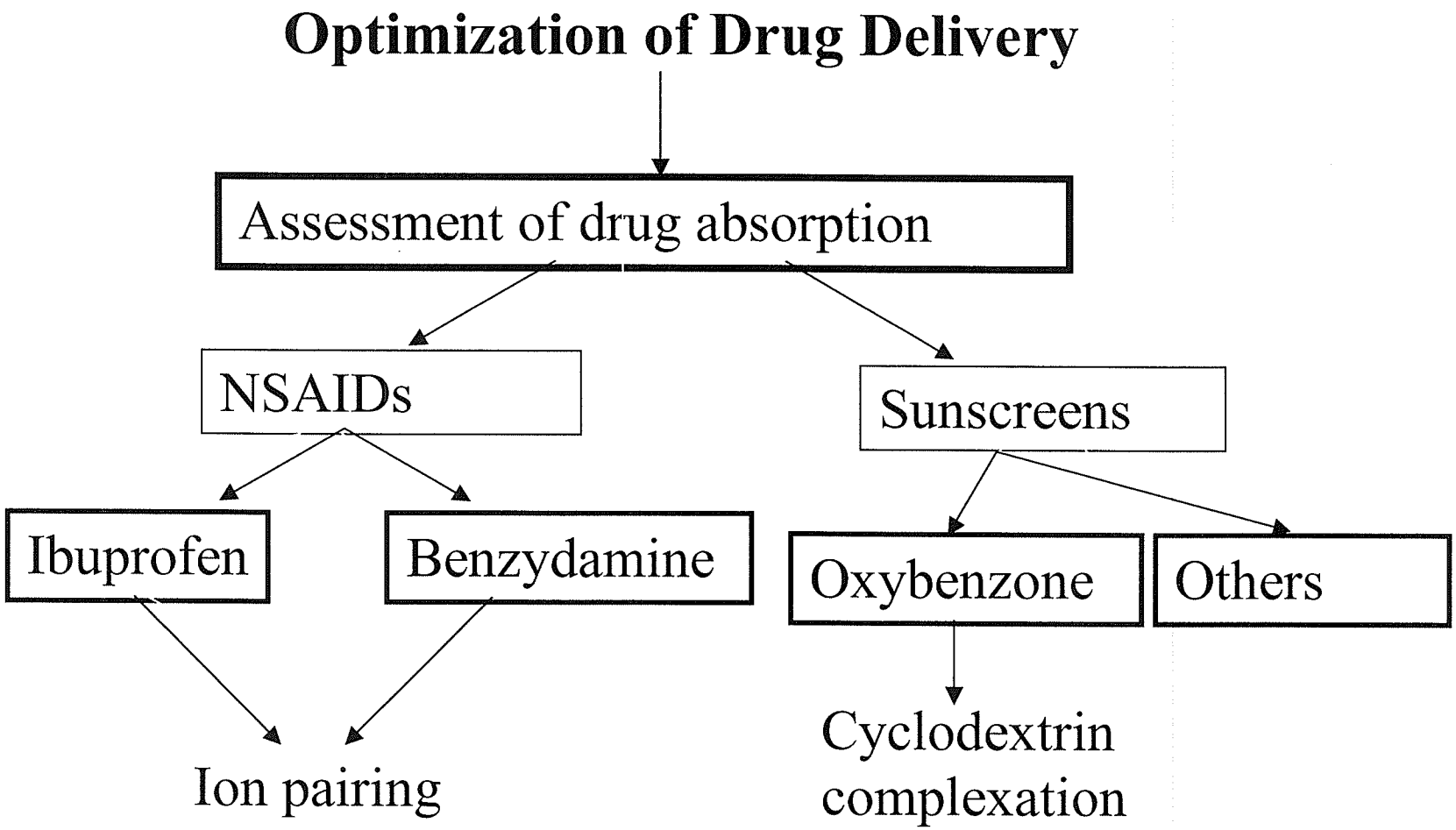


Figure 7.1. Optimization of drug delivery

Chapter 2. describes the percutaneous penetration of ibuprofen sodium across poly-dimethylsiloxane [PDMS] membrane at different pH values. It was observed that flux of ibuprofen across the membrane increased with the increase in solubility in the vehicle. This was evident with increased flux with increase in solubility from pH 4 to 7. However, the solubility at pH 8 was similar to that at pH 7, the flux was significantly lower. This can be explained by increased ionization at pH 8. The permeability coefficient, which is an absolute measure of permeability of the drug, increases with amount of unionized drug and the permeability coefficient increased with the decrease in pH. This suggests that at higher pH the lower permeability of the ionized species was more than compensated by increased solubility, which is consistent with the previous findings (Valenta *et al.* 2000). NMR measurements suggested that the tertiary amine has the largest degree of ion pair formation and interaction with ibuprofen is in agreement with the changes observed in the diffusion rates for primary, secondary and tertiary salts. Triethylamine, which was shown to pair best with ibuprofen, was observed to show maximum flux (Sarveiya *et al.* 2004).

In Chapter 3 the ion pair technique is utilized to enhance the penetration of benzydamine, a basic NSAID. Diffusion of benzydamine hydrochloride through polydimethylsiloxane (PDMS) was measured at different pH values 5.0, 6.0, 7.0, 7.6, 9.0. The flux for benzydamine hydrochloride increased significantly with the amount of unionized base. Permeation of benzydamine hydrochloride across excised human skin was measured at pH 5.0, 6.0 and 7.0 (chosen to simulate an appropriate range of physiological conditions). Again benzydamine flux increased with increase in pH and degree of unionisation. The octanol/water partition coefficient was directly related to steady state flux. Since the permeation of benzydamine increased with the amount of unionized base, ion pairing is expected to partially neutralize the charge over the molecule and interact to increase the lipophilicity of the molecule thereby enhancing membrane penetration. To study the effect of ion pairing on penetration, benzydamine was paired with ibuprofen, benzoate or octane sulphonate ions. ^{1}H and ^{13}C NMR measurements were performed to determine the interaction and ion pair formation between benzydamine and the respective counter ions. The chemical shift changes were more significant when benzydamine was paired with ibuprofen or benzoate, which

suggested that benzydamine was able to interact more strongly with ibuprofen and benzoate rather than with octane sulphonate. This interaction was evident by the large chemical shift changes in the benzydamine and respective counter ions. This was also reflected in the permeation characteristics, where ibuprofen and benzoate ions significantly increased the membrane permeation of benzydamine. The flux for benzydamine hydrochloride alone was 0.087 ± 0.016 $\mu g/cm^2/h$; while it was 12.54 ± 0.094 $\mu g/cm^2/h$ and 11.31 ± 0.83 $\mu g/cm^2/h$ in the presence of ibuprofen and benzoate ions ($p<0.0005$; multiple regression with pair-wise multiple comparison). Octane sulphonate did not significantly enhance permeation of benzydamine (0.121 ± 0.08 $\mu g/cm^2/h$).

We conclude that the degree of permeation enhancement of a drug is associated with its lipophilicity, extent of ion pairing, the nature of molecular interaction between the two ions, and the reduction in the charge over the drug molecule. Our studies suggest that ion pairing is a good technique to increase the permeation of drug without changing the molecule itself. Further studies could be undertaken with ibuprofen and benzydamine using other counterions to further optimize the penetration and tissue distribution.

The penetration of lipophilic sunscreens was also investigated. To facilitate and quantify the amount of sunscreens in different biological matrices after topical application to human volunteers, a liquid chromatographic assay was developed (Sarveiya *et al.* 2004). Chapter 4 describes the development of the assay procedure for four of the most common sunscreens. Most of the sunscreen agents are highly lipophilic and present similar retention times and therefore difficult to resolve. HS is especially problematic because it presents two peaks corresponding to two isomeric forms (Chisvert *et al.* 2001a). The method described in Chapter 4 successfully resolves oxybenzone, octylmethoxycinnamate, octylsalicylate and homosalate using a Symmetry C_{18} column using methanol-water as the mobile phase. The assay permits analysis of the sunscreen agents in biological fluids, including bovine serum albumin (BSA) solution, plasma and urine, and in human epidermis. The HPLC assay and extraction procedures developed are sensitive, simple, rapid, accurate and reproducible. This method was used

in the pharmacokinetic study for the analysis of sunscreens in skin, plasma and urine following topical application to human volunteers.

A volunteer study was done in order to assess the penetration of sunscreens into and across the skin after topical application for a commercial sunscreen formulation (Chapter 5). Further, the effect of anatomical site of topical application on percutaneous absorption of sunscreen was measured. The volunteer study confirmed the systemic absorption of one of the sunscreen component, oxybenzone. Oxybenzone was detected in plasma and in the urine. Small amounts of oxybenzone (<130 ng/mL) were observed in the plasma, whereas other sunscreens were below the limits of detection. Up to approximately 1% of the applied dose of oxybenzone and its metabolites was detected in the urine. Appreciable amounts were also detected in the stratum corneum through tape stripping. A comparison of the skin penetration of sunscreen at different anatomical sites (face and back) demonstrated that as much as twice the amount is present or retained in facial stratum corneum. This suggests a smaller amount of sunscreen needs to be applied to the face to produce the same local amount of sunscreen, and presumably sun protection as would be required on the back.

Oxybenzone was the only sunscreen found to be absorbed across the skin There have been suggestions that certain sunscreen agents may have mutagenic effects if absorbed into the skin to the viable cells of the epidermis and beyond (Knowland *et al.* 1993). Studies by the National Toxicology Program suggested a possible decrease in sperm motility, an increase in abnormal sperm formed, and alteration in sperm production with oxybenzone (National Toxicology Program 1991; National Toxicology Program 1991). Benzophenone is listed among 'chemicals suspected of having endocrine disrupting effects' by the World Wildlife Fund, the National Institute of Environmental Health Sciences in the USA and the Japanese Environment Agency. Infact, oxybenzone and its metabolite DHB have been recently suggested as endocrine modulators (Schlumpf *et al.* 2001; Nakagawa and Suzuki 2002). The oxybenzone metabolite, DHB is reported to be more toxic to rat hepatocytes and may be more prone to free radical formation than the parent compound (Nakagawa and Moldeus 1992). DHB has been detected in urine after topical application of a sunscreen formulation containing oxybenzone to human volunteers (Hayden *et al.* 1997; Sarveiya *et al.* 2003;

Sarveiya *et al.* 2004). Oxybenzone has also been recently reported in human plasma after topical application (Sarveiya *et al.* 2004). Moreover, oxybenzone has been reported to be rapidly metabolized to DHB and other metabolites, further increasing the concern (Okereke *et al.* 1993). It is clear through *in vitro* (Jiang *et al.* 1999) and *in vivo* studies (Hayden *et al.* 1997; Sarveiya *et al.* 2003; Sarveiya *et al.* 2004) that oxybenzone penetrates into and across the skin in appreciable amounts. In addition, recent studies from our laboratory suggest increased penetration of oxybenzone in the presence of the insect repellant deet and *vice versa* (Kasichayanula *et al.* 2002). This further raises the potential for toxicity. There may be additional concern for young children who have less well-developed processes of elimination, and a large surface area per body weight than adults, with systemic availability of a topically applied dose. Further, the absorption is likely to increase after repeated application.

It was clear that a new formulation strategy was required to modify the skin permeability and retention of oxybenzone in order to optimize sunscreen protection and minimize the risk of toxicity. In an attempt to decrease the permeation across the skin oxybenzone was complexed with HPβCD. HPβCD increased the solubility of oxybenzone by several fold, thereby increasing the affinity of oxybenzone towards the vehicle, which resulted in decreased thermodynamic activity of the solute in the vehicle. This decreased the release of solute from the vehicle, and thereby reducing permeability. Complexation of oxybenzone with HPβCD was demonstrated in the solid state by thermal analysis (DSC and TGA) and in solution by phase solubility and NMR spectroscopic studies. It was concluded that HPβCD can reduce the release and membrane diffusion of oxybenzone whilst retaining its efficacy as a sunscreen agent. This formulation strategy may be useful in controlling skin penetration of sunscreens and other topically applied chemicals. It is possible that inclusion of oxybenzone in HPβCD may also be beneficial in limiting interaction of UV filter with the skin and thus reducing irritation and allergic side effects. Further studies on human epidermis are currently being undertaken. In addition, the influence of cyclodextrin complexation on oxybenzone oxidation (Schallreuter *et al.* 1996) and formation of toxic photoproducts following UVR exposure should be investigated, as this may be a further advantage.

Overall, it can be concluded that ion pair and complexation are good techniques to modify the permeation characteristics of a drug or chemical after topical application. NMR spectroscopy can be is useful to enhance the knowledge to demonstrate the existence of ion pairs and also to rationalize permeation behavior in terms of molecular interactions.

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Appendix

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Liquid chromatographic assay for common sunscreen agents: application to in vivo assessment of skin penetration and systemic absorption in human volunteers

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Abstract

The purpose of the present study was to develop a reverse-phase high-performance liquid chromatographic (HPLC) assay for quantifying four common sunscreen agents, namely 2-hydroxy-4-methoxybenzophenone, 2-ethylhexyl-*p*-methoxycinnamate, 2-ethylhexylsalicylate (octylsalicylate) and salicylic acid 3,3,5-trimethcyclohexyl ester (homosalate) in a range of biological matrices. This assay was further applied to study the skin penetration and systemic absorption of sunscreen filters after topical application to human volunteers. Separation was achieved utilizing a Symmetry C_{18} column with methanol–water as the mobile phase. The assay permits analysis of the sunscreen agents in biological fluids, including bovine serum albumin (BSA) solution, plasma and urine, and in human epidermis. The assay was linear ($r^2 > 0.99$) with minimum detectable limits of 0.8 ng for oxybenzone, 0.3 ng for octylmethoxycinnamate, and 2 ng for homosalate and octylsalicylate. The inter- and intra-day variation for the four sunscreens was less than 3% at the upper end of the linear range and less than 6% at the lower end. Recoveries of sunscreens from plasma, 4% (w/v) BSA solution and epidermal membranes were within the range of 91–104%. Recoveries from urine of the four sunscreens, and oxybenzone with its metabolites were more than 86%. Up to approximately 1% of the applied dose of oxybenzone and its metabolites was detected in the urine. Appreciable amounts were also detected in the stratum corneum through tape stripping. The HPLC assay and extraction procedures developed are sensitive, simple, rapid, accurate and reproducible. Results from the preliminary clinical study demonstrate significant penetration of all sunscreen agents into the skin, and oxybenzone and metabolites across the skin.

Keywords: Skin penetration; Sunscreen agents

1. Introduction

The use of sunscreens has increased with the awareness of the detrimental effects of sun exposure on human skin such as erythema, skin aging and cancers. Sunscreen products are formulated to provide a specific sun protection factor (SPF) and to absorb a broad spectrum of ultraviolet radiation (UVR). In addition to traditional sunscreen products, sunscreen chemicals are also incorporated into a wide range of everyday hair and skin products and may therefore be used without the wearer making a conscious decision to apply a sunscreen.

The actives used in topical formulations are generally classified as either chemical or physical sunscreens. Physical sunscreens comprise of particles that act by scattering, reflecting, or absorbing the passage of radiation. Chemical sunscreens act by absorbing incident UVR and then dissipating it as longer wavelength energy, thereby protecting the skin from potentially damaging UVR. The efficiency of sunscreens is estimated by the sun protection factor, which depends on the content of UV filters in the formulation. The necessity to provide high SPF and screening efficiency against both UV-A (320–400 nm) and UV-B (290–320 nm) wavelengths has led to the development of sunscreen

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preparations containing many different sunscreen chemical combinations. Benzophenones, dibenzoylmethanes and anthranilates are the most common UV-A filters, whereas the UV-B filters include *p*-aminobenzoic acid (PABA) derivatives, salicylates, cinnamates, digalloyl triolate, lawsone, acrylates and benzimidazole derivatives. Of the approved sunscreen chemicals, oxybenzone (OX, benzophenone-3), octylmethoxycinnamate (OMC), butylmethoxydibenzoylmethane (BDM), octylsalicylate (OS) and homosalate (HS) are some of the most common active ingredients used in sunscreen formulations.

Recent studies have provided evidence that some sunscreens are absorbed systemically following topical application to the skin [1–3]. These studies involved determination in skin layers only or measurement of urinary excretion of absorbed sunscreens and their metabolites. Neither provided a full pharmacokinetic analysis, as only a single measure of absorption or excretion was assessed. It would be advantageous to quantify penetration within the skin tissue and systemic distribution of sunscreen agents following topical application. This would aid in the determination of the exposure of viable tissues to sunscreen chemicals, provide a better understanding of the potential for toxicity both locally and systemically, and facilitate design of novel formulations to target the outer skin layers.

In addition to in vivo studies, skin penetration of chemicals and drugs is frequently investigated using in vitro techniques. The in vitro technique utilizes diffusion cells, which consist of a receptor and donor phase separated by a synthetic or skin membrane. Where lipophilic solutes are investigated, as is the case for many sunscreens, bovine serum albumin (BSA) or other solubility modifiers are used as receptor fluids to provide adequate solubility and ensure sink conditions [4,5]. A suitable extraction procedure and high-performance liquid chromatographic (HPLC) assay is required to facilitate these studies.

Many of the HPLC assays published for sunscreen agents are designed for product evaluation and determination of concentrations in cosmetic formulations [6–8]. Few assays for evaluation of sunscreens in biological samples have been reported [5,9–11]. A reliable analytical method for the quantitative determination of the common sunscreen chemicals in biological fluids will facilitate the evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetic data.

The aim of this study was to develop simple, rapid and reliable operating procedures for quantification of sunscreen chemicals in a range of biological matrices. Butylmethoxydibenzoylmethane, octylmethoxycinnamate, octyldimethyl PABA, octylsalicylate, oxybenzone and homosalate are the most common sunscreen agents. Most of them present similar retention times in previously published assays and are therefore difficult to resolve. HS is especially problematic because it presents two peaks corresponding to two isomeric forms [6]. This paper provides a reproducible and accurate assay, by which four of the most common sunscreen agents

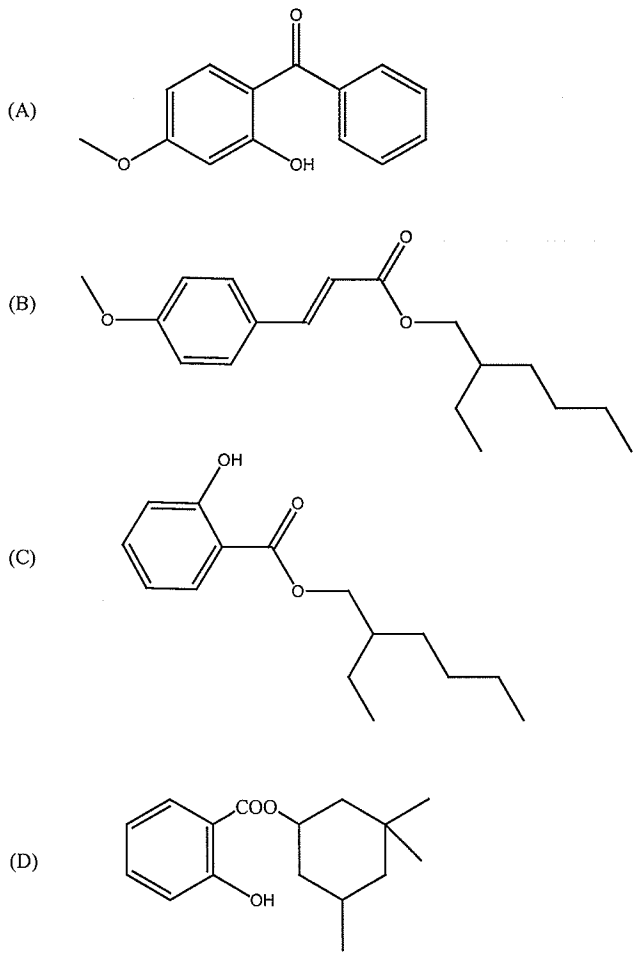

Fig. 1. Structures of the sunscreen agents: (A) oxybenzone; (B) octylmethoxycinnamate; (C) octylsalicylate; (D) homosalate.

(Fig. 1), including HS, can be resolved simultaneously. Using this assay procedure, a preliminary investigation of the penetration into the skin tissues, plasma and excretion in the urine of four common sunscreens, as active ingredients in a commercially available sunscreen product, was studied. This paper also provides procedures for the extraction of sunscreens from tape strips, skin tissue and biological matrices including plasma, urine and bovine serum albumin.

2. Experimental

2.1. Materials and methods

OX, OS and BSA were purchased from Sigma–Aldrich (USA). OMC and HS were gifts from BASF Corporation (NJ, USA) and EM Industries (Germany), respectively. Coppertone sunblock lotion (Schering-Plough Health Care Products Inc.) was the commercially available sunscreen product used for the study. HPLC grade methanol was from Fisher Scientific (USA). De-ionized water (Milli-Q, Waters Inc.,

USA) was used and all other chemicals used were analytical reagent grade.

2.2. HPLC instrumentation and conditions

An Alliance liquid chromatographic system (Waters Inc.) equipped with a 2690 separations module and 996 photodiode array detector was used. Separation was achieved at ambient temperature on a Symmetry C_{18} column (5 μm, 3.9 mm × 150 mm i.d., Waters Inc.) with an inline pre-filter. Integration was undertaken using a personal computer equipped with Millenium 4.0 version software.

The mobile phase consisted of methanol–water, filtered through a 0.45 μm membrane filter (Durapore, Millipore, USA). Gradient flow from 75:25 methanol–water to 92:8 methanol–water was used from 0 to 4 min, thereafter the flow was isocratic with 92:8 methanol–water. The solvent composition was returned to initial conditions after 11 min. The mobile phase was continuously degassed before and during use. The flow rate was 1.0 ml/min. To obtain a satisfactory UV response for all the analytes, each chemical was measured at its wavelength of maximum absorbance: oxybenzone at 289 nm, octylmethoxycinnamate at 310 nm, homosalate and octylsalicylate at 237 nm. Injection volumes of 10 μl were used for the assay.

Stock solutions were prepared by accurately weighing the agents (OX, OS, OMC and HS) and dissolving in methanol. Three working solutions of the four sunscreens were freshly prepared from their stock solutions by 1:10 dilution. Appropriate dilution of these working solutions gave concentrations of 0.1–0.5 μg/ml. The entire procedure was repeated on six different days to test inter-day variation and repeated six times at low and high concentrations to test intra-day variation.

The minimum detectable limits were measured by diluting the sunscreen agents with methanol to give an appropriate range from 0.01 to 20 μg/ml. Aliquots of 10 μl of the samples were injected onto the HPLC column.

2.3. Sample treatment and preparation

2.3.1. Plasma and BSA

The four sunscreen standards were spiked into human plasma and 4% (w/v) BSA in phosphate buffer (pH 7.4) at low and high concentrations (0.5 and 5.0 μg/ml). The sample solutions were stirred for 30 min following spiking to ensure complete dissolution of the sunscreen agents. After protein precipitation with two sample volumes of acetonitrile (200 μl acetonitrile to 100 μl of sample), the samples were centrifuged at 10,000 × g for 10 min. The supernatant was injected onto the HPLC system. Six replicates were performed at each concentration. Blank plasma and BSA in phosphate buffer were treated identically and injected onto the HPLC system to ensure that there were no peaks interfering with the sunscreen active substances.

2.3.2. Urine

The four sunscreen standards were spiked (5 μg/ml) into fresh human urine. To 1 ml of this solution was added an equal volume of phosphate buffer (pH 6.8). The sample was then treated with beta-glucuronidase enzyme (600 units) and heated at 37 °C for 24 h. The reaction was stopped with an equal volume of acetonitrile resulting in protein precipitation. It was vortex mixed and centrifuged at 5000 × g for 10 min. The sample was evaporated to dryness then re-suspended in methanol and 20 μl of the supernatant was then injected onto the HPLC column.

2.3.3. Skin tissue

Human epidermal membranes were immersed in 5 ml of standard sunscreen solution for 24 h in dark conditions at 25 °C. The formulation residue was removed from the epidermal tissue by rinsing with 5 ml distilled water three times and drying. Retained sunscreen was then extracted with 2 ml of absolute methanol for 30 min. The extraction procedure was repeated three times with methanol. After centrifuging each extract at 10,000 × g for 10 min, the resultant supernatants were diluted appropriately and quantified by HPLC.

2.4. Preliminary investigation of in vivo absorption of sunscreen

Ethical approval was obtained from the Health Research Ethics Board at the University of Manitoba and St. Boniface Hospital Ethics Committee. A commercially available sunscreen product, Coppertone Colorblok for kids (SPF 30) was applied at a dose of 2 mg/cm^2 to the arms and back of three female human volunteers, aged 22–42 years. This constituted an application of approximately 1.7 g of the sunscreen formulation applied to a total area of approximately 860 cm^2. This lotion contains 8% HS, 7.5% OMC, 6% OX, and 5% OS as active ingredients. Baseline blood and urine samples were collected prior to sunscreen application. Permeation of sunscreen into the skin, systemic absorption and urinary elimination were monitored for up to 48 h following application. At 30 min after application, a small area of the skin was wiped with Kleenex tissue and skin strip samples taken by application and removal of Scotch® crystal clear tape (3 cm × 1.9 cm). Tape stripping is a relatively non-invasive technique, which permits samples of stratum corneum (0.5–1 μm thickness) to be collected from the treated area. The tapes were applied to the treated areas by application of a consistent pressure generated by stroking the thumb 10 times along the tape. The stratum corneum was sequentially stripped up to 16 times and the 16 strips taken from each site were grouped into four groups for subsequent analysis of sunscreen content (group 1: strip 1; group 2: strips 2–6; group 3: strips 7–11; group 4: strips 12–16). This procedure was repeated on a separate site at 4 and 8 h. The stratum corneum was removed by 16 sequential strips, focussing on the upper layers of the stratum corneum and not affecting layers underneath the stratum

corneum. It has been found that on the flexor surface of the forearm about 30 tape strips are needed to strip off most of the horny layer [12]. Complete removal of the stratum corneum was not possible even after 30–40 strippings [13], and a certain barrier function in the tissue so treated remains [14,15]. Ohman and Vahlquist showed that after 100 tape strippings, the entire stratum corneum could be removed [16]. The stripping procedure was not normalized, since the inconsistent cohesion of the corneocyte layers means that reproducible amounts of SC (within and between subjects) cannot be removed [17]. The product was washed off the skin at 8 h post-application. Blood samples were taken from all subjects at pre-application baseline and at 1, 2, 4, 6, 8, and 24 h post-application. Urine output of all subjects over the 48 h post-application period was collected. All blood and tape strip samples were analyzed as described.

Sunscreen in the stratum corneum tape strip samples was extracted by a two-step procedure adapted from Potard et al. [18]. This involved overnight contact of the tape with isopropanol to destructure the polymeric glue, followed by dissolution of the polymeric glue and the hard polymeric tape support by acetonitrile. The solvent was then evaporated and the residue re-suspended in 1 ml methanol for analysis of sunscreen content by HPLC.

3. Results and discussion

3.1. Chromatography and resolution

HPLC chromatograms of the four sunscreen agents after sample preparation from an extract of 4% (w/v) BSA, plasma and tape strips are shown in Fig. 2. Many of the HPLC assays published are designed for product evaluation and determination of concentrations in cosmetic formulations [6–8,19]. The assay method previously published for evaluation of sunscreens in biological samples is useful but offers certain limitations in terms of sensitivity, especially with octylsalicylate [5]. Increased sensitivity is particularly important while measuring biological samples. Moreover, the method does not include the UV-B filter homosalate, which is present in many of the commercially available sunscreen formulations. Homosalate is particularly difficult to measure due to the low extinction coefficient, and the presence of two peaks corresponding to two isomeric forms. The two peaks due to homosalate are H1 and H2 (Fig. 2). H2 was used for calibration and quantitation. The present method, which includes homosalate, also provides increased sensitivity and resolution for many of the sunscreens measured compared to previous published methods. The procedure is relatively rapid with a run time of only approximately 10 min.

3.2. Linearity

Table 1 reports the results for calibration plot linearity. Excellent linearity was obtained over the range 0.1–5.0 μg/ml for the four sunscreen agents.

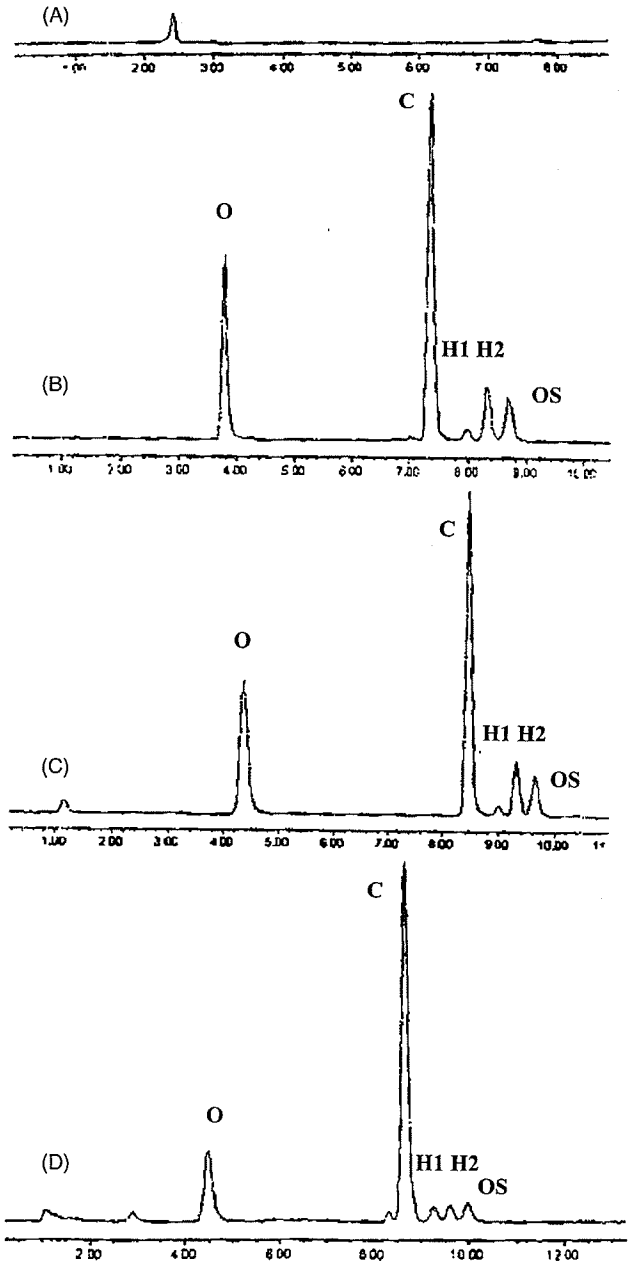

Fig. 2. Chromatograms of a blank of 4% (w/v) BSA (A), an extract from 4% (w/v) BSA in phosphate buffer (B), plasma (C) and skin strips (D). Peaks: (O) oxybenzone; (C) octylmethoxycinnamate; (H1 and H2) homosalate; (OS) octylsalicylate.

3.3. Assay precision

Calibration graphs were constructed by plotting the peak area versus concentration of standards injected. The best straight lines were determined using the method of least squares. To obtain a satisfactory UV response for all the analytes, each chemical was measured at its wavelength of maximum absorbance: oxybenzone at 289 nm, octylmethoxycinnamate at 310 nm, homosalate and

Table 1
Quantitative results for HPLC assay of sunscreens

	OX	OMC	OS	HS
Wavelength (nm)	289	310	237	237
Linear range (μg/ml)	0.1–5.0	0.1–5.0	0.1–5.0	0.1–5.0
Slope ($\times 10^6$)	3.53 ± 0.037	4.42 ± 0.20	2.01 ± 0.026	1.81 ± 0.026
Intercept ($\times 10^4$)	−(5.76 ± 2.1)	(2.15 ± 0.31)	−(3.78 ± 1.3)	−(3.47 ± 1.95)
Correlation coefficient	0.9999	0.9998	0.9998	0.9998
Minimum detection limit (ng)	0.8	0.3	2	2
Inter-day variation, mean ± S.D. (%CV)				
At 0.1 μg/ml	0.092 ± 0.002 (2.72)	0.1002 ± 0.004 (4.139)	0.101 ± 0.005 (5.439)	0.983 ± 0.005 (5.594)
At 5 μg/ml	4.976 ± 0.0304 (0.612)	5.01 ± 0.0357 (0.713)	4.99 ± 0.027 (0.557)	5.02 ± 0.037 (0.7457)
Intra-day variation, mean ± S.D. (%CV)				
At 0.1 μg/ml	0.095 ± 0.002 (2.47)	0.099 ± 0.005 (5.55)	0.105 ± 0.004 (4.15)	0.107 ± 0.005 (4.94)
At 5 μg/ml	4.985 ± 0.058 (1.16)	4.965 ± 0.146 (2.95)	4.947 ± 0.058 (1.176)	4.953 ± 0.048 (0.998)

R.S.D. of peak area ($n = 6$).

octylsalicylate at 237 nm. The intra- and inter-day variation for the four sunscreens was less than 3% at the upper end of the linear range and less than 6% at the lower end (as summarized in Table 1). There was no significant difference between day-to-day analysis (slopes evaluation, $P < 0.001$).

3.4. Minimum detectable limits

The lower limits of quantitation calculated as greater than 10 times the baseline noise in the assay were 2 ng (0.2 μg/ml) for oxybenzone, 1 ng (0.1 μg/ml) for octylmethoxycinnamate and 4 ng (0.4 μg/ml) for homosalate and octylsalicylate. The minimum detectable limits, calculated as greater than three times the baseline noise level in the assay, were 0.8 ng (0.08 μg/ml) for oxybenzone, 0.3 ng (0.03 μg/ml) for octylmethoxycinnamate, and 2 ng (0.2 μg/ml) for homosalate and octylsalicylate.

The limits of detection in a previously published assay by Jiang et al. were 0.1 ng (0.01 μg/ml) for oxybenzone, 1 ng (0.1 μg/ml) for octylmethoxycinnamate and 5 ng (0.5 μg/ml) for octylsalicylate [5]. Chisvert et al. reported detection limits of 1.7 μg/ml for oxybenzone, 2.2 μg/ml for octylmethoxycinnate, 2.3 μg/ml for homosalate and 1.5 μg/ml for octylsalicylate [6]. These are not directly comparable with the current data as the method for determination of minimum detection limits is different. Jiang et al. argued that lower sensitivity, particularly for octylsalicylate was because of compromise wavelength [5]. A similar wavelength was used by Chisvert et al. [6]. This has been resolved in our method, leading to increased sensitivity. In addition to the use of multiple wavelengths, gradient flow in our method favored better peak shape and separation. Therefore, this method would be more useful for measuring sunscreen agents, especially in biological samples.

3.5. Recovery study in human skin, plasma, urine and 4% (w/v) BSA in phosphate buffer

The recovery of the four sunscreen agents from plasma and 4% (w/v) BSA solution is summarized in Table 2. Recoveries were within the range of 90–104%. The coefficients of variation calculated from the six replicates were all less than 5%. Extraction of the sunscreens from the epidermal membranes and urine is also summarized in Table 2. Recoveries were within the range of 98–100 and 86–92%, respectively. Data for oxybenzone and its metabolites are summarized in Tables 2 and 3.

3.6. Preliminary volunteer study—absorption and distribution of sunscreens following topical application

This preliminary study demonstrates the application of the assay and extraction procedures developed. Sunscreens are regularly applied to large areas of the body and therefore it is essential to have an understanding of their safety.

Table 2
Recovery of sunscreens from human plasma, 4% (w/v) BSA in phosphate buffer and urine spiked with 0.5 or 5 μg/ml of each sunscreen

	OX	OMC	OS	HS
0.5 μg/ml plasma				
Recovery (%)	99.29	103.83	97.33	95.92
%CV	2.75	3.78	2.22	4.10
5 μg/ml plasma				
Recovery (%)	98.4	96.38	92.61	90.76
%CV	3.47	2.53	2.13	4.41
0.5 μg/ml of 4% (w/v) BSA				
Recovery (%)	102.1	99.47	102.31	97.95
%CV	2.56	1.90	2.22	3.28
5 μg/ml of 4% (w/v) BSA				
Recovery (%)	100.91	101.25	100.88	98.30
%CV	1.45	2.38	1.18	2.70
5 μg/ml urine				
Recovery (%)	90.03	89.22	86.82	92.25
%CV	3.05	4.45	4.32	3.22
Epidermal membranes				
Recovery (%)	98.84	99.20	98.49	99.55
%CV	2.53	1.82	3.07	3.20

Mean of six extractions.

Table 3
Recovery of oxybenzone and its metabolites from urine spiked with 5 μg/ml of each chemical

	OX	DHMB	DHB	THB
Recovery (%)	89.88	94.88	92.76	93.21
%CV	2.84	2.39	2.49	4.13

DHMB: 2,2′-dihydroxy-4-methoxybenzophenone; DHB: 2,4-dihydroxybenzophenone; THB: 2,3,4-trihydroxybenzophenone. Mean of six extractions.

In particular, it is necessary to quantify the skin penetration and distribution of sunscreen agents. Even if the degree of penetration is low, as the sunscreen product may be applied to a large surface area on a regular basis the total amount absorbed may be significant and the potential consequences should be considered. To date, a systematic investigation of sunscreen absorption and the influence of formulation has not been undertaken. In addition, as anatomical site has been shown to influence drug absorption, the difference in skin penetration after topical application to the arms and back was also considered. A significant amount of sunscreen penetrates the epidermal barrier (Fig. 3 and Table 4), a finding which is consistent with previously published in vitro and in vivo research [3]. Higher amounts of sunscreens were recovered from the upper layers of stratum corneum at 30 min post-application. At 4 and 8 h post-application, similar depth of penetration profiles were obtained but with overall lower sunscreen concentration (Table 4). There was no significant difference in the absorption of sunscreens

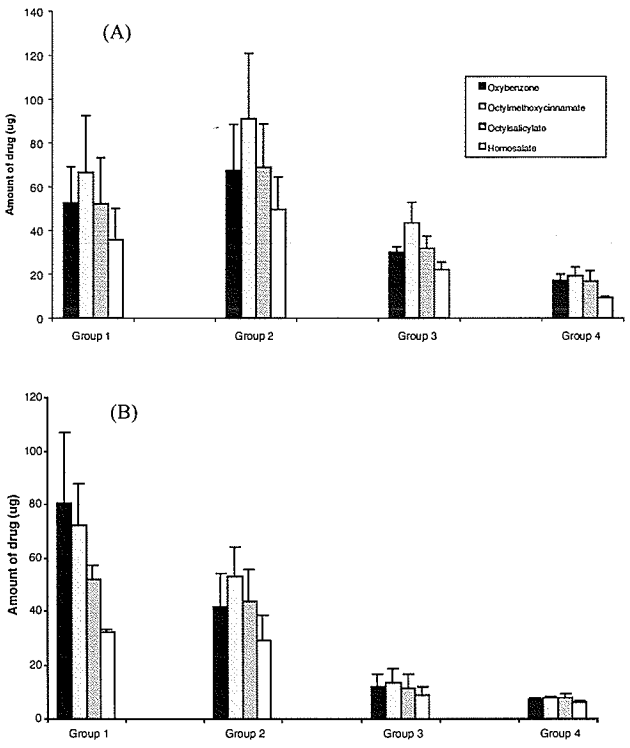

Fig. 3. Amount of sunscreen in the tape strips from the arms (A) and back (B) of volunteers after 30 min of application of the sunscreen formulation.

Table 4
Amount (μg per pool of strippings) of UV filters recovered from the tapes ($n = 3$)

Stripping group	After 30 min		After 4 h		After 8 h	
	Arm	Back	Arm	Back	Arm	Back
Group 1						
Oxy	52.39 ± 16.87	80.33 ± 26.44	**8.69 ± 3.61**	31.76 ± 6.49	19.78 ± 14.44	21.56 ± 4.10
OMC	66.59 ± 25.88	72.42 ± 15.22	**10.91 ± 2.76**	40.07 ± 5.18	25.67 ± 13.20	27.95 ± 4.62
OS	52.21 ± 21.12	51.925 ± 5.10	**9.88 ± 1.10**	32.20 ± 4.34	10.89 ± 3.39	24.44 ± 7.43
HS	35.71 ± 14.32	32.21 ± 1.24	**6.36 ± 1.82**	21.32 ± 7.25	13.28 ± 8.12	14.49 ± 4.06
Group 2						
Oxy	67.29 ± 21.37	41.37 ± 12.71	50.78 ± 32.60	29.42 ± 7.61	25.53 ± 16.59	16.09 ± 2.39
OMC	91.07 ± 29.75	52.74 ± 11.17	67.66 ± 41.45	35.93 ± 8.26	38.51 ± 24.53	21.12 ± 5.45
OS	69.01 ± 19.70	43.41 ± 11.95	54.21 ± 31.21	29.24 ± 4.69	26.93 ± 17.94	19.79 ± 9.15
HS	49.55 ± 14.86	29.24 ± 9.36	36.84 ± 21.36	19.6 ± 4.68	16.27 ± 12.53	11.9 ± 2.68
Group 3						
Oxy	**30.14 ± 2.32**	12.03 ± 4.81	16.49 ± 8.53	8.25 ± 3.60	23.24 ± 8.62	7.42 ± 1.22
OMC	**43.46 ± 9.30**	13.63 ± 5.20	22.59 ± 11.07	11.17 ± 3.35	12.30 ± 6.36	10.05 ± 2.33
OS	**31.89 ± 5.49**	11.67 ± 5.10	18.905 ± 9.71	10.12 ± 3.92	10.41 ± 4.61	9.23 ± 3.09
HS	22.12 ± 3.39	8.66 ± 3.39	13.67 ± 7.71	6.09 ± 2.02	6.35 ± 4.26	5.43 ± 0.64
Group 4						
Oxy	**17.12 ± 2.88**	7.18 ± 0.67	11.98 ± 6.05	4.86 ± 0.64	6.29 ± 1.65	4.58 ± 2.10
OMC	**19.29 ± 3.94**	8.02 ± 0.54	12.61 ± 2.54	7.27 ± 3.53	10 ± 1.91	6.77 ± 4.95
OS	16.86 ± 4.71	7.9 ± 1.42	10.17 ± 1.68	7.51 ± 5.7	7.5 ± 1.56	6.65 ± 5.63
HS	9.27 ± 0.63	6.00 ± 0.84	7.43 ± 2.04	4.14 ± 1.94	4.23 ± 1.01	3.63 ± 2.56

Oxy: oxybenzone; OMC: octylmethoxycinnamate; OS: octylsalicylate; HS: homosalate. Group 1: strip 1; group 2: strips 2–6; group 3: strips 7–11; group 4: strips 12–16. Bold numbers are significantly different ($P < 0.05$). Values for arms are compared with back at a particular time.

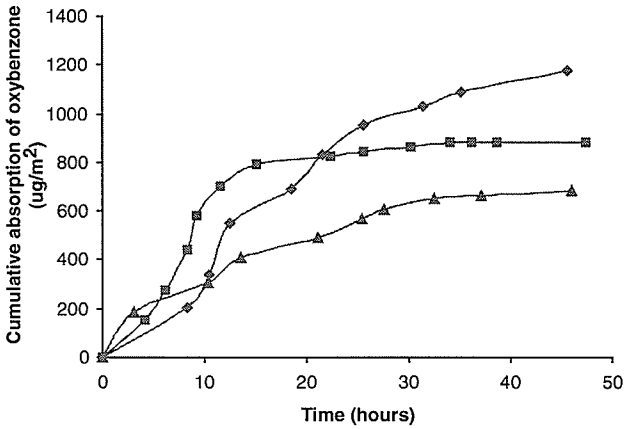

Fig. 4. Systemic absorption of oxybenzone following topical application of a commercial sunscreen product to three healthy human volunteers; absorption was determined from urinary excretion of oxybenzone and its metabolite.

into the skin after application to the arms and back, though slight differences were observed (shown in bold in Table 4). This is consistent with previous reports of similar absorption kinetics through the arms and back after topical application of other drugs [20,21]. In addition, the amount quantified in the first strip (group 1) seems to predict which components will have better substantivity for the stratum corneum. From Fig. 3, it appears that oxybenzone, octylmethoxycinnamate and octylsalicylate have more affinity towards the stratum corneum when compared to homosalate.

Systemic absorption of oxybenzone was confirmed through detection of oxybenzone in the blood/plasma and in the urine. The plasma was only measured for the four sunscreens, excluding their metabolites. Small amounts of oxybenzone (<130 ng/ml) were observed in the plasma, whereas other sunscreens were below the limits of detection. Up to approximately 1% of the applied dose of oxybenzone and its metabolites were detected in the urine (Fig. 4). The major metabolite was 2,4-dihydroxybenzophenone (DHB), whereas 2,3,4-trihydroxybenzophenone (THB) was detected only in trace amounts. The low levels of oxybenzone in the blood may be due to rapid metabolism and distribution, as has been demonstrated previously in rats [22]. Okereke et al. further reported that oxybenzone and its metabolites were found in liver, kidney, spleen, heart and even testes. Since the area of application of sunscreen in our study was approximately 864 cm^2, which is almost half the area that one could apply in beach sunbathing situation, the total amount of systemic absorption of oxybenzone could be higher in practice. In addition, tissue and systemic levels of sunscreens may be greater in young children who have less well-developed processes of elimination, and a larger surface area per body weight ratio than adults. Sunscreens are recommended to be applied frequently throughout the day, therefore the amount used in practice is likely to exceed the application amount of 2 mg/cm^2, also promoting penetration.

This assay provides an efficient means of quantifying the most common sunscreens in a range of biological matrices relevant to both in vitro and in vivo assessment of skin penetration. As such, it will facilitate the development of novel sunscreen products with high SPF and substantivity (skin retention) but also minimal absorption to deep tissues or the systemic circulation.

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Ion-pairs of ibuprofen: increased membrane diffusion

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Abstract

The purpose of the present study was to determine the influence of pH and ion-pairing on the permeation of ibuprofen across polydimethylsiloxane (PDMS) membrane. The solubility of ibuprofen sodium was determined at a range of pH values. Saturated solutions were then used to determine the influence of pH on diffusion across PDMS as a model membrane. The apparent partition coefficient of ibuprofen sodium between n-octanol and phosphate buffer at various pH values was also investigated. Organic salts of ibuprofen using ethylamine, diethylamine, triethylamine and ethylene diamine as counter-ions were synthesized and the influence of these counter-ions on the permeation of ibuprofen was studied. The presence of ion-pairing was confirmed using ^{1}H NMR and ^{13}C NMR. Diffusion studies at different pH values (4.0, 5.0, 6.0, 7.0 and 8.0) indicated that ibuprofen sodium flux increased significantly with increasing pH from 4.0 to 7.0. Above pH 7.0 a decrease in diffusion was observed. The permeability coefficient increased with an increase in the amount of unionized acid. The apparent partition coefficient was directly related to the steady-state flux. The steady-state flux of ibuprofen increased up to 16-fold using different counter-ions. The highest flux was measured from ibuprofen triethylamine. The flux of ibuprofen salts across a lipophilic membrane can be increased by formation of ion-pairs. The extent of enhancement is associated with the lipophilicity, extent of ion-pairing and reduction in charge over the drug molecule.

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Introduction

Most of the clinically accepted drugs for delivery through the skin are of low molecular weight, lipophilic and effective at low doses. However, the majority of drugs are weak acids or bases, and are ionized under normal physiological conditions. The human stratum corneum acts as a significant barrier for the skin penetration of these hydrophilic ionizable drugs. Charged species are known to be poor penetrants across skin, other biological membranes and non-porous polymers. Their permeation coefficient has been estimated to be about 10^4 times smaller than for the respective uncharged species (Swarbrick et al 1984). Many strategies, including the use of penetration enhancers, have been exploited to increase the penetration of drugs through the stratum corneum (e.g. Walters & Hadgraft 1993; Hadgraft 1999; Asbill et al 2000; Barry 2001). However, as many of the skin penetration enhancing chemicals have the potential to cause skin irritation (e.g. Kanikkannan & Singh 2002), more effective and safer penetration enhancement techniques need to be developed.

The formation of ion pairs has been investigated for the enhancement of membrane permeability and hence bioavailability of hydrophilic ionized molecules. The theory is that when oppositely charged molecules interact, this association reduces or neutralizes the overall electrostatic charge of the ion-pair molecule that is formed. The consequent increase in lipophilicity of the ion-pair compared to the ion results in increased permeation of the molecule through a membrane (e.g. intestinal, skin or synthetic).

Early studies on ion-pair transport focused on absorption from the gastrointestinal tract. Wilson and Wiseman were among the first to test the ion-pair hypothesis for the lipophilization of the ionic drug tropsium (Wilson & Wiseman 1954). They reported an enhanced transfer rate across everted intestine using alkylsulphonates as counter-ions. Gasco and colleagues reported an increase in the bioavailability of propranol in the presence of taurodeoxycholate (Gasco et al 1984). Furthermore, hexylsalicylate was found to be capable of enhancing the bioavailability of hydrophilic drugs such as pholedrine and bretylium after oral and rectal application respectively.

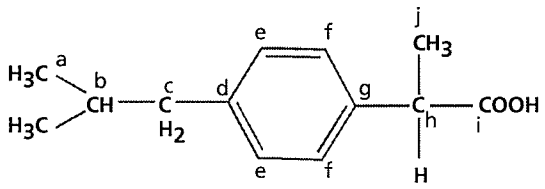

Figure 1 Structure of ibuprofen molecule.

The concept of forming ion-pairs to increase the skin permeability of hydrophilic drugs has also been reported (Hadgraft et al 1985, 1986; Young et al 1988; Pedersen 1990). Kadono and colleagues (1998) reported increased penetration through shed snakeskin of salicylate by ion-pair formation with alkylamines. Megwa and colleagues also found increased skin penetration and local tissue deposition of salicylate in the presence of alkylamines (Megwa et al 2000a). In a further study, these researchers showed that secondary, tertiary and quaternary amines increased the permeation of salicylates through human epidermal membranes in vitro (Megwa et al 2000b). Permeability enhancement was greatest with tertiary amines and was found to increase with alkyl chain length. Increase in skin penetration of lignocaine (Valenta et al 2000) and ondansetron (Takahashi & Rytting 2001) by ion-pair formation has also been reported. Although most of these studies describe the ion-pair approach as the means to increase the permeation of drugs across biological and synthetic membranes, few have provided direct evidence of ion-pair formation.

The objective of this study was to determine the significance of ion-pair formation on the permeation of ibuprofen (Figure 1). The solubility of ibuprofen sodium was measured over a range of pH values. The saturated solutions obtained from the solubility determination were used to measure the diffusion at various pH values through a polydimethylsiloxane (PDMS) membrane. In further investigations, the effect on membrane permeability of a number of amine counter-ions was examined. Nuclear magnetic resonance (NMR) spectroscopy was used to identify the presence of ion-pair formation between ibuprofen and the respective amine counter-ion. In addition, the partition characteristics of ibuprofen sodium and various organic ibuprofen salts were examined using an n-octanol–aqueous buffer system.

Materials and Methods

Materials

Ibuprofen sodium, ethylamine hydrochloride, diethylamine hydrochloride, triethylamine hydrochloride and ethylene diamine dihydrochloride were from Sigma (St Louis, MO). PDMS membrane (thickness 0.005″) was from Pillar Surgical (CA). HPLC-grade acetonitrile and methanol (Fisher Scientific, USA) were used, and all other chemicals were of analytical grade.

High-performance liquid chromatography

A Waters liquid chromatographic system equipped with a model 717 plus auto sampler, model 600S controller and 996 photodiode array detector was used. Separation was achieved on a Symmetry C_{18} column (5 μm, 3.9 × 150 mm i.d., Waters Inc., MA) at ambient temperature with an in-line pre-filter. Integration was undertaken using Millenium$^{3.2}$ software.

The mobile phase consisted of dilute phosphoric acid adjusted to pH 2.2:acetonitrile (40:60) filtered through a 0.45 μm membrane filter (Durapore membrane filter, Millipore). The mobile phase was continuously degassed before and during use. The flow rate was 1.0 mL min^{-1} and the detection wavelength was 220 nm. The retention time for ibuprofen was ~4.8 min. Calibration curves were calculated on peak area measurements.

NMR spectroscopy

^{1}H and ^{13}C NMR spectra were recorded at 300 and 75 MHz, respectively, using a Bruker Avance 300 spectrometer (Karlsruhe, Germany). Samples were dissolved in deuteromethanol and chemical shifts (δ) for hydrogen and carbon resonance reported in ppm relative to TMS.

Solubility–pH profile

An excess of ibuprofen sodium was added to phosphate buffers with pH values of 4.0, 5.0, 6.0, 7.0 and 8.0 in screw-capped vials and stirred in the dark at 25 °C for 48 h. The pH of the resultant mixture was determined during this period and adjusted to the required value by adding phosphoric acid or KOH. The mixtures were then centrifuged at 10 000 g for 10 min and the supernatants analysed for ibuprofen content using HPLC. The pH of the solutions was confirmed after centrifugation. All experiments were repeated four times.

Synthesis of ibuprofen ion-pairs

Equimolar amounts of ibuprofen sodium and amine hydrochloride (ethylamine hydrochloride, diethylamine hydrochloride, triethylamine hydrochloride or ethylene diamine dihydrochloride) were dissolved in methanol and stirred for 24 h. The cloudy mixture was then filtered through a 0.45 μm membrane filter (Durapore). The precipitate was collected, weighed and was proven to be sodium chloride (NaCl) by reaction with silver nitrate. The molar yield of sodium chloride was more than 90% of expected amount in every synthesis. The solvent from the clear filtrate solution obtained after filtration was evaporated and the residue was dried in vacuo for 24 h with P_2O_5 as a drying agent. The salts were dissolved in deuteromethanol and ^{1}H NMR and ^{13}C NMR used to confirm the presence of ion-pair in solution.

Permeation experiments

In-vitro permeation studies across PDMS membrane were performed in Pyrex glass Franz-type diffusion cells. The

membrane was immersed in deionized distilled water for 1 h before use. PDMS membrane (cross-sectional area 1.18 cm^2) was then mounted between the donor and receptor compartments of diffusion cells and the assembly held in place with a plastic clamp. The diffusion unit was immersed in a water bath at 37 °C. Phosphate buffer pH 7.0 (approx. 3.5 mL) was the receptor fluid. For permeation at different pH values the donor phase was 1 mL of the saturated solution at that particular pH. After equilibration with the buffer, 1.0 mL of the donor solution was added to the donor cell. A magnetic stirrer driven by an external magnet continuously stirred the receptor compartment at the same speed for all cells. Samples of the receptor phase were withdrawn and replaced by drug-free buffer at appropriate times throughout the 6 h period of the experiment. The ibuprofen content in the receptor phase was determined using HPLC. Experiments were repeated four times.

The cumulative amount of drug released through the PDMS membrane, Q(t), was determined from Q = (CV)/A, where C is the concentration of ibuprofen (sodium) in the receptor compartment in $\mu g\,mL^{-1}$ for the corresponding sample time t, V is the volume of fluid in the receptor phase and A is the diffusional area of the membrane. The flux of ibuprofen through the membrane into the receptor from each of the formulations was determined from the slope of the plot of cumulative amount in the receptor phase vs time and expressed as $\mu g\,cm^{-2}\,h^{-1}$. Permeability coefficients were calculated for ibuprofen for each formulation.

Apparent partition coefficient

The apparent partition coefficients were investigated between n-octanol and phosphate buffers at various pH values. Each phase had been pre-saturated with the other by equilibration overnight before the experiments. A known amount of ibuprofen sodium was dissolved in buffers of different pH values to which n-octanol was added. The mixture was stirred continuously for 24 h at 25 °C. After phase separation, the ibuprofen content in the buffer was analysed by HPLC. Since the initial amount of ibuprofen sodium was known, the amount in the organic phase was determined by difference.

Diffusion and partition of ibuprofen ion-pairs

The apparent partition coefficients and the diffusion studies were performed as described above. Phosphate buffer pH 7.0 was used as an aqueous phase for the determination of the apparent partition coefficient. The diffusion studies were conducted containing 2% solutions of ibuprofen or its equivalent of the amine salt, using propylene glycol as the solvent. The ibuprofen content was analysed using HPLC.

Statistical analysis

The difference between the flux of ibuprofen for the infinite dose application (saturated solutions) at different pH values and for different ibuprofen salts was assessed using multiple regression with pair-wise comparison. One-way ANOVA, followed by Tukey's HSD post-hoc test, was used for assessing the difference in solubility due to pH or salts.

Results and Discussion

Solubility

Ibuprofen is relatively non-polar and accordingly its highest solubility is obtained in solvents of lower solubility parameter values, such as acetone, ethyl acetate and lipophilic alcohols. Solubility decreases in most polar solvents. By replacing the acidic proton by sodium, the region of maximum solubility is shifted to larger solubility parameter values as compared to the parent acid (Bustamante et al 2000). As expected, the solubility of ibuprofen sodium (pKa = 4.45; Avdeef et al 1998) increases with increasing pH. The solubility profile is summarized in Table 1. The solubility at pH 7 and 8 is significantly greater than at pH 4, 5 and 6 ($P < 0.001$). There is no significant difference in solubility from pH 7 to 8.

Solvent–membrane interactions

Percutaneous absorption involves partitioning of a solute from its vehicle into the skin and subsequent diffusion of solute through the skin. Identical solute flux would be expected from solutions in which the solute had equal

Table 1 Solubility, log P values (n-octanol:buffer), permeability coefficient and steady-state flux through PDMS membrane for ibuprofen sodium at different pH values.

pH	Solubility ($mg\,mL^{-1}$)	log P	k_p ($cm\,h^{-1}$)	Fraction unionized	Flux ($\mu g\,cm^{-2}\,h$)
4	0.028 ± 0.0007		2.2977	73.81	64.131 ± 1.8
5	0.156 ± 0.008	3.28 ± 0.007	0.398	21.98	62.36 ± 0.91
6	1.0 ± 0.05	2.42 ± 0.02	0.187	2.74	187.10 ± 12.3*
7	340.51 ± 31.3	0.92 ± 0.04	0.00081	0.28	277.23 ± 4.23*
8	299.035 ± 21.4	0.63 ± 0.01	0.0001262	0.03	37.834 ± 3.5*

Values represent the mean ± s.d. (n = 4). *Significantly different from value at pH = 4; $P < 0.002$.

thermodynamic activity. Non-ideal behaviour is a result of solute and/or solvent interaction with the membrane (Twist & Zatz 1988a; Jiang et al 1998). In this situation the physicochemical properties of the barrier will change depending on the interaction involved. It is therefore often difficult to interpret results because of the highly complex nature of the stratum corneum. A synthetic membrane, such as PDMS membrane, offers advantages concerning the physicochemical properties of the diffusional barrier, including perm selectivity, high diffusivity, thickness control, and less stringent storage and handling requirements. Moreover, Twist & Zatz (1988b) suggest that solvents (e.g. water, glycerin, propylene glycol and polyethylene glycol 400) are not sorbed to a significant extent by this material and behave as ideal vehicles; permeation from these vehicles is therefore considered to be a function only of permeant activity. Hence to determine the appropriate permeant activity and to eliminate membrane–solvent–solute interaction, PDMS membrane was chosen for the studies. Propylene glycol and water were used as solvents since they are not significantly sorbed by this membrane. The flux through PDMS membranes is usually faster as compared to the human skin barrier, but the permeability relationships and trend are very similar (Valenta et al 2000).

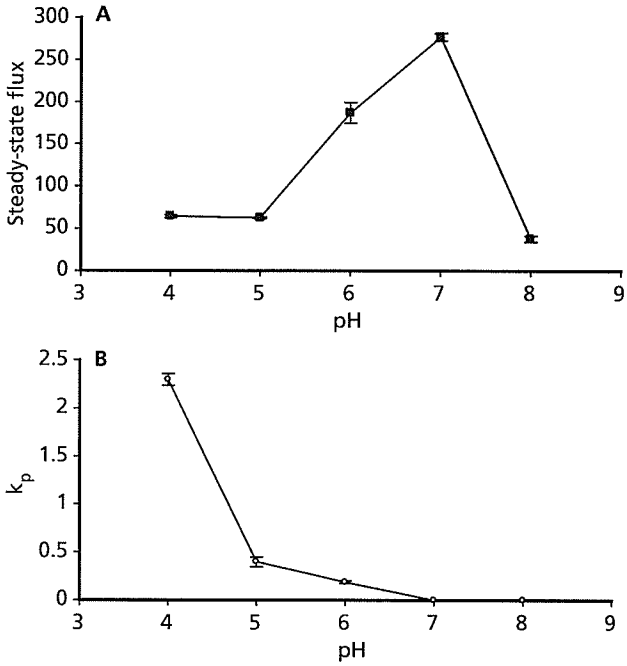

Figure 2 Relationship between steady-state flux (A) and permeability coefficient k_p (B) with respect to change in pH.

pH and penetration

Ibuprofen is a non-steroidal anti-inflammatory drug (NSAID) that has been formulated into a number of topical preparations. Its solubility and diffusion parameters as a function of pH have been documented (Watkinson et al 1994; Hadgraft & Valenta 2000). The low solubility of ibuprofen is one of the factors responsible for its reduced transfer across the skin. Sodium salts are more soluble than the parent drug, thereby increasing the amount of drug in solution in the aqueous vehicle. However, increasing the polar nature of the permeant reduces its tendency to permeate the lipophilic stratum corneum. The overall effect on permeation is a combination of these effects.

Donor depletion was observed at pH 4, 5 and 6, therefore flux was calculated using the cumulative amounts from the first four sampling times. The highest flux was determined at pH 7 (Table 1). The flux increased as the pH increased from pH 4 to pH 7 and then decreased as shown in Figure 2. The total flux (J_{tot}) of a permeant through a membrane is a composite term, contributed to by the diffusion of both the ionized and unionized moieties. The transport across the membrane can be described by the permeabilities of the ionized and unionized species and their respective concentrations $k_{p(ion)}$, $k_{p(union)}$, c_{ion}, and c_{union} (Hadgraft & Valenta 2000):

$$J_{tot} = (k_{p(union)} \times c_{union}) + (k_{p(ion)} \times c_{ion})$$

The ambient pH and the pK_a give the relative amounts of ionized and unionized species. In the case of ibuprofen sodium, flux at lower pH is dominated by the first term (unionized species), whereas at higher pH it is dominated by the second term (ionized species). Figure 3 shows the relationship between permeability coefficient as a function of the percentage of unionized ibuprofen present. The percentage of drug that was ionized or unionized at a particular pH was calculated using the Henderson–Hasselbach equation.

A linear relationship ($y = 31.984x + 1.322$; $r^2 = 0.978$) between the permeability coefficient and fraction ionized suggests that the diffusion was mostly as a result of partition and transfer of unionized ibuprofen present in the donor phase, and the insignificant intercept indicates the contribution of ionized species. A plot of permeability as a function of pH (Figure 2) also follows a reasonable trend that would be expected of an acidic compound. The permeability coefficient of the ibuprofen sodium increases

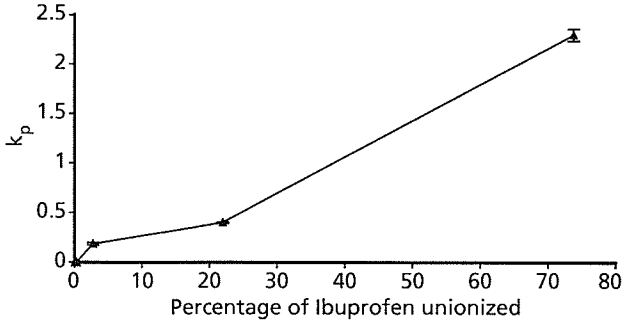

Figure 3 Relationship between percentage of ibuprofen unionized and permeability coefficient.

with decrease in pH. The highest permeability coefficient was determined at pH 4 (Figure 2), when more than 50% of ibuprofen sodium is unionized ($pK_a = 4.45$, (Avdeef et al 1998)). It is interesting to note that the steady-state flux of ibuprofen sodium is greater at higher pH, whereas its permeability coefficient is higher at lower pH when the fraction of unionized species is greater. This suggests that at higher pH the lower permeability of the ionized species is more than compensated for by the increased solubility, which is consistent with the findings of previous studies (Watkinson et al 1994).

Partitioning experiments

The apparent partition coefficients between n-octanol (representative of skin lipids) and phosphate buffers at various pH values are presented in Table 1. As expected, the octanol:buffer partition coefficient increased with decreasing pH from pH 8 to 5. The drug concentration in the aqueous phase at pH 4 was below the limit of detection, indicating a very high partitioning into octanol for the unionized species.

NMR spectroscopy

The goal of both the ^1H and ^{13}C NMR measurements (see Tables 2, 3a and 3b) was to obtain evidence for the presence of ion-pair formation between ibuprofen (Figure 1) and respective amine salts from the chemical shift changes to protons and carbons near the cationic and anionic charges. Ion-pair formation is an indicator of increased permeability of the salt through skin.

Comparison of the proton spectra of ibuprofen and ibuprofen sodium, and the amine hydrochloride and ibuprofen salts in CD_3OD showed no significant changes in chemical shifts (Table 3a). Relative to the chemical shifts in the corresponding amine, the ^1H NMR spectra of the primary, secondary, tertiary and quaternary ethylamine hydrochloride and ibuprofen cations show small deshielding shifts in both the methylene and methyl protons adjacent to the nitrogen (Table 2). The protons on the carbon adjacent to the nitrogen are recognized by their downfield position in the salt as compared to the free amine. The deshielding effect for primary, secondary and tertiary amine salts is smaller in the ibuprofen salts than in the corresponding hydrochlorides, which is indicative of greater charge neutralization and ion-pair formation. The largest downfield chemical shift was observed in the methylene group in the quaternary salts where charge separation is greatest, and therefore charge neutralization least, because of steric hindrance.

^{13}C NMR provided significant evidence for ion-pair formation between ibuprofen and the corresponding amine. The carboxylic acid group in ibuprofen (RCOOH) shows a chemical shift of carbon (i) at 178.55 ppm while the sodium salt ($RCOO^-Na^+$) shows the carboxylate anion at 186.67 ppm (Table 3b). Based on these values for complete protonation (or deuteration) in the acid and complete ionization in the sodium salt, intermediate chemical shift values reflect the degree of charge neutralization resulting from interaction between the carboxylate anion and the nitrogen cation in the ibuprofen amine salts. The ammonium salt (181.96 ppm) indicates considerable charge neutralization, which may be partly stabilized by solvation of an ion-pair, including hydrogen bonding interactions as well as electrostatic attraction. Less charge neutralization occurs in the quaternary salt (183.39 ppm), where hydrogen bonding interactions cannot occur and close contact between the charges is sterically hindered. The degree of neutralization in the quaternary salt is equivalent to that observed for the primary amine (183.56 ppm) but must result from a different mode of interaction in each case. As the lipophilic character resulting from increased ethyl substitution of the amine salts increases, ion-pair formation, as indicated by chemical shift changes as a measure of charge neutralization, is favoured. This is shown by a decrease in the chemical shift values from the primary (183.56 ppm), to

Table 2 ^1H and ^{13}C chemical shifts of amines, amine hydrochloride and ibuprofen amine salt.

	^1H		^{13}C	
Amine	CH_2	CH_3	CH_2	CH_3
1°	2.69	1.12	37.20	18.39
2°	2.63	1.13	44.64	14.95
3°	2.58	1.07	47.25	11.51
4°	–	–	–	–
Amine hydrochloride	CH_2	CH_3	CH_2	CH_3
1°	3.05 (+0.36)	1.35 (+0.23)	36.44 (−0.76)	13.31 (−5.08)
2°	3.09 (+0.46)	1.35 (+0.22)	43.77 (−0.87)	11.88 (−3.07)
3°	3.27 (+0.69)	1.38 (+0.31)	48.02 (−0.77)	9.63 (−1.88)
4°	3.34	1.32	53.50	7.87
Ibuprofen amine salt	CH_2	CH_3	CH_2	CH_3
1°	2.91 (+0.22)	1.24 (+0.12)	36.05 (0.39)	13.31 (−5.08)
2°	2.96 (+0.33)	1.25 (+0.12)	43.49 (−0.28)	11.77 (−3.18)
3°	3.21 (+0.63)	1.33 (+0.26)	–	9.39 (−2.12)
4°	3.30	1.30	53.44	7.79

Table 3a ^{1}H NMR chemical shift (δ) of ibuprofen and its salts for proton on carbon.

	RCOOH	Na^+ $RCOO^-Na^+$	1° $RCOO^-N^+H_3Et$	2° $RCOO^-N^+H_2Et_2$	3° $RCOO^-N^+HEt_3$	4° $RCOO^-N^+Et_4$	Diamine $RCOO^-N^+H_3$ $CH_2CH_2NH_2$
a	0.92	0.92	0.89	0.89	0.91	0.89	0.90
b	1.87	1.84	1.84	1.84	1.85	1.83	1.84
c	2.47	2.45	2.44	2.44	2.46	2.44	2.44
e	7.11	7.07	7.06	7.07	7.1	7.05	7.06
f	7.24	7.32	7.27	7.27	7.23	7.28	7.27
h	3.61	3.61	3.57	3.58	3.68	3.56	3.58
j	1.46	1.46	1.42	1.43	1.44	1.41	1.42

Table 3b ^{13}C NMR spectra of ibuprofen and its salts.

	RCOOH	Na^+ $RCOO^-Na^+$	N^+H_4 $RCOO^-$ N^+H_4	1° $RCOO^-N^+H_3Et$	2° $RCOO^-N^+H_2Et_2$	3° $RCOO^-N^+HEt_3$	4° $RCOO^-N^+Et_4$	Diamine $RCOO^-N^+H_3$ $CH_2CH_2N\ H_2$
a	22.90	22.91	22.90	22.94	22.96	22.87	22.89	22.93
b	46.15	46.21	46.24	46.26	46.26	46.19	46.25	46.25
c	31.48	31.54	31.61	31.64	31.64	31.58	31.65	31.63
d	139.74	140.25	140.92	140.57	140.74	139.96	140.42	140.74
e	128.33	128.39	128.44	128.47	128.49	128.40	128.52	128.46
f	130.34	129.87	130.16	130.07	130.13	130.41	129.97	130.13
g	141.54	142.85	141.81	142.83	142.45	141.67	143.08	142.39
h	46.34	49.97	–	–	–	–	–	–
i	178.55	186.67	181.96	183.56	182.79	178.74	183.39	183.126
j	19.23	20.21	19.86	20.21	20.08	19.26	20.27	20.06

the secondary (182.79 ppm) and to the tertiary amine (178.74 ppm). The similarity of the chemical shifts for ibuprofen and the tertiary amine salt indicates a high degree of charge neutralization, which is consistent with extensive close ion-pair formation. Tertiary amines have been previously reported to form more stable ion-pairs when compared with primary and secondary amines (Megwa et al 2000b). Similar, but smaller, chemical shift changes are observed in the methyl group (j) and the aromatic ring carbon (g). Correlation of the chemical shifts of the amine salts of ibuprofen with the carbon (h) adjacent to the carboxylate anion is not possible because this signal is obscured by the solvent protons. The similarity of the chemical shift for the carboxylate carbon in the primary amine salt with the mono ibuprofen salt of ethylene diamine is consistent with a similar degree of interaction between these primary amine salts and the carboxylate anion.

Relative to the corresponding amines, the ^{13}C NMR spectra of the cations in the primary, secondary, tertiary and quaternary hydrochloride and ibuprofen salts show significant deshielding in the cation of the methylene carbon and increased shielding of the methyl carbon (Table 2). This reversal of shielding may be due to the conformational location of the methyl groups with respect to the C–C bonds. Nevertheless there is a consistent correlation between the chemical shift changes observed in all four series of salts. Similar, but smaller, chemical shift differences are observed in the methylene carbon of the ibuprofen salts compared with the hydrochloride salts, again indicating greater charge neutralization and ion-pair formation. No significant change is observed in the methyl carbons between the ibuprofen and hydrochloride salts. These measurements therefore provide evidence for the existence of an ion-pair between ibuprofen and amines in solution. The conclusion based on NMR measurements that the tertiary amine has the largest degree of ion-pair formation is in agreement with the changes observed in the diffusion rates for primary, secondary and tertiary salts.

An ion-pair is a pair of oppositely charged ions held together by Coulomb attraction without formation of a covalent bond. Experimentally, an ion-pair behaves as one unit in determining conductivity, kinetic behaviour, osmotic properties, etc. Following Bjerrum, oppositely charged ions with their centres closer together than a distance of:

$$q = (8.36 \times 10^6\ z^+z^-)/(\varepsilon_r T)\ \text{pm}$$

are considered to constitute an ion-pair ('Bjerrum ion pair'). z^+ and z^- are the charge numbers of the ions, and ε_r is the relative permittivity (or dielectric constant) of the medium. Solvents with lower dielectric constant favour formation of an ion-pair. The distance q increases with temperature and high-temperature solvents behave in this respect as solvents of lower dielectric constants, permitting long-range ion-pairing (Yizhak 1999). Since the dielectric constant for propylene glycol is lower than

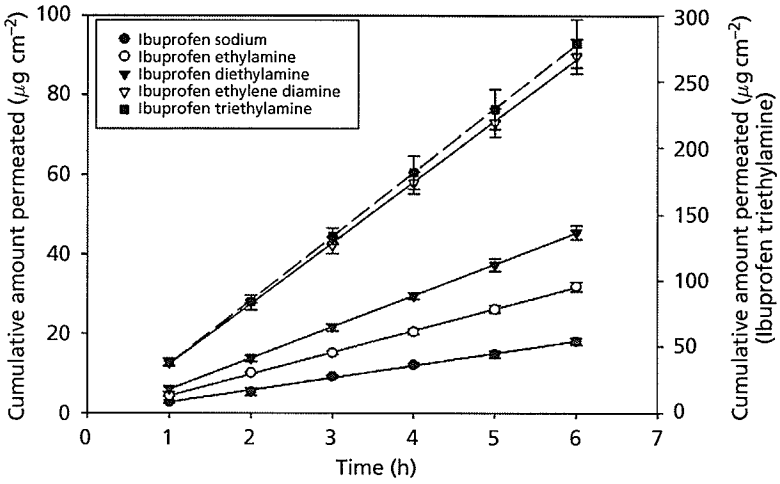

Figure 4 Comparison of diffusion profiles of different ibuprofen ion-pairs from propylene glycol through PDMS membrane.

Table 4 Steady-state flux and log P values of ibuprofen ion-pairs.

Salt type	log P	Flux
Ibuprofen sodium	0.92 ± 0.04	3.09 ± 0.091
Ibuprofen ethylamine	0.967 ± 0.02	5.42 ± 0.092*
Ibuprofen diethylamine	1.12 ± 0.03	7.91 ± 0.14*
Ibuprofen triethylamine	1.18 ± 0.03	48.14 ± 1.34*
Ibuprofen ethylene diamine	1.11 ± 0.02	15.31 ± 0.35*

Each value represents the mean ± s.d. (n = 4). *Significantly different from ibuprofen sodium, $P < 0.0005$.

methanol and we have evidence for ion-pair formation in methanol, we assumed ion-pair formation of ibuprofen with various amine counter-ions in propylene glycol. Propylene glycol has been used as the solvent for studying the penetration experiments, as specified in the methods section.

Ibuprofen salts and diffusion through PDMS membrane

Propylene glycol was used as a solvent since it is not significantly sorbed by the PDMS membrane (Twist & Zatz 1988b). The measured apparent partition coefficients of the salts into n-octanol seems to be in agreement with their lipophilicity (Table 4). Ibuprofen showed the highest partition coefficient with triethylamine as a counter-ion, followed by diethylamine and ethylamine. The partition coefficient of ibuprofen ethylene diamine was similar to that of diethylamine.

All the salts showed higher steady-state flux for ibuprofen through the PDMS membrane as compared to the sodium salt (Figure 4). Ibuprofen ethylene diamine is plotted on the right-hand y-axis, whereas the other salts are plotted on the left-hand y-axis. The highest steady-state flux was measured from ibuprofen triethylamine, followed by ibuprofen ethylene diamine, ibuprofen diethylamine and ibuprofen ethylamine. Although the partition coefficient of ibuprofen ethylene diamine was similar to ibuprofen diethylamine, its steady-state flux is almost twice that of ibuprofen diethylamine.

Conclusions

The results of this study suggest that it is possible to enhance the flux of salts across lipophilic membranes using an ion-pair approach. The degree of enhancement is associated with the lipophilicity, extent of ion-pairing and reduction in the charge over the drug molecule.

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