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**CHEMICAL AND PHYSICAL METHODS OF ENHANCING THE
PERCUTANEOUS ABSORPTION OF ANTIMICROBIAL AGENTS**

TINA AMINI

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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April 2001

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Submitted for the degree of Doctor of Philosophy, 2001

SUMMARY

Contrary to previously held beliefs, it is now known that bacteria exist not only on the surface of the skin but they are also distributed at varying depths beneath the skin surface. Hence, in order to sterilise the skin, antimicrobial agents are required to penetrate across the skin and eliminate the bacteria residing at all depths. Chlorhexidine is an antimicrobial agent with the widest use for skin sterilisation. However, due to its poor permeation rate across the skin, sterilisation of the skin cannot be achieved and, therefore, the remaining bacteria can act as a source of infection during an operation or insertion of catheters. The underlying theme of this study is to enhance the permeation of this antimicrobial agent in the skin by employing chemical (enhancers and supersaturated systems) or physical (iontophoresis) techniques. The hydrochloride salt of chlorhexidine (CHX), a poorly soluble salt, was used throughout this study.

The effect of ionisation on *in vitro* permeation rate across the excised human epidermis was investigated using Franz-type diffusion cells. Saturated solutions of CHX were used as donor and the variable studied was vehicle pH. Permeation rate was increased with increasing vehicle pH. The pH effect was not related to the level of ionisation of the drug. The effect of donor vehicle was also studied using saturated solutions of CHX in 10% and 20% ethanol as the donor solutions. Permeation of CHX was enhanced by increasing the concentration of ethanol which could be due to the higher concentration of CHX in the donor phase and the effect of ethanol itself on the membrane.

The interplay between drug diffusion and enhancer pretreatment of the epidermis was studied. Pretreatment of the membrane with 10% Azone /PG demonstrated the highest diffusion rate followed by 10% oleic acid/PG pretreatment compared to other pretreatment regimens (ethanol, dimethyl sulfoxide (DMSO), propylene glycol (PG), sodium dodecyl sulphate (SDS) and dodecyl trimethyl ammonium bromide (DTAB). Differential Scanning Calorimetry (DSC) was also employed to study the mode of action of these enhancers.

The potential of supersaturated solutions in enhancing percutaneous absorption of CHX was investigated. Various anti-nucleating polymers were screened in order to establish the most effective agent. Polyvinylpyrrolidone (PVP, K30) was found to be a better candidate than its lower molecular weight counterpart (K25) and hydroxypropyl methylcellulose (HPMC). The permeation studies showed an increase in diffusion rate by increasing the degree of saturation.

Iontophoresis is a physical means of transdermal drug delivery enhancement that causes an increased penetration of molecules into or through the skin by the application of an electric field. This technique was employed in conjunction with chemical enhancers to assess the effect on CHX permeation across the human epidermis. An improved transport of CHX, which was pH dependant was observed upon application of the current. Combined use of iontophoresis and chemical enhancers further increased the CHX transport indicating a synergistic effect. Pretreatment of the membrane with 10% Azone/PG demonstrated the greatest effect.

Keywords: Chlorhexidine dihydrochloride, chemical enhancers, differential scanning calorimetry, supersaturation, iontophoresis.

To my parents and my brother

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Abbreviations

AUFS	absorbance units full scale
CFUs	Colony-forming units
CHX	Chlorhexidine dihydrochloride
cm	centimetre
DSC	differential scanning calorimetry
DTAB	dodecyltrimethylammoniumbromide
g	grammes
h	hours
HPLC	High-Performance Liquid Chromatography
HPMC	hydroxypropylmethylcellulose
I.S.	internal standard
IR	infra-red
J	flux
k_p	permeability coefficient
K_{sp}	Solubility product
M	molar
mg	milligrammes
min	minutes
ml	millilitres
mM	millimolar
n	Sample size
NaCl	sodium chloride
OA	Oleic acid
ODS2	octadecylsilane (C ₁₈)
P	partition coefficient
PG	Propylene glycol
pKa	$-\log_{10}$ dissociation constant
PrP	propyl paraben (propyl p-hydroxy benzoate)
PVP(K25)	Polyvinylpyrrolidone (average molecular weight of 24000)
PVP(K30)	Polyvinylpyrrolidone (calculated molecular weight of 40000)

r	correlation coefficient
s.d.	standard deviation
SDS	Sodium dodecyl sulphate
T	temperature
UV	ultraviolet
v/v	Volume in volume (ml/100 ml)
w/v	weight in volume (g/100 ml)
%	percentage
λ	wavelength (nm)
°C	degrees Celcius
μg	microgrammes
μl	microlitres

CHAPTER ONE

INTRODUCTION

THEORY AND METHODS OF INVESTIGATING TOPICAL DRUG DELIVERY

Introduction

Intravascular catheters are a major part of medical care. However, catheter-related infection has become recognised as a significant clinical problem and is a cause of morbidity and mortality in hospitalised patients (Elliot *et al.*, 1997). Elliot and co-workers (1997, 1993) studied the source of microbial contamination and suggested that the most likely source of contamination was the organisms on the patient's skin. They showed that organisms were still present on the skin of 67% of patients despite the use of aseptic techniques including thorough skin disinfection and antibiotic prophylaxis. They suggested that the bacteria present on the site of insertion might have been transferred from the patient's skin onto the surface of the various components of the insertion equipment.

Several approaches to prevent catheter-related infections have been developed. These methods include electrically charged catheters that will repel organisms from their surface (Crocker *et al.*, 1992) and catheters containing antimicrobial agents that reduce catheter colonisation (Tebbs and Elliot, 1993). The use of prophylactic antibiotics at the time of catheter insertion has also been proposed (Elliot, 1993; Mackinnon *et al.*, 1987). Although all these approaches have reduced catheter-related infection rates, none has been able to prevent the infections.

Care of the catheter insertion site (skin sterilisation), using topical antiseptics, seems more promising as this approach eliminates the cause of the infection (skin bacteria) rather than the prevention of the infection. In this study, this approach has been investigated and, since the elimination of the skin bacteria by using topical formulations requires an understanding of the type and location of these bacteria, the anatomy and biochemistry of the skin and the available antimicrobial agents, these parameters have been described in details in this section. Furthermore, various physicochemical parameters, such as solubility and partition coefficient, and physiological factors that affect the transport of the topically applied chemicals have also been discussed.

1.1.1 Epidermis

The epidermis is a thin sheet providing the outer covering of the body. It generally ranges in thickness from 0.075 to 0.15 mm, except on the palm and sole where it can be up to 0.6 mm thick. Epidermal cells undergo a rapid process of division, maturation, and shedding which takes on average 15 to 30 days (Montagna *et al.*, 1992). Epidermis is composed of a superficial stratum corneum (non-viable epidermis) and stratum malpighii (viable epidermis). A diagram of the epidermis is presented in figure 1.2.

The viable epidermis has a density near that of water (Flynn, 1979) and contains no blood vessels or nerve endings and obtains all of its nutrition and waste exchange by diffusion from the dermis. The stratum malpighii (viable epidermis) is divided into stratum germinativum (basal), stratum spinosum, stratum granulosum and stratum lucidum.

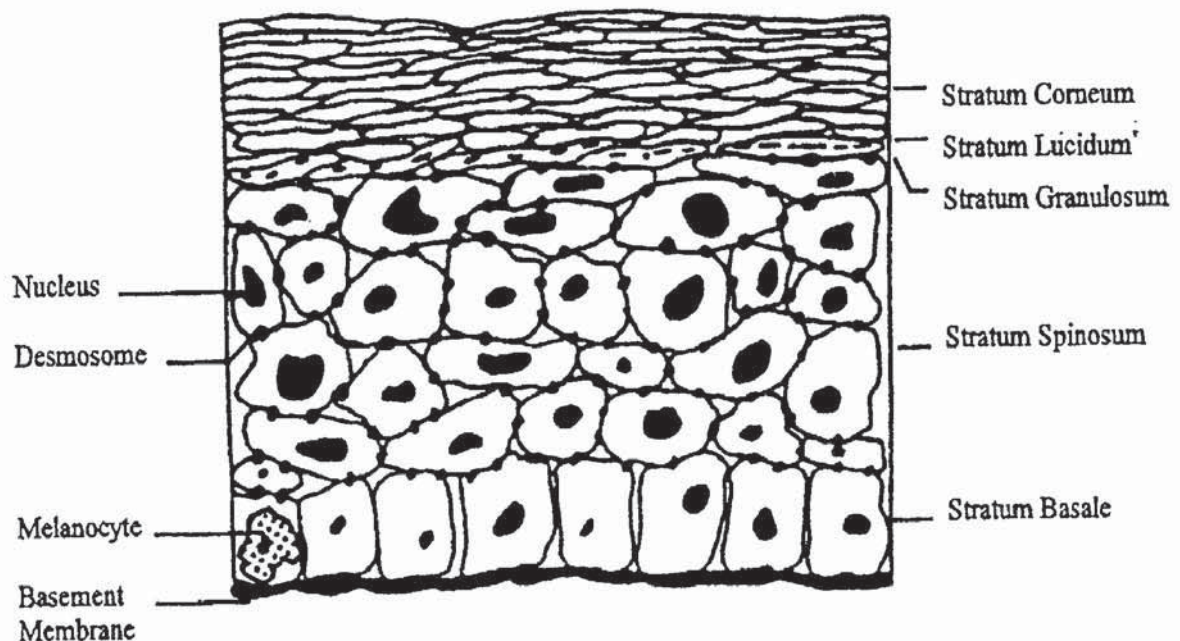


Figure 1.2 Structure of epidermis

1.1 Skin Structure

Skin is a large multilayered organ that in the average adult covers a surface area of 2 m² and receives about one-third of the blood circulating through the body. It forms a complex barrier to the external environment, maintaining body fluids within our system. No part of the skin remains free of a resident microbial population, the composition of which is continually changing to reflect alterations in the status of the skin.

Anatomically, the skin has many histological layers but, in general, it is described in terms of three tissue layers: the epidermis, the dermis, and the subcutaneous tissues (hypodermis) (Figure 1.1).



Figure 1.1 Schematic cross section of the skin (De Bruijn and Den Breejen, 1995)

Basal Cells

Sitting on the dermal-epidermal junction is a one-cell-thick layer of oval-shaped cells, the basal cells. The cells are much like cells in other body tissues. They have typical intracellular organelles (nucleus, mitochondria, ribosomes) and possess the expected metabolic machinery. The basal cells are anchored to the basal lamina and to each other by desmosomes. These protein structures occur irregularly on the cell periphery and bridge the gap between cells for attachment. As the cells undergo their process of division and maturation (differentiation), the desmosomes are repeatedly broken and reformed. At all times, though, the cells are in intimate contact with their neighbours.

Mucopolysaccharides are also found between cells. They serve as a cell coat or glycocalyx. The function of this coat is unclear but could be related to cell adhesion, maturation, and cell-cell recognition, among others. The membranes of basal cells are rich in phospholipid and cholesterol. The lipids are arranged in a classical bilayer structure with protein molecules embedded in or extending through the membrane. The proteins function in cell adhesion and active transport. Within the cells is a complex cytoskeleton of filaments and tubules. These appear to control cell shape and positioning of cell organelles for many of the cell functions. In the epidermis, there is an abundance of keratin filaments within the cytoplasm. These filaments change in composition as the cell differentiate.

In normal skin, the rate of cell production in the basal layer is balanced by shedding at the skin surface. Cells are shed at the rate of one cell layer per day in man. When a basal cell divides, on average one daughter cell remains in the basal layer. The other daughter leaves the basal layer and enters the differentiating layers.

Stratum Spinosum

As the basal cells move upwards, they change their shapes into a more rounded form with spiny projections and appear as stratum spinosum. Though the nucleus is still present, a spinous cell does not divide. These cells begin to synthesise specific proteins. This synthesis continues throughout much of the differentiating epidermis. The protein aggregates into masses called keratohyalin granules. Also synthesised in this layer and granular layers are lamellar granules or membrane-coating granules. In the upper differentiating epidermis, these granules migrate toward the cell periphery and their

contents are extruded into the intercellular space (Wertz and Downing, 1982). The contents of the granules are components of the soon-to-be-formed stratum corneum intercellular space and cell envelope.

Stratum Granulosum

After the germinal layer has raised 12-15 layers above its point of origin, it becomes flattened and the basophilic nuclear material is dispersed throughout the cell, as granules. This layer is referred to as stratum granulosum. During the transition, these granules develop to fill the interior space of the keratinocytes with keratin filaments surrounded by an amorphous matrix of sulphur-rich proteins. These filaments and proteins are probably responsible for the hexagonal shape of these cells. The cell membrane also undergoes dramatic changes. The phospholipid content is markedly reduced and ceramides are greatly increased in abundance. Also present is a newly synthesized protein called involucrin which is the protein precursor of the proteinaceous stratum corneum cell envelope (Goldsmith, 1983).

Stratum Lucidum

The stratum lucidum layer, which lies below the stratum corneum, is the site of an increase in the stage of nuclei disintegration and keratinisation. The cell plasma membrane becomes thickened with protein deposited on its inner surface to form the stratum corneum cell envelope (Farbman, 1966). In addition to these changes, lipid composition, elements and water profiles change. Phosphorus, sodium, and potassium levels drop 10 to 20-fold in the stratum corneum.

Stratum Corneum

The stratum corneum (SC), or horny layer, consists of compacted, dead, flattened, keratinised cells embedded in an intercellular lipid matrix. The majority of the protein of the stratum corneum is composed of intracellular keratin filaments which are cross-linked by inter-molecular disulphide bridges (Steven and Steinert, 1994). These horny cells have lost their nuclei and are formed and continuously replenished by the slow upward migration of cells produced by the basal cell layer of the stratum germinativum. The lipid matrix is organised in lamellar bilayers (Fartasch, 1996). These bilayers are formed by

rearrangement and fusion of lamellar discs that are extruded into the intercellular regions from the uppermost cells of the stratum granulosum (Lavker, 1979). Figure 1.3 illustrates the proposed structure of the lipid bilayers throughout the intercellular domains of human SC by Barry (1991a).

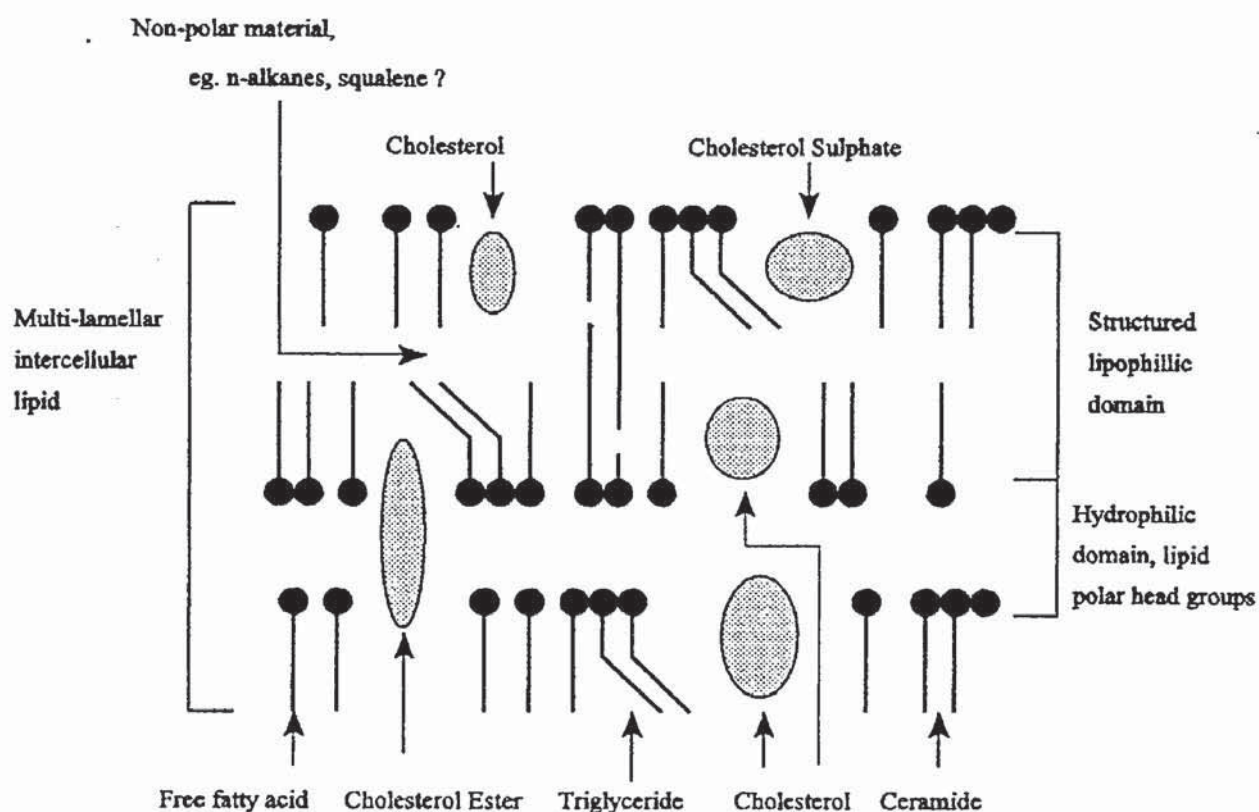


Figure 1.3 Proposed structure of the lipid bilayers of the intercellular domains of human stratum corneum (Barry, 1991a)

These lamellae consist primarily of cholesterol, free fatty acid and ceramides and cholesterol-3-sulphate (Wertz and Van den Bergh, 1998; Melnik *et al.*, 1989) (figure 1.4). Six classes of ceramides (designated ceramides 1 to 6) have been isolated and identified (Brain and Walters, 1993). Their structures contain long-chain bases, such as sphingamine and 4-hydroxysphingamine, N-acetylated by different fatty acids. Because of its unique structure, ceramide 1, an acylceramide, may act as a stabilizer of the intercellular lipid lamellae. This molecule consists of the usual sphingosine base with an amide-linked ω -hydroxyacid ester linked to the ω -hydroxyl group (Wertz and Downing, 1989). It is possible that ceramide 1 functions as a molecular rivet in the intercellular lipid lamellae of the stratum corneum (Wertz and Van den Bergh, 1998). There is also strong evidence to indicate that lamellae are further stabilised by chemical bonds between the long-chain ceramides and glutamate residues on the corneocyte protein envelope (Lazo *et al.*, 1995).

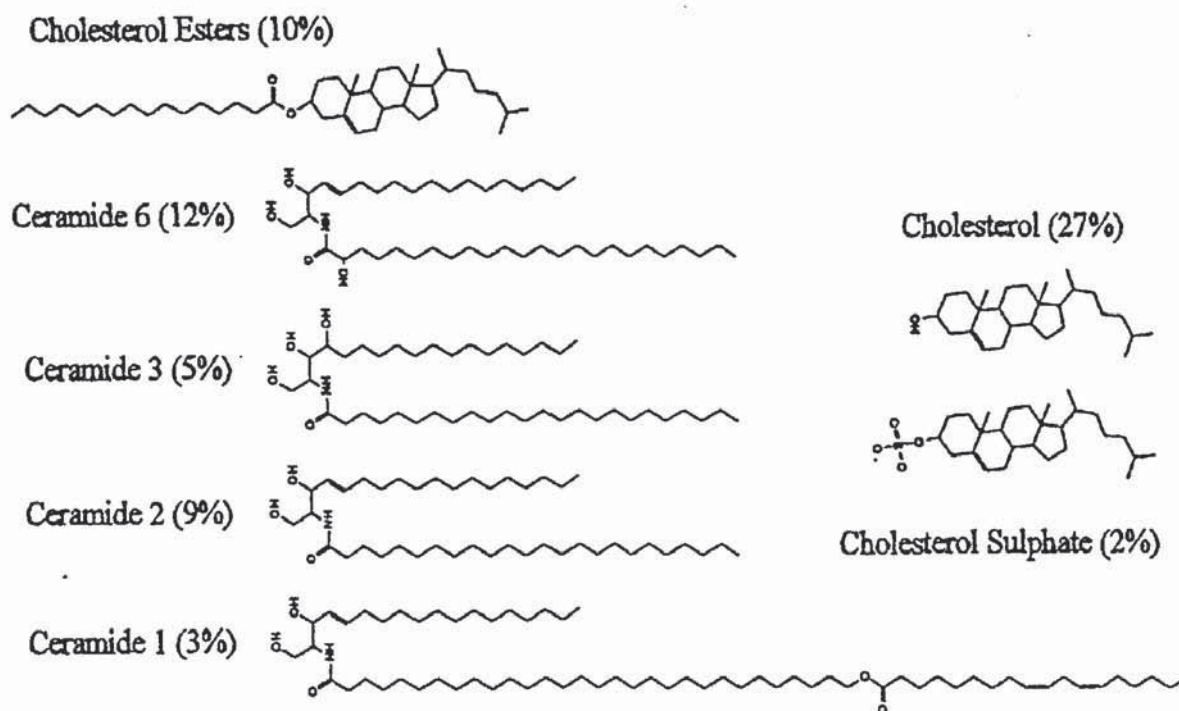


Figure 1.4 Structure of lipids of the intercellular regions of the human stratum corneum (Brain and Walters, 1993)

The stratum corneum is the rate-limiting barrier that restricts the inward and outward movement of chemical substances. Evidence for this is the compromising of the skin barrier caused by damage to the stratum corneum or its removal from the skin with adhesive tape (Tregear, 1966). Malkinson (1958) found that normal skin admits only 1 to 2% of ^{14}C -labelled hydrocortisone, whereas up to 90% was absorbed through stripped skin. Marzulli (1962) also found that stripped sheets of stratum corneum have approximately the same resistance to permeability as the entire skin. The stratum corneum exhibits regional differences in thickness over the body. Over most of the body, it is about $10\mu\text{m}$ thick when dry, increasing to about 40 to $50\mu\text{m}$ when fully hydrated; however, the thickness may be several hundred micrometers on palms of the hands and soles of the feet (Holbrook and Adland, 1974).

The lipid compositions of epidermal layers are shown in table 1.1.

Component (% w/w)	Basal & Spinous	Granular	SC
Phospholipid	62	65	0
Sterols (unesterified)	9	21	20
Sterols esters	1	2	2
Ceramides	1	10	52
Glucosyl ceramides	7	8	0
Triacylglycerols	8	2	2
Fatty acids	7	16	22
Hydrocarbons	2	9	0
Others	3	7	2

Table 1.1 Lipid composition of epidermal layers (Yardley and Summerly, 1981)

1.1.2 Dermis

The dermis is approximately 0.2-0.3 cm thick and consists of 80% protein, mainly collagen and elastin in a matrix of mucopolysaccharide ground substance (Woodbourne, 1973). Dermis is divided into two distinct zones: a superficial finely structured thin papillary layer adjacent to the epidermis and a deeper coarse reticular layer consisting of bundles of collagenous and elastic fibres. Numerous blood vessels, lymphatics, and nerves are supported within the dermis and there are dermal capillary loops closely opposed to the lower epidermis and the surface area of contact is large due to the convoluted interface between the two layers. This arrangement facilitates both epidermal metabolism and the transport of topically absorbed compounds to the vasculature of the dermis. It also contains the epidermal appendages, such as the hair follicles, sebaceous glands, and sweat glands (Flynn, 1979). The dermis provides flexibility with strength.

1.1.3 Hair Follicles

Hair follicles are distributed over the entire skin surface with the exception of the soles of the feet, the palms of the hands, the red portion of lips, and part of the sex organs. The follicles consist of concentric layers of cellular and non-cellular components. The hair (shaft) originates from the hair follicle and is formed of keratinised cells, which has a very high content of α -helical structure, compacted together into plates or scales (Flynn, 1979). The cross-linked protein structure of hair imparts its great strength. Each hair follicle is associated with one or more sebaceous glands (figure 1.5). These glands vary in size from 200 to 2000 μm in diameter (Woodbourne, 1973) and secrete an oily material, known as sebum, which acts as a skin lubricant and a source of stratum corneum plasticising lipid (Flynn, 1979). Sebum is reportedly a mixture of triglycerides, free fatty acids, waxes, sterols, squalene, and paraffins. The free fatty acids give sebum bactericidal and fungicidal activities. The composition of sebum and skin surface lipid is shown in table 1.2.

Component (% w/w)	Sebum	Surface lipid
Squalene	12	10
Sterols (Unesterified)	<1	2.5
Sterol esters	0	1.5
Wax esters	23	22
Triacyl glycerols	60	25
Di-and mono-acyl glycerol:	0	10
Fatty acids	0	25
Unidentified	5	4

Table 1.2 Sebum and skin surface lipid composition (Nicolaidis, 1984)

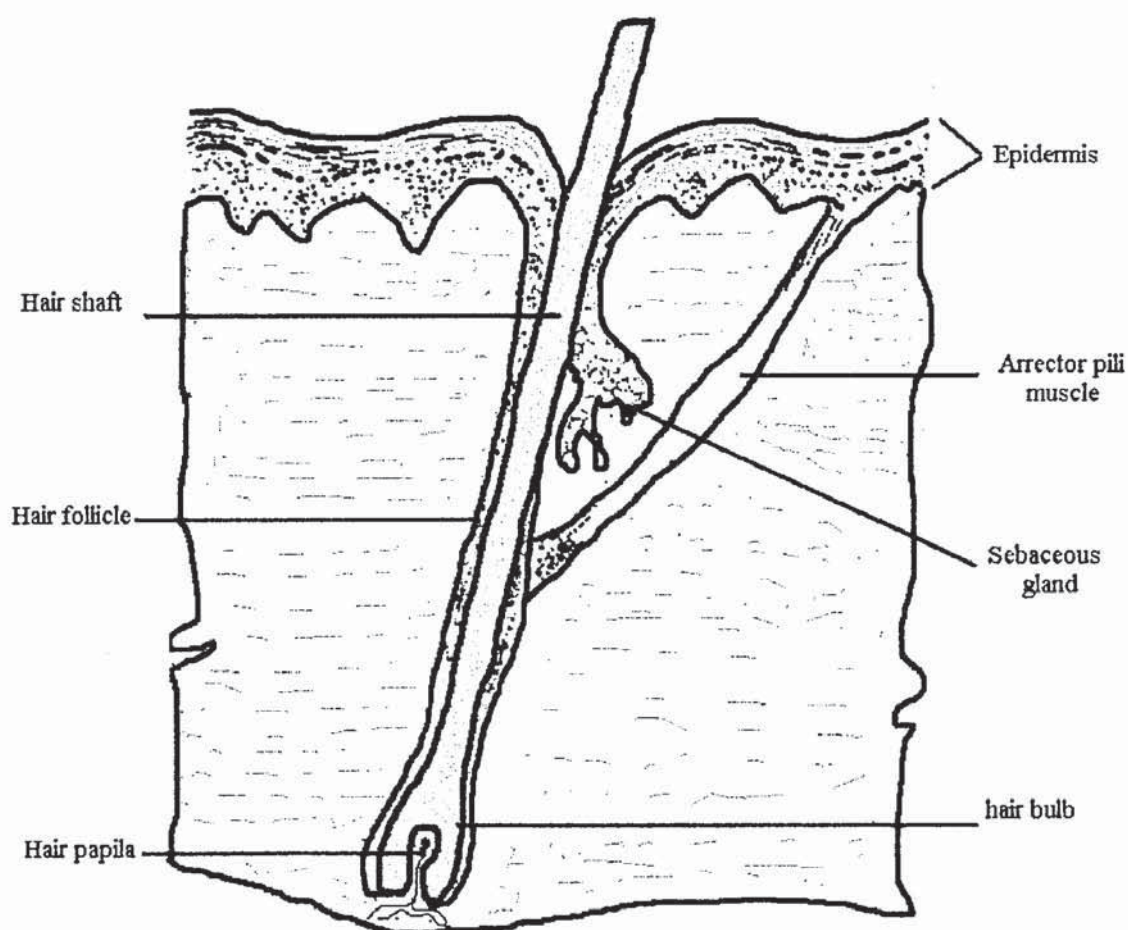


Figure 1.5 Anatomy of the hair follicle

1.1.4 Sweat Glands

There are two types of sweat glands in the skin, eccrine and apocrine. The major type of sweat gland in human, the eccrine sweat gland, is distributed over practically all parts of the body. The deepest secretory portion of the gland is a coiled structure situated in the reticular dermis and the excretory portion spirals through the papillary dermis to open onto the surface at the pore. Eccrine glands produce a watery secretion of acid pH containing electrolytes, trace elements and organic substances, which is conveyed to the surface of the skin where its evaporation plays an important thermoregulatory role (Barry, 1988).

The apocrine glands are much less numerous, present as little as one tenth the density of eccrine glands. They are most numerous in areas such as the underarms, genitals, breasts, head, and abdomen. The ducts from the gland empty into the hair follicle. These glands secrete only a small volume of fluid and are slow to respond to stimuli. The secretion composition varies with body site.

1.1.5 Subcutaneous Layer

The dermis rests on the subcutaneous tissue or fat layer consisting of loose connective tissue or adipose cells. It manufactures and stores fat and acts as a heat insulator and a shock absorber (Katz and Pulse, 1971).

1.2 Percutaneous Absorption of Chemicals

Therapeutic agents have been applied to the skin for the purpose of local or systemic effects for decades. However, only few drugs or a fraction of the applied dose readily permeate the lipids of the stratum corneum, which is believed to be the main barrier to percutaneous absorption (Guy and Hadgraft, 1985). Therefore, numerous approaches have been investigated to increase skin permeation, such as physical techniques, chemical enhancers, and increased drug loading.

To utilise diffusion process successfully, it is necessary to understand the process and the pathways of percutaneous absorption and also to establish a mathematical model to describe and predict skin penetration.

1.2.1 Percutaneous Absorption Process and Pathways

The percutaneous absorption process includes diffusion or transport of penetrant to the skin surface, partitioning of the chemical into the stratum corneum, diffusion through the intercellular lipids of the stratum corneum, partitioning of the chemical from the lipophilic stratum corneum into the aqueous viable epidermis, diffusion through the viable epidermis and upper dermis and finally uptake of the penetrant into a cutaneous blood vessel and systemic access (figure 1.6).

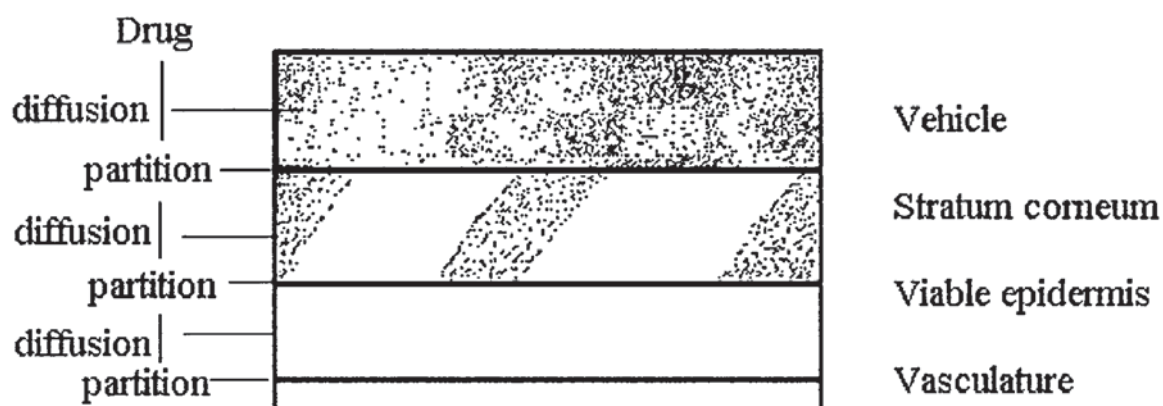


Figure 1.6 Drug transport across the skin (Guy and Hadgraft, 1989)

The penetration routes are either transfollicular (*via* hair follicles, sweat ducts, and secretory glands) or transepidermal (across the stratum corneum transcellularly and intercellularly) (figure 1.7). Transepidermal route occurs when a permeant is transported across corneocytes and intercellular lipid bilayers of the SC. However, Praustnitz (1997) suggested that this route does not generally occur as it involves the crossing of approximately 100 intercellular bilayers, which requires significant amount of energy. In the intercellular route, drugs are transported along multilamellar bilayers rather than crossing them.

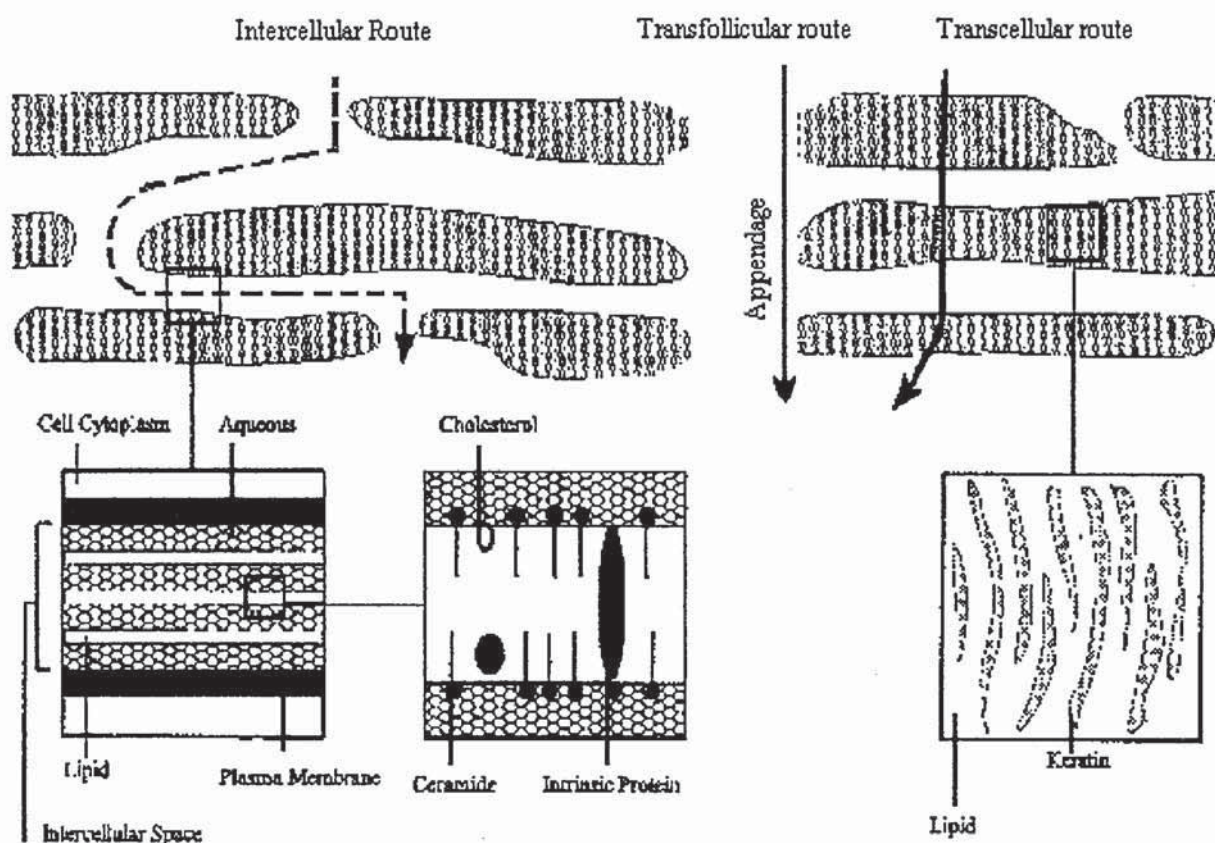


Figure 1.7 The brick and mortar model of the stratum corneum illustrating possible routes of drug penetration through human skin (adapted from Barry, 1991)

1.2.2 Mathematics of Skin Penetration

It would be very useful to be able to identify the essential parameters in percutaneous absorption and predict the rate at which materials penetrate the skin to assess potential toxicological hazards, and also to improve the way in which drugs are administered topically. Therefore, a model describing skin penetration will be of benefit. Percutaneous absorption, whether *via* the intercellular or intracellular route, is essentially a passive diffusional process. Diffusion is defined as a process of mass transfer of individual molecules of a substance, brought about by random molecular motion and associated with a concentration gradient. The theoretical diffusion profile of a drug through the skin is shown in figure 1.8. It is characterised by a period of non steady-state condition followed by steady-state penetration.

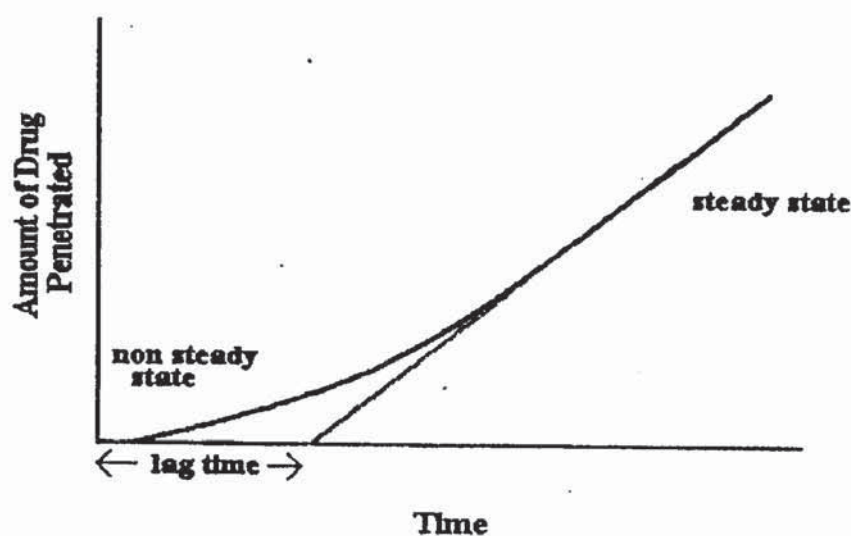


Fig 1.8 Typical profile of amount transported versus time for drug diffusion through the epidermis (infinite dose)

The simplest way of modeling the process of skin absorption is to employ the Fick's first law of diffusion which describes the steady-state flux of the drug through the skin (J),

$$J = -D \frac{dC}{dx} \quad (1.1)$$

where, D is diffusion coefficient and dC/dx is the concentration difference across the skin.

The negative sign signifies that diffusion occurs in the direction of decreasing concentration of penetrant, thus the flux is always a positive quantity.

The classical description of the transport process is shown in figure 1.9.

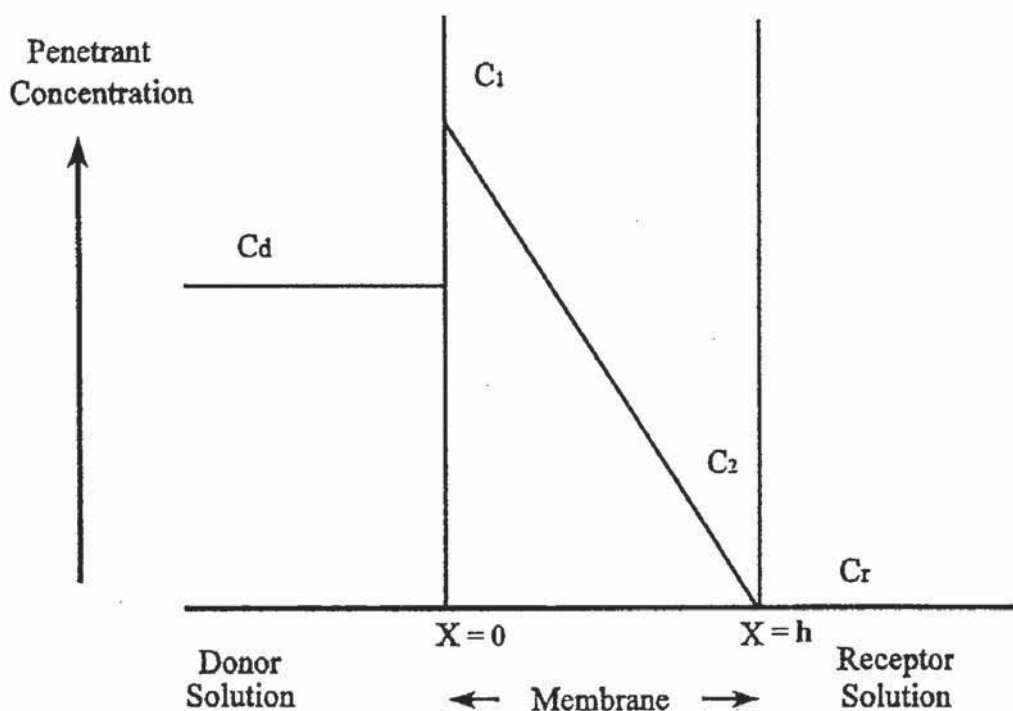


Figure 1.9 Concentration profile across homogenous membrane at steady-state: zero order flux (Barry, 1983)

If we consider the interactions that exist between the drug, the vehicle and the skin, which is not an inert barrier to diffusion, we can see that the partition behaviour is very important. The partition coefficient can be calculated as:

$$K = \frac{C_1}{C_d} = \frac{C_2}{C_r} \quad (1.2)$$

Therefore, the flux (J) across the membrane can be described as (Cooper and Berner, 1985):

$$J = \frac{DK(C_d - C_r)}{h} \quad (1.3)$$

if sink conditions hold in the receptor compartment, solute concentration at any time (t), within the innermost membrane layer ($x = h$), is assumed to be negligible ($C_d \gg C_r$), hence

$$J = \frac{DKC_d}{h} \quad (1.4)$$

Flux of the penetrant can be calculated from the slope of the steady-state diffusion curve. In some cases, it is not possible to determine D , K , or h independently, therefore, they can be collected into a single variable defined as:

$$k_p = \frac{KD}{h} \quad (1.5)$$

where k_p is the permeability coefficient and substitution into equation 1.4 gives,

$$J = k_p C_d \quad (1.6)$$

k_p can be determined by dividing the steady-state slope by the initial concentration of drug applied to the donor phase.

When the steady-state portion of the line is extrapolated to the time axis, the point of intersection is known as the lag time, t_L , which is dependent on the membrane diffusion coefficient (D) and the thickness of the membrane (h),

$$t_L = \frac{h^2}{6D} \quad (1.7)$$

This is the time required for a penetrant to establish a concentration gradient within the membrane and its measurement allows an estimation of the diffusion coefficient, providing there is no binding.

It must be remembered, however, that the above equations are only applicable for steady-state conditions and will not be valid if there are significant interactions, such as binding between the drug and components of the skin. Provided diffusion is from an infinite dose, Scheuplein (1978) suggested the following equation that defines the entire absorption process rather than the steady-state segment:

$$M = CK \left\{ \frac{Dt}{h} - \frac{h}{6} - \frac{2h}{\pi^2} \sum_{n=1}^{\infty} \frac{(-1)^n}{n^2} \exp \left[\frac{-dn^2\pi^2t}{h^2} \right] \right\} \quad (1.8)$$

Where M is the cumulative amount of drug in the receptor phase.

1.3 Factors Affecting Percutaneous Absorption

Skin absorption is affected by physiological and physiochemical factors.

Physiological Factors

The predominant physiological factors that influence percutaneous absorption are age, regional sites, species variation, skin integrity, temperature, hydration and cutaneous drug metabolism of the skin. Singh and Singh (1993) showed that the absorption of topical steroids occurs more readily in children than adults. They explained that this result was due to the higher water content of children's skin. *In vivo* permeation studies carried by Roberts *et al.* (1982) showed the effect of regional sites on the permeability of methyl salicylates. Higher permeability was seen in the abdominal area compared to the forearm site. Barry (1983) reported that skins of guinea pig, rat and rabbit are more permeable than human skin. Wester and Maibach (1991) have also showed that the skin of hairless mouse is more permeable compared to human skin. The rate of diffusion can increase with increasing temperature. Guy and Hadgraft (1980) suggested that the thickness of the applied vehicle would affect diffusion of the molecule through the vehicle to the skin. Typically, with viscous formulations, as the temperature increases the viscosity decreases thereby aiding diffusion through the vehicle. The state of hydration of the SC is also an important factor in determining the rate of percutaneous absorption of a compound. It has been reported that the permeability coefficient of several phenols is directly related to SC water-content (Roberts, 1991). This effect could be due to the general swelling of the bricks in a bricks-and-mortar model which results in the loosening of the mortar and increasing the movement of the chemical through the intercellular keratin route. High water-content can also affect the solubility of the chemical. Finally, cutaneous drug metabolism caused by catabolic enzymes may also affect percutaneous absorption. Steinstrasser and Merkle (1995) showed the effect of the endopeptidases on proteins. They reported that this protease is capable of metabolising the proteins by splitting bonds inside peptide chains making up proteins.

Physiochemical Factors

According to equation 1.4, partition coefficient, diffusion coefficient, and concentration of the permeant are the main characteristics which influence its absorption. Due to the

existence of lipid and polar regions in the SC, a penetrant must be able to partition into both regions. Hence, it should possess a reasonable octanol/water partition coefficient. Hadgraft and Walters (1995) have suggested a value of about 100 ($\log P = 2$) for the optimal permeability.

Increasing the concentration of drug applied increases the escaping tendency of permeant molecules or their thermodynamic activity and enhances percutaneous absorption. Optimum permeation can be achieved employing a saturated solution which can be further increased using supersaturated systems (Iervolino *et al.*, 2000)

The selection of vehicle or solvent is another important consideration in the design of topical dosage forms. Drug solubility, therefore, the applied concentration, and affinity of the solute for the vehicle and skin are dependent on the vehicle. If affinity for the solvent is high, the permeant will tend to remain in the vehicle rather than partitioning into the stratum corneum. In addition, solvents can exert more specific interactive effects. They can affect the stratum corneum and alter the barrier properties of it. The permeation of the solvent itself may also affect drug solubility within the skin, thus influencing partition behaviour and penetration (Mollgaard and Hoelgaard, 1983)

According to the pH-partition theory (Swarbrick *et al.*, 1984), the unionised form of the ionisable drugs penetrates the skin more rapidly than ionised form. Therefore, by adjusting the vehicle pH of aqueous solutions, the absorption may be improved. Parry *et al.* (1990) showed that only the undissociated species of benzoic acid partitions into and permeates across the skin. Inagi *et al.* (1981) studied the effect of pH on the penetration of indomethacin ($pK_a=4.5$) through guinea-pig skin. At low values of pH, at which the drug was essentially unionised, the flux value was maximal. Increasing the pH, increased the fraction of ionised indomethacin and resulted in a decrease in flux as expected, however at pH 6.2, the flux of indomethacin was higher than expected based on permeation of unionised drug only.

However, some of the ionised compounds exhibited significant permeability. Hadgraft and Valenta (2000) investigated the effect of pH on the permeation of ibuprofen and

lignocaine through human skin. They showed that the maximum flux through the skin occurred at a pH where ionisation was high.

1.4 Permeation Enhancement

As the stratum corneum is the rate-limiting barrier of the skin, transdermal research has focused on facilitating drug transport across this barrier. Several enhancement techniques have been developed to overcome the impervious nature of the SC through physical approaches, such as iontophoresis (Angibogu *et al.*, 2000), electroporation (Wang *et al.*, 1998), phonophoresis (Asano *et al.*, 1997), and application of supersaturated systems (Davis and Hadgraft, 1991); biochemical means such as prodrugs (Bando *et al.*, 1997) and liposomal vesicles (Cevc, 1996); and use of chemical enhancers. In addition, the physical nature of the dermatological product influences drug penetration characteristics. For example, some transdermal delivery systems produce a state of occlusion, which causes enhancement.

Chemical enhancers, supersaturated systems and iontophoresis will be discussed and reviewed in more detail later in the relevant chapters.

1.5 Permeation studies

The effect of many of the physicochemical and physiological factors on the topical bioavailability can be assessed by a variety of percutaneous penetration experiments. *In vitro* and *in vivo* methods can be used to evaluate chemical absorption through the skin.

In vitro studies are carried out on isolated human or animal skin, or artificial membranes mounted in diffusion cells while *in vivo* studies employ the skins of living humans or animals *in situ*, generally by monitoring the appearance of the drug in body compartments or the disappearance of the drug from a surface depot following topical application. *In vitro* methods can not exactly duplicate the *in vivo* scenario as the absence of dermal vascularisation and enzymatic metabolism may modify the clearance and therefore the flux of the drug. However, its main advantage lies on the fact that it allows

control of the laboratory environment and so the elucidation of the individual factors which modify drug penetration.

Undoubtedly, the most satisfactory membrane is human skin that can be obtained from surgical procedures or from cadavers and kept frozen (below -20°C). It is often assumed that the SC diffusional resistance cannot be altered in the *in vitro* situation. However, physiological factors such as skin age, site, and condition contribute to large inter- and intra-specimen variabilities in the barrier properties of human skin that cannot be strictly controlled, especially as availability is often limited.

To circumvent these problems, many scientists have employed animal skin in their studies. A great deal of work has been performed correlating absorption through animal skin and human skin (Bronaugh and Maibach, 1985; Bronaugh *et al.*, 1982). Pig and monkey skin appear to be good models for human skin, whilst rat, rabbit, and mouse skin tend to be more permeable (Wester and Maibach, 1991).

Artificial membranes have also been used for permeation studies and have a number of advantages such as being readily available, therefore, less time-consuming and being reproducible. Different type of artificial membranes have been employed like composite or laminate sheets (Yamaguchi, 1997), zeolites, which are rigid three-dimensional aluminosilicate with having 50% of their volume filled with water (Dyer *et al.*, 1979), dimethylpolysiloxane or silicone membranes, which are lipophilic in nature (Walkow and McGinity, 1987a,b), hydrophilic microporous cellulose acetate membranes (Barry and Brace, 1977), and cellulose nitrate filters impregnated with lipids (Hadgraft and Ridout, 1988). However, the fact that skin, particularly human skin, is more complex than these membranes of fixed pore size makes these type of membrane not the best choice.

In *in vitro* studies, the membrane is then mounted in diffusion cells to measure the passage of the compounds from the SC side through to a fluid bath.

1.6 Removal and Separation of Skin

In *in vivo* transdermal drug delivery, the whole thickness of skin must be considered, however, it is generally accepted that the stratum corneum is the principal barrier. The dermis is vascularised *in vivo* and drugs penetrating to this layer would be carried away by the systemic circulation in a continual sink condition. When used *in vitro*, the dermis can provide a substantial barrier. Hence, epidermis or stratum corneum alone is usually a better choice for *in vitro* permeation studies. There are several techniques for separating the epidermis from the underlying tissue.

Traditionally, chemical methods, enzyme digestion and subcutaneous injections of staphylococcal epidermolytic toxin were used to separate the epidermis from the dermis. Manschel in 1925 utilised maceration in acetic acid. Others suggested the immersion of skin in various salt solutions, such as potassium chloride (Griesmer and Gould, 1954) and sodium bromide (Scott *et al.*, 1986). Luis (1977) treated human skin with 2 N sodium thiocyanate for 3-5 hours while Walker *et al.* (1977) suggested ammonium chloride. A crude pancreatic extract was used by Medawar (1941) which was later replaced by the purified trypsin (Fan, 1958). Elias *et al.* (1974) employed the subcutaneous injection method to separate the epidermis from the dermis. However, this method can only be used on fresh tissues and might also damage the water barrier properties of SC (Smith *et al.*, 1982).

The traditional methods have been recently replaced by heat separation and mechanical methods. Although heat separation method was introduced by Kligman and Christopheris in 1963, it is still the method of choice by many researchers (Hirvonen *et al.*, 1998; Lin *et al.*, 1996). The procedure involves removing the excess fatty and connective tissues and immersing the skin in heated distilled water (60°C) for 2 minutes. The skin should be then blotted dry and the epidermis can be teased away from the underlying dermis by using forceps. However, this method is only useful for tissues without much hair as, unlike the chemical methods, the hair remains in the dermis and during separation these leave holes in the epidermis. Furthermore, denaturation of some SC proteins is also possible (Walker *et al.*, 1977).

Mechanical method is another technique used by recent investigators. Using an electric dermatome, controllable thickness of skin can be obtained (Grewal *et al.*, 2000; Cornwell *et al.*, 1998; Bronaugh *et al.*, 1981). However, this method does not ensure complete removal of the dermis.

In order to separate the SC from the viable epidermis, purified trypsin has been used by most researchers (Al-Saidan *et al.*, 1998; Bahatia and Singh, 1998)

Upon preparation of skin samples, the membrane can be either used immediately in diffusion studies or stored for future use. The method of choice for storing skin is freezing it at -20°C. It has been shown that skin samples stored frozen for 12 months did not show a significant change in water permeation (Bronaugh *et al.* 1986). The tissue sample can be wrapped in aluminium foil and then sealed in parafilm and frozen. When required, the skin can be thawed at room temperature.

1.7 Skin Microflora

The body surfaces of humans are populated by microorganisms. The various anatomical sites have different proportions and total number of organisms, which are usually non-pathogenic but can cause disease under appropriate conditions. The composition and growth of skin microflora depends on skin temperature, humidity, pH, and the availability of nutrients (Noble, 1993). Nutrients are derived mainly from eccrine and sebaceous gland secretions. The distribution of skin microbes is determined by species-specific nutrient demand, as well as the presence of cutaneous glands and the physical characteristics of skin sites. All these factors might give rise to large intra- and inter-individual differences in composition of the residential skin microflora (Braks *et al.*, 1999).

The dominant microbial populations on the skin surface are Gram-positive bacteria, which are normally relatively resistant to desiccation compared to Gram-negative bacteria. *Staphylococcus* and *Micrococcus* are the most abundant microorganisms on the skin surface because they are generally salt tolerant and can utilise the lipids present on the skin surface. Other Gram-positive bacteria usually found as part of the normal microflora

of the skin surface include *Corynebacterium*, and *Propionibacterium*. Gram-negative bacteria generally occur primarily in the moister regions of the skin surface, such as in the armpits and between the toes. Relatively few fungi are included in the normal microflora of the skin surface. Two yeasts, *Pityrosporum ovale* and *Pityrosporum orbiculare*, which are able to metabolise the lipids found on the skin surface, normally exist on scalp tissues (Ronald, 1988). Table 1.3 presents the most important families of the resident skin microflora.

Group	Family	Genus	Example Species
Gram positive cocci	<i>Micrococcaceae</i>	<i>Micrococcus</i> <i>Staphylococcus</i>	<i>M. luteus</i> <i>S. epidermis</i>
Diphtheroids-like species	<i>Coryneforms</i>	<i>Brevibacterium</i> <i>Corynebacterium</i>	<i>B. epidermis</i> <i>C. diphtheria</i>
	<i>Propionibacteriaceae</i>	<i>Propionibacterium</i>	<i>P. acnes</i>
Fungi	<i>Yeast</i>	<i>Malassezi</i> <i>Pityrosporum</i>	<i>M. fur fur</i> <i>P. ovale</i>

Table 1.3 The most important families of the resident skin microflora (Braks *et al.*, 1999)

1.8 Microbiological Sampling Techniques

The purpose of microbiological sampling is to allow statements of density, types and locations of microorganisms which reside in different layers of the skin. These statements can then be used to fulfil our goal, which is the elimination of bacteria in or on the skin.

It was generally believed that bacteria are confined to the surface of the stratum corneum (Kligman, 1965) although Updegraff (1964) showed that even after fourteen tapes stripping at the same site, bacteria were still present at that site. Microscopic studies have demonstrated dense microbial growth both on the skin surface around pilosebaceous

follicles, and in the depths of pilosebaceous units (Montes and Wilborn, 1969). Their findings were supported with the work of Selwyn and Ellis (1972), who also showed deep flora in the hair follicles. Kearney *et al.* (1984) used a cryostat sectioning procedure to determine quantitative viable counts of microorganism both on the surface and in the successive layers of human skin biopsies. They showed that two types of bacteria, staphylococci and propionibacteria, were distributed at varying depth beneath the skin surface. In 1963, Williams and later in 1978, Hoizle and Kligman (1978) provided evidence, which suggests that sweat ducts may normally contain bacteria. This claim was proved by the work of Malcolm and Hughes (1980).

However, to this date, the precise location of bacterial species is the subject of controversy. The differences in the reported location of bacteria in the skin could be explained by the fact that those scientists were examining different sites of the human body and also using different sampling techniques as each technique may give a different answer. In addition, the distribution of microorganisms on human skin varies from site to site and person to person as the environment mostly determines the microbial inhabitants. Therefore, there is no universally applicable sampling technique and the investigator must choose a method most likely to answer a defined question.

1.8.1 Swabbing

Swabbing is one method in which a defined area of the skin is rubbed with a moistened swab, which is then transferred to a dispersion fluid and plated out for viable cell counts. This technique samples a variable and low percentage of surface microorganisms, depending on swab fibre, time and pressure of rubbing. This method is therefore useful for qualitative work. Its advantages are simplicity, speed and use for pathologically affected skin and sites such as toe-webs.

1.8.2 Washing

The second method is washing which itself can be divided into two groups. The first is where part of the body, e.g. the hands, is washed and the fluid used for microbiological analysis (Michaud *et al.*, 1976). This method is simple and, because a fluid sample is

obtained, many quantitative and qualitative bacteriological tests can be done, but it has great variability and only samples surface microorganisms. However, this method cannot be used to examine microflora over small areas of skin.

The second type, the cylinder-scrub procedure, is the most commonly used for quantitative work. At first, a buffered non-ionic detergent, Triton X-100, is pipetted into a cylinder which is pressed vertically on the skin site to be sampled. The skin is rubbed with a blunt-ended rod and then the fluid is removed for bacterial analysis.

By this method, defined areas of skin can be sampled and greater recoveries of microorganisms obtained. However, it does not remove follicular microorganisms and can not be used on some skin sites, such as toe-webs and nose.

1.8.3 Surface Distribution

Surface distribution is another technique. Various solid vehicles can be used to transpose the microorganisms from the skin for identification and viable cell counting. The main methods are solid nutrient medium, velvet pad, and self-adhesive tape stripping. In velvet pad method, samples of velvet are bonded to ebonite discs and autoclaved. Then the velvet surface is pressed firmly on the skin and then briefly on agar. In self-adhesive tape stripping method, the adhesive surface is firmly applied to the skin, and is then pressed on agar. The agar plate is then incubated overnight at 37°C and the bacteria colonies are identified and counted the following day. This method can detect intrafollicular microorganisms if repeated samples are taken from the same site.

These methods underestimate the total flora present at the site as there may be failure to replicate a part of the total area, either during sampling or in transferring to the nutrient medium and they cannot detect intrafollicular microorganisms (except for the self-adhesive tape stripping method). These methods also provide a rough map of skin bacteria. The advantages of these methods are speed and simplicity, they can be used to sample diseased skin which may be sensitive.

Sampling of follicles is difficult. Holland *et al.* (1974) used a cyanoacrylate glue to remove follicular contents. Material from follicles could be processed and analysed. Although the method is simple, the degree of removal of follicular material is variable. The most accurate method was developed by Puhvel *et al.* (1975). In this method, a skin punch biopsy is processed to separate the epidermis and the intact follicles from the dermis. The follicles are then dissected from the epidermal disc under the stereomicroscope and investigated.

1.9 Antimicrobial Agents

Antimicrobials and antiseptics are terms commonly used to designate chemical compounds that have varying degrees of antimicrobial properties, in that they are either inhibitory or lethal to microorganisms (Poulson, 1990).

The field of antiseptics and disinfectants had its beginning with the discovery that germs were associated with infection and that the use of certain chemicals prevented the spread of germs, resulting in fewer cases of postoperative infection. Lister's use of phenol around operating theatres, and on surgical dressings and Semmelweis' use of chlorinated hand-washing were the beginning of the use of germicides as disinfectants and antiseptics. Since then, several compounds have been used for this purpose, such as alcohols, aldehydes (especially glutaraldehyde and formaldehyde), chlorine-releasing agents, organic mercurials, quaternary ammonium compounds, phenolic compounds, iodine, hydrogen peroxide, chlorhexidine, and triclosan.

Since the number of chemicals in use is too great, only those that have the widest use have been discussed here.

1.9.1 Alcohols

Alcohols are today the classic but still effective and important surgical disinfectants. Inexpensive, readily obtainable, and bacteriologically potent, their chief deficiency lies in an inability to kill spores. Buchholtz was probably the first to investigate the antimicrobial activities of ethanol on a scientific basis in 1875 and to relate these to the

possible application for antiseptic purposes (Rotter, 1996). Preoperative skin disinfection with alcohol was first carried out in the latter half of the 19th century by Nelaton using alcohol soaks at the site of operation. Furbringer was the first to recommend alcohol in 1888 for hand disinfection of surgeons and medical staff. Price (1939) found that 60 - 90% of all ethanol solutions proved strongly and rapidly bactericidal, and that much of the killing effect apparently takes place during the first few seconds of contact. Isopropyl and n-propyl alcohols are more effective bactericidal than ethanol (Kelsey and Maurer, 1972). They are miscible with water in all proportions, but isopropanol has a less objectionable odour than n-propanol, and is considered as a suitable alternative to ethanol in various products (Bandelin, 1977). However, recently due to flammability and their drying effect on the skin, alcohols are seldom used as antiseptics. Alcohols are also used in combinations with iodine and chlorhexidine (Jeffrey, 1995).

The mechanism for the disinfection effect of alcohols is generally believed to originate from the coagulation of proteins (Morton *et al.*, 1983), although the bacteriostatic actions of alcohols is due to the inhibition of cell metabolites. The antimicrobial activity of alcohols is highly dependent on their concentration and on the status (dry or moist) of the microorganism. Some water is required for alcohols to be most effective. Ethanol and the propanols are the best antiseptics among this group as they are miscible with water, hardly toxic, nonallergenic, fast-acting, and microbiocidal rather than microbiostatic, if used in the correct range of active concentration (60-70%). Propanol has slightly greater bactericidal activity than that of ethanol but is also about twice as toxic (Hugo and Russell, 1998).

In appropriate concentrations, alcohols provide the most rapid and greatest reduction in microbial counts on intact skin (Larson, 1991). Alcohols should be allowed to evaporate thoroughly from the skin to be fully effective and to decrease irritation (Larson, 1995). Table 1.4 gives example of the times necessary to kill various bacterial species in ethanol at the optimum concentrations of 60-80% v/v. Except for *Streptococcus pyogenes*, all species listed are killed within 1 min.

Percutaneous absorption of alcohols does occur and increases with growing chain length of the alcohol. The acute toxicity of ethanol and propanol when used on the skin, has been reported to be very low (Rotter, 1998).

Bacterial species	Killing time (s)		
	60%	70%	80%
<i>Staphylococcus aureus</i>	15	15	10
<i>Staphylococcus epidermis</i>	30	30	
<i>Streptococcus pyogenes</i>			90
<i>Pseudomonas aeruginosa</i>		10	
<i>Escherichia coli</i>	60	30	30

Table 1.4 Bactericidal efficacy of ethanol at various concentrations (%v/v) (Rotter, 1996)

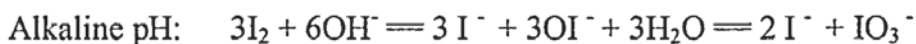
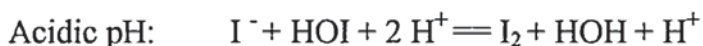
1.9.2 Iodine and Iodine Compounds

The first reference to the use of iodine in wounds was made in 1839 (Gottardi, 1991). Although, for many years, solutions of iodine in alcohol (as iodine is only slightly soluble in water) and iodine in potassium iodide have been used, these solutions have now largely been replaced by the solubilised preparation of iodine known as iodophor, most particularly solutions of iodine in polyvinylpyrrolidone. The advantages of these iodophor formulations are: less corrosive, less irritating, and nonstaining, compared to aqueous and alcoholic solutions (Bloomfield, 1996).

Iodine undergoes a number of reactions in aqueous solution. The chemistry of aqueous iodine solution is described by the following equations (Gottardi, 1991):



Hence, it can be seen that iodine may exist in several forms in an aqueous solution, depending on pH:



However, only hypiodic acid (HOI) and molecular iodine (I_2) play a role in the disinfection process (Boothe, 1998). Therefore, the disinfecting efficiency of iodine increases with decrease in pH even though iodine exerts its antimicrobial effect over a wide range (Gottardi, 1985). Molecular iodine has superior sporicidal and cysticidal properties compared with hypiodic acid.

Iodine acts by decreasing the oxygen requirements of aerobic microorganisms (Maris, 1995). Iodine also interacts preferentially with the proteins of the cytoplasmic membrane (Maris, 1995; Gottardi, 1985). Dunn (1952) studied the actions of halogens on enzymes and stated that they may oxidise -SH groups in the enzymes to -S-S- groups.

Iodine formulations show a broad-spectrum activity that includes fungi, mycobacteria, protozoa, and viruses, as well as vegetative bacteria. Pre-surgical skin disinfection of surgeon and patient, disinfection of mucous membranes, and wound disinfection are the most common uses of iodophors (Boothe, 1998). Bacterial resistance to iodine seems to be uncommon (Goldenheim, 1993), with no reports of bacterial resistance to povidone-iodine preparations.

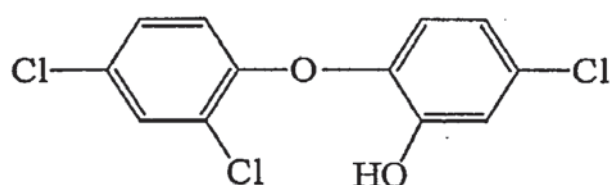
In the study reported by Gershenfeld (1957), the bactericidal efficiency of povidone-iodine, even in dilutions up to 1:10, was adequate to kill some Gram-negative and Gram-positive bacteria, such as *Mycobacteria pyogenes*, *Salmonella typhosa*, *Streptococcus hemolyticus*, and *Pseudomonas aeruginosa*, within 15 seconds. Lowbury and co-workers (1964), in their study on alternative methods for the removal of the transient flora, reported that povidone-iodine surgical scrub caused a reduction of 99.97% in mean count of samples from sites experimentally contaminated with *Staphylococcus aureus*. Although *in vitro* studies have shown povidone-iodine to be highly effective against

selective bacteria, including *Staphylococcus aureus* (Goldenheim, 1993), *in vivo* studies provide conflicting data regarding efficacy (Doughty, 1994).

Disadvantages in the use of iodine in skin antisepsis are staining of skin and fabrics coupled with possible sensitising of skin and mucous membranes. As mentioned above, these problems can be circumvented by using iodophors as the iodine in iodophors is bound to a carrier of high molecular weight such as polyvinylpyrrolidone. Therefore, they can provide a sustained-release reservoir for active iodine and consequently, avoiding the harmful side-effects of high iodine. However, when used for antisepsis, iodophors should be allowed to remain on the skin for a minimum of 2 minutes to obtain the full advantage of the sustained-release iodine (Hugo and Russell, 1998).

1.9.3 Triclosan

Triclosan (2,4,4'-trichloro-2'-hydroxydiphenyl ether) is a non-ionic, broad spectrum antimicrobial agent with the following structure:



Bhargava and Leonard (1996) reported that the primary site of its action is the cytoplasmic membrane and is also directed toward RNA and protein synthesis in bacteria.

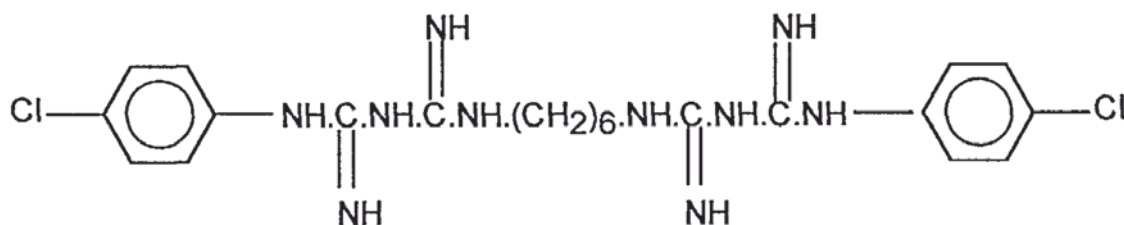
The poor water-solubility of triclosan (0.6 mg/ml, 25°C) limits its usable concentration. Various methods can be used to increase the solubility of triclosan; these include the use of solvents, surfactants and also by pH adjustment (pK_a of triclosan is 7.9), thus an enhancement in clinical effect may be expected. However, increasing the pH has an unfavourable effect on the antimicrobial activity of triclosan. At high pH, the increase in triclosan solubility is due to the hydrophilicity of the phenol anion, the charge which reduces the effectiveness of the triclosan.

Triclosan has been used in personal care products and dermatological and topical preparations for protection of skin (Bhargava and Leonard, 1996). Triclosan has also been used in the formulation of a new antiseptic called I.V.Prep, which has been used in the preparation of skin for invasive procedures. This preparation containing 90% isopropyl alcohol, 0.25% triclosan and film formers (Citraflex and Gantrez) that provide a thin protective coating when applied to the skin, has been shown to have higher efficacy compared to conventional methods (Gabel-Hughes *et al.*, 1991). The antimicrobial activity of triclosan is concentration and formulation dependent. It is effective in low concentrations against a broad spectrum of gram-negative and gram-positive bacteria, notably *Proteus vulgaris*, *Salmonella sp.*, *Mycobacteria*, anaerobic bacteria and fungi (Regos *et al.*, 1979). In a study by Bartzokas *et al.* (1984) on a 2% triclosan detergent against methicillin-resistant *Staphylococcus aureus* in surgical units, the triclosan was found to be effective against both resistant and susceptible *S. aureus*.

The assessment of dermal toxicity of different products of triclosan at concentrations ranging from 0.0005 to 1% showed no skin sensitisation in human (Kanetoshi *et al.*, 1992). Concentrations of 3% triclosan in 3% propylene glycol, administered dermally to rabbits for 13 weeks, resulted in some skin irritation, but no other toxic effect was observed (Steinkjer and Braathen, 1988).

1.9.4 Chlorhexidine

Chlorhexidine is a cationic bisbiguanide that was first synthesised in 1950 (Denton, 1991). Chlorhexidine has the following structural formula :



Chlorhexidine is a strong base with pKa values of 2.2 and 10.3. It readily forms salts, such as hydrochloride, acetate, and gluconate, from which a range of pharmaceutical preparation have been formulated. Acetate and gluconate salts of chlorhexidine may be

used interchangeably in aqueous preparations, however, because of its economy and convenience, the gluconate is much preferred over the acetate. Moreover, in aqueous solution chlorhexidine diacetate gives an odour of acetic acid. Solubilities of these salts in water and ethanol are shown in table 1.5.

Chlorhexidine salt	Solubility in	
	Water	Alcohol
Gluconate	miscible	1 in 5
Acetate	1 in 55	1 in 15
hydrochloride	Sparingly soluble (0.6% w/v)	Slightly soluble

Table 1.5 Solubilities of chlorhexidine salts in different vehicles (Martindale, 1999)

Chlorhexidine is available as a solution and as a scrubbing agent. Chlorhexidine solution is used principally as a topical antiseptic on skin wounds and mucous membranes, but it is also used as a pharmaceutical preservative. Chlorhexidine scrubs contain alcohol and are used to prepare the surgeon and patient before surgery. This antimicrobial agent in combination with silver sulfadiazine has also been used to coat the catheters in order to prevent the catheter-related infections (Sheng *et al.*, 2000; Heard *et al.*, 1998). Patients using catheters impregnated with antiseptic agents (chlorhexidine and silver sulfadiazine) had less microorganism colonization compared with conventional catheters.

Chlorhexidine exhibits a broad spectrum of antibacterial activity, strong binding to the skin, ability to adsorb to negatively charged surfaces in the mouth, persistence, low toxicity, and a minimal negative effect on activity by blood or other organic material (Larson, 1995).

Chlorhexidine has its major antibacterial action by interference with the function of cellular membranes, with the primary site of action being the cytoplasmic membrane

(Maris, 1995; Barrett-Bee *et al.*, 1994). Chlorhexidine has a very rapid bactericidal effect as well as persistence of action (Maris, 1995). Chlorhexidine is both inhibitory and lethal to Gram-positive and Gram-negative bacteria at relatively high dilutions according to Davies *et al.* (1954) though Gram-negative bacteria are less sensitive. They stated that concentrations as low as 0.5 µg/ml would inhibit the growth of gram positive organisms. At bacteriostatic concentrations the bacterial cell membrane is damaged with loss of cell contents and inhibition of enzyme proteins. At higher concentrations, precipitation of cellular proteins and nucleic acids occurs resulting in effective bactericidal action (Davies and Field, 1969; Heard and Ashworth, 1968; Emilson *et al.*, 1973). Animal studies indicate that, at high concentrations, chlorhexidine binds to rat liver microsomes interfering with enzyme metabolism and causing protein precipitation (Christensen and Jensen, 1974).

The *in vitro* bactericidal activity of 0.05% chlorhexidine gluconate was determined by Denton (1991) and is presented in table 1.6. The results are reported as a log₁₀ reduction factor. The higher value represents the lower colony count (higher antimicrobial effect).

Test organism	No.of strains	Mean log ₁₀ reduction at		
		20 seconds	1 min	10 min
<i>Staphylococcus aureus</i>	16	0.4	0.7	2.5
<i>Staphylococcus epidermis</i>	41	2.2	3.4	>5.1
<i>Streptococcus pyogenes</i>	9	1.2	1.8	>3.7
<i>Cornebacterium spp.</i>	8	1.1	1.4	3.7
<i>Propionibacterium acne</i>	2	0.7	1.8	3.6
<i>Escherchia coli</i>	14	3.2	5	>6.4
<i>Pseudomonas aeruginosa</i>	15	1.7	2.7	4.9

Table 1.6 Bactericidal activity of 0.05% chlorhexidine gluconate (Denton, 1991)

The potential for the development of bacterial resistance to this antimicrobial agent seems low but has been reported (Larson, 1995). The optimum range of pH for activity of the chlorhexidine is 5.5 to 7. Despite being an effective antiseptic, its use has limitations, as chlorhexidine is incompatible with anionic detergents and anionic sites.

During the extensive use of chlorhexidine as a skin antiseptic, very few adverse reactions, such as skin sensitivity, to the active component have been reported. The degree of percutaneous absorption of chlorhexidine is extremely low (Case, 1980) although it was detected in low concentrations in the venous blood of infants after bathing with a preparation containing chlorhexidine gluconate 4% however, No adverse effects due to percutaneous absorption of chlorhexidine were reported (Cowen *et al.*, 1979).

1.10 Comparison of Antimicrobial Agents

Comparative studies provide valuable information about the efficacy of each antimicrobial agent and would allow the choice of the most appropriate agent for a particular purpose. In the following section, a review of comparative studies carried out by other researchers has been given.

A recent trial comparing povidone-iodine and iodine tincture antiseptics showed a substantially lower rate of blood culture contamination with use of iodine tincture (Strand *et al.*, 1993). However, use of iodine tincture is limited because repeated exposure to high concentrations of iodine can be toxic. Therefore, an alternative antiseptic should be considered. Based on their findings, Mimoz *et al.* (1999) proposed alcoholic chlorhexidine solution as the substitute for iodine tincture. They showed that the single application of an alcoholic solution of 0.5% chlorhexidine could reduce the blood culture contamination more than an aqueous solution of 10% povidone-iodine. Maki *et al.* (1991) compared the effect of three antiseptics for disinfection of patients' central venous and arterial catheter sites before insertion and for site care every other day. They showed that with 70% v/v propan-2-ol and povidone-iodine solution, infections occurred in 7.1 and 9.3% of patients respectively, while chlorhexidine gluconate caused a lower incidence of infection (2.3%). Their findings were supported by the work of Mimoz *et al.* (1996) and

Garland *et al.* (1995), who also showed that chlorhexidine is superior to povidone-iodine and alcohol when is used for catheter care.

Goldblum *et al.* (1983) compared the effect of repeated application of 4% chlorhexidine detergent solution and povidone-iodine for skin preparation of haemodialysis patients and personnel. The chlorhexidine reduced total bacterial count and eradicated *Staphylococcus aureus* at both 2 and 4 hours after disinfection to a significantly greater extent than did the povidone-iodine. Lowbury and Lilly (1973) reported that 2 minutes of washing with 4% chlorhexidine caused a significantly greater mean of immediate reduction (86.7%) in the bacterial count than was found with povidone-iodine surgical scrub (68%). A number of studies on the efficacy of skin disinfection has shown that solutions containing chlorhexidine, and, to a lesser extent, isopropanol, demonstrate a sustained antibacterial effect on the skin flora, in contrast to povidone-iodine (Rotter, 1981; Ayliffe *et al.*, 1988). In another study, the cumulative efficacy of 4% chlorhexidine gluconate in detergent and 1.5% triclosan in natural soap handwash preparations in eliminating *Serratia marcescens* from hands was evaluated (Bartzoaks *et al.*, 1987). It was shown that chlorhexidine was more efficient in reducing the colony counts (mean log₁₀ reduction factor of 4.15 compared to the value of 3.78 for triclosan). Larson (1989) compared the antimicrobial effect of triclosan with those of alcohol and chlorhexidine in handwashing studies. Triclosan was found to be milder with greater sustained activity but less effective in the immediate reduction of microbial counts than the other antimicrobial agents studied.

These comparative works indicate the higher antimicrobial activity of chlorhexidine preparations in the most scenarios.

1.11 Aims and Objectives

Despite preparing patients' skin before insertion of catheters and improvement in catheter design (such as charged catheters or catheters impregnated with antimicrobial agents), catheter-related infections continue to be a significant problem in the hospitalised patients. Elliot *et al.* (1997) studied the source of microbial contamination and suggested that the most likely source of contamination was the organisms on the patient's skin. They showed that even after the disinfection of the skin with the antiseptic, organisms were

still present in the skin of 67% of patients. Other researchers have also showed the failure of antiseptics in the elimination of entire skin microflora (Heard *et al.*, 1998; Selwyn and Ellis, 1972). This failure could be due to the existence of bacteria at other sites of the skin than just on the surface, such as pilosebaceous units, sweat ducts and skin cervices, where lipids and superficial cornified epithelium protect them and therefore they are beyond the reach of antiseptics.

These results indicate that the complete elimination of bacteria (skin sterilisation) can only be achieved by delivering the antiseptics into the skin. However, most skin antiseptics have low degree of diffusion through the skin. To overcome this problem, we can either introduce new topical antimicrobial agents with higher degree of permeation or alternatively, enhance the diffusion of available antimicrobial chemicals. The latter strategy was adapted in this study.

Taking together the high efficacy of chlorhexidine with the safety and wide use of this antiseptic in practice, it seemed appropriate to choose chlorhexidine in this study for the purpose of skin sterilisation on the insertion site and thus preventing the insertion-related infections. To evaluate the ability of this antiseptic in removing the skin microflora residing in all depth of the skin, diffusion studies was first carried out and then different techniques such as, chemical (chemical enhancers and supersaturated systems) and physical (iontophoresis) techniques were employed to enhance the diffusion of this antimicrobial agent into the areas of microbial residence.

CHAPTER TWO

GENERAL METHODS

2.1 Materials

Chlorhexidine dihydrochloride (CHX), chlorhexidine digluconate (CHD), 1-heptanoic acid sodium salt, propyl paraben, sodium bromide, potassium bromide, sodium benzoate, sodium caprylate, trypsin solution (type III from bovine pancrease) and trypsin inhibitor solution (type II from soybean) were purchased from Sigma chemicals. Diethyl ether was purchased from BDH.

HPLC grade solvents were used for the preparation of HPLC mobile phases and other chemicals were reagent grade as appropriate. Distilled water was used throughout for the preparation of solutions. Human skin was obtained from Selly Oak Hospital (Birmingham, UK) and Stephen Kirby Skin Bank (London, UK).

2.2 Equipment

2.2.1 High-Performance Liquid Chromatography (HPLC)

HPLC is a liquid chromatography method for separating molecular mixtures that depends on the differential affinities of the solutes between two immiscible phases. One of the phases is a fixed bed of large surface area (stationary phase), while the other is a fluid (mobile phase) which moves through, or over the surface of, the fixed phase. In HPLC the mobile phase is forced through the packed column under high pressure.

In this study, high-performance liquid chromatography (HPLC) analyses were performed using a Waters Chromatography station which comprised a Waters 600E model pump (Millipore), Waters 700 Satellite WISP auto-injector system (Millipore), reversed-phase column (ODS2, Jones Chromatography) and ultraviolet detector (Waters 484 Tunable Absorbance Detector, Millipore). A sensitivity of 0.001 AUFS, which was the highest sensitivity, was used during all runs. A reversed-phase HPLC system was used for analysing CHX where the stationary phase is non-polar, a hydrocarbon, and the mobile phase is polar. The mobile phase consisted of methanol-water mixture with the proportion of 75:25. Since CHX exists as a dicationic species in a neutral environment and therefore, readily forms ion pairs, the use of an ion-pairing agent with opposite charge in the mobile

phase is necessary. Sodium heptane sulphonate was used for this as it has minimal absorbance at the UV maxima of CHX, 254 nm; the wavelength at which CHX was monitored. 0.1% diethylamine was also added to the mobile phase for further reduction of peak tailing and the final pH was adjusted to 4, in which CHX exists in ionic form, with glacial acetic acid. The mobile phase was filtered to remove particulates which may clog the system and it was also degassed using sonication or sparging with helium to eliminate outgassing in the pump or detector and prevent any baseline drift (Li Wan Po and Irwin, 1980).

An internal standard is desirable because it compensates for possible errors in the injection step. Propyl paraben (PrP) was chosen as the internal standard as it could be adequately separated and detected under the HPLC conditions employed for CHX.

Peak area ratios of CHX:PrP were used for quantification of sample peak responses.

The HPLC system used may be quantified by a number of mathematical parameters which define the chromatographic performance in terms of retention and resolution. The column capacity factor, k' , a measure of the sample retention is probably the most useful separation parameter and may be calculated using the following equation:

$$k' = \frac{(t_1 - t_0)}{t_0} \quad (2.1)$$

where t_1 is the retention time of the chemical of interest and t_0 is the retention time of the unretained solute, which is the solvent in our system. For good isocratic separation, k' should be in the range of 1 to 10. Values outside this range should be avoided since small capacity factors, less than 1, indicate inadequate separation from the solvent front whilst large values of k' , greater than 10, are associated with long retention times and broadened peaks.

The number of theoretical plates, N , gives a measure of the column efficiency which is dependent on the degree of band broadening relative to the time taken to elute. N can be defined by the equation:

$$N = 16 (t_1/W_1)^2 \quad (2.2)$$

Where W_1 is the base width of the first peak. N is usually expressed per meter of column length (derived by dividing the above equation by column length in meter) and for typical systems, should be in the range of 2500 to 10000. Columns with large N values will produce narrow peaks and better resolution than those with lower N values.

The efficiency may also be expressed as the height equivalent of a theoretical plate, H :

$$H = \frac{L}{N} \quad (2.3)$$

where L is the column length in μm . Values of H should lie between 25 and 100 μm .

The resolution, R_s , of a system is a measure of the efficiency of the separation of different components and may be represented by the equation:

$$R_s = \frac{2 (t_2 - t_1)}{(W_1 + W_2)} \quad (2.4)$$

where W_2 is the base width of the second peak. An R_s value of 1 indicates a satisfactory separation with $\approx 2\%$ overlap, whilst a value of 1.5 represents almost total separation.

In this study, optimised chromatographic parameters were: mobile phase 0.005M sodium heptane sulphonate, 0.1% diethylamine, glacial acetic acid for pH adjustment (pH=4), in methanol-water (75:25); flow-rate 1.2 ml/min.; detector wavelength 254 nm; sensitivity 0.001 AUFS. An ODS2 column (octadecylsilane, C_{18}) was selected as it has a high carbon loading which will result in a greater degree of endcapping and consequently, less peak-tailing. Quantitation was thus achieved via the use of a calibration curve. A stock solution of CHX and a set of standards (10^{-7} – 4×10^{-5} g/ml) by serial dilution were used. For this concentration range, the injection of a 100 μl sample gave a linear response (regression equation $y = 0.07067x - 0.0231$, correlation coefficient = 0.999). An example

of a typical chromatogram is illustrated in figure 2.1. Values of t_1 , t_2 , k' , N , H and R_s , quantifying the chromatographic efficiency of the employed system, are summarised in table 2.1.

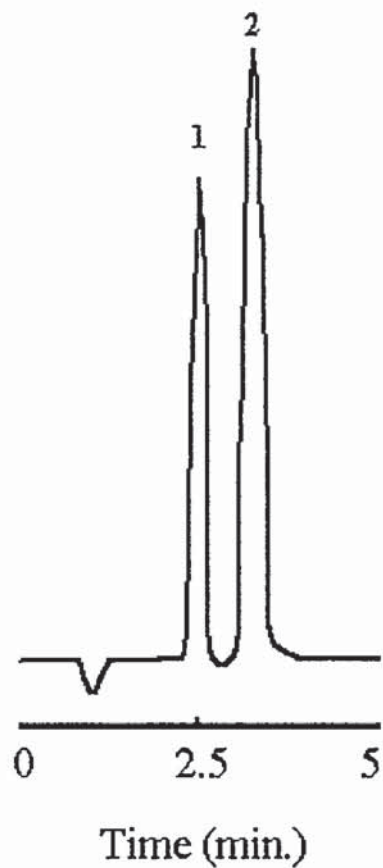


Figure 2.1 Example of a typical chlorhexidine dihydrochloride chromatogram (peak 2) with propyl paraben (peak 1) as internal standard.

t_1 (min)	t_2 (min)	k'	$N(m^{-1})$	$H(\mu m)$	R_s
2.5	3.4	1.5	4166.66	36	1.8

Table 2.1 HPLC parameters of CHX

2.2.2 Diffusion Cells

Different types of diffusion cells are available. Basic variations include orientation (horizontal and vertical) and receptor compartments (static or flow-through). Coldman *et al.* (1969) designed the first vertical cell and other workers modified this design later (Gummer *et al.*, 1987; Chien and Valia, 1984; Chowhan and Prichard, 1978). Vertical cells comprise two chambers, the top one (donor) holds the solution under investigation and the bottom part contains the receptor solution that can be a buffer solution or just water. The receptor compartment has a sampling port for taking samples for analysis and is continuously agitated using a magnetic stirrer bar and is maintained at a constant temperature *via* a water jacket. The membrane is mounted horizontally between two halves of the cell. This kind of orientation of the membrane means that the donor chamber may be exposed to ambient temperature and humidity, while the lower chamber can be maintained at physiological temperature, thus mimicking *in vivo* conditions.

In horizontal diffusion cells, the membrane is placed in the vertical position and they contain similar sized donor and receptor compartments. In this cell type, the donor solution can be stirred to ensure the homogeneity of the formulation under investigation. However, a large volume of donor solution is required to cover the membrane surface completely. Samples of vertical and horizontal cells are illustrated in figure 2.2 and 2.3 respectively.

The degree of agitation is one of the most important aspects of *in vitro* diffusion cells. It must be sufficient enough to minimize the diffusion boundary layers at the interfaces between the donor and receptor phase and the absorption barrier. In comparative work, the rate of agitation should be monitored and maintained at a uniform rate to eliminate boundary layer effects (Ackerman and Flynn, 1987; Sato and Win Kim, 1984).

Finite and infinite dose of dermatological products can be used in permeation studies. A delivery system that employs constant delivery may justify infinite dose, however, this type of experiment has little in common with the delivery of drugs from topical applications. To more adequately depict true clinical situations, finite doses have been used which can reasonably reflect the usage situation. To test the usefulness of the finite

dose, Franz (1975) studied the *in vitro* diffusion of various compounds from thin solid deposited through human skin. Data obtained were compared with those previously obtained in *in vivo*. Good agreement was reported between the two sets of data.

In this study, vertical cells (Franz cells) with an average diffusional surface area of 1.65 cm^2 and receptor volumes of approximately 29 ml were used for passive diffusion studies while horizontal ones with an average diffusional area of 1.7 cm^2 and receptor volumes of 10 ml were used in iontophoresis studies.

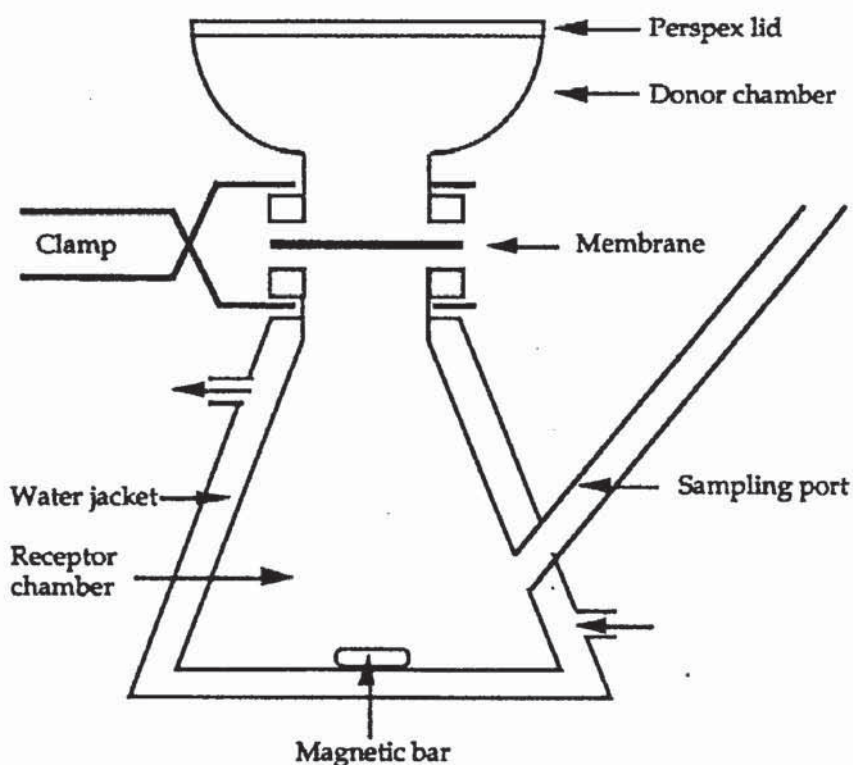


Figure 2.2 Schematic representation of a vertical Diffusion cell

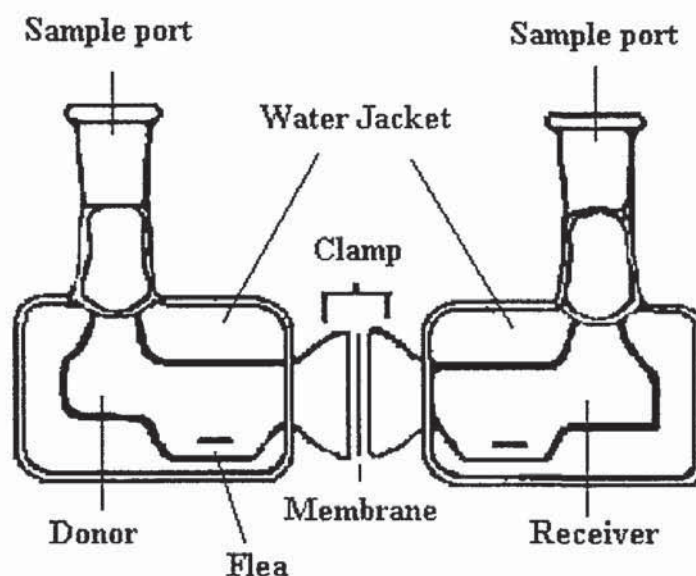


Figure 2.3 Schematic representation of a horizontal diffusion cell

2.2.3 Differential Scanning Calorimetry (DSC)

DSC has traditionally been used to study the thermal transition in crystalline solids such as melting, dehydration, complexation and polymorphic changes (Otero-Espinar *et al.*, 1992). However, it is now being employed to study the structure and function of proteins, lipids, and water in a variety of biologically relevant barriers and can provide important information on structural alterations within the sample. It is a differential technique in which sample is placed in the sample cell and the reference cell is empty. The energy difference between two cells maintained at a constant temperature is then measured. If the sample undergoes a thermally induced transition, heat must be applied to or withdrawn from the sample in order to maintain the same temperature in both sample and reference compartments. The instrument measures the heat flow into the sample relative to the reference and this differential heat flow is recorded as a function of temperature.

A hypothetical DSC thermogram is shown in figure 2.4. As a sample is heated, its integral energy increases, with a resultant increase in the vibrational and rotational motion of the molecular constituents. The temperature of the sample is raised proportionately, with the magnitude of the increase being governed by the heat capacity of the sample at that temperature. Hence, if the internal energy of the sample is plotted against temperature, then the slope of the curve at any temperature provides the heat capacity (C_p):

$$C_p = \frac{1}{m} \times \frac{\partial H}{\partial T} \quad (2.5)$$

where m is the mass of substance being heated, H is the heat content, and T is the temperatures. That is, an infinitesimal change in integral energy brings about an infinitesimal change in temperature, and the constant of proportionality is the heat capacity. Heat capacities vary with temperature; however, since the variation is quite small over narrow temperature ranges, they are treated as almost independent of temperature, being defined as the amount of heat (per gram or per mole) required to raise the temperature of the sample by 1°C (or K). Subsequent to appropriate calibration, the rate of heat input into the sample ($\text{J min}^{-1}\text{g}^{-1}$) is measured by the scanning calorimeter and, given the scanning rate ($^{\circ}\text{C min}^{-1}$); the parameter recorded is usually C_p ($\text{J}^{\circ}\text{C}^{-1}\text{g}^{-1}$) as a function of temperature. The phase transition temperature, T_m , represents the temperature at which the heat capacity, and consequently the excess specific heat absorbed by the system, reaches a maximum. This heat is required by the molecule to execute a conformational change.

In this study, DSC (Perkin-Elmer DSC-4) was employed to examine the thermal behaviour of human stratum corneum. Human epidermis was prepared by the heat separation method described in section 1.6 with a slight modification (the immersion time was reduced from 2 minutes to 45 seconds). The epidermis was then spread flat on filter paper soaked in 0.1% trypsin solution and stored in a refrigerator at 4°C overnight. Skin was removed and placed in an oven at 37°C for an hour after which the SC was teased away from the remaining underlying epidermis carefully. Each SC sheet was then washed

in an aqueous 0.1% trypsin inhibitor solution (type II) by gentle shaking for 30 seconds. Sheets of SC were subsequently rinsed in double distilled water and left to dry at room temperature. Dried SC samples were hydrated (50% w/w) again since dry samples do not exhibit all of the peaks (Goodman and Barry, 1985). Hydrated samples (approx 6 mg) were placed into hermetically sealed pans to prevent interference from water at 100°C. The pan was placed in the DSC oven together with a blank. The blank was prepared in the same way but did not contain the sample. The sample and blank pans were continuously purged with nitrogen gas and scanned between 0-110°C at 5°C min⁻¹.

The Thermal Analysis Data Station (TADS) was used for data collection, handling and presentation. Initial temperature calibration was made with an indium standard which had an onset temperature of 156.9°C.

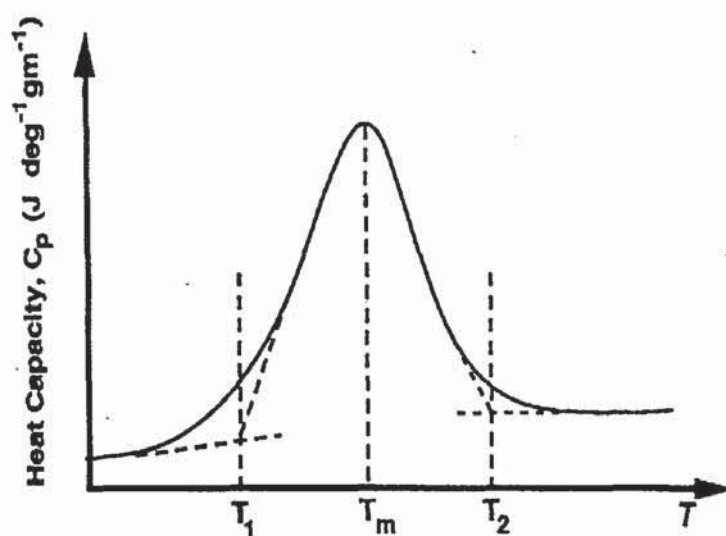


Figure 2.4 A hypothetical DSC profile for an endothermic transition
(T_m is the transition midpoint)

2.3 Experiments

2.3.1 Solubility determination

Solubility of chlorhexidine dihydrochloride (CHX) was determined in different solvents (distilled water, 10% ethanol aqueous solution, and 20% ethanol aqueous solution) as follows:

An excess of chlorhexidine hydrochloride (20 mg) was suspended in 20 ml of the solvent at room temperature ($22\pm1^{\circ}\text{C}$). These suspensions were stirred for 3 days to equilibrate. An aliquot of supernatant fluid was filtered through a pre-saturated 0.2 μm membrane filter (GA-8S, Gelman Sciences). 1 ml of the filtrate was diluted 100-fold in a 100 ml volumetric flask using distilled water. 100 μl of the diluted solution was then injected to HPLC and analysed as discussed in section 2.2.1.

To investigate the effect of pH on solubility of the CHX, 20 ml phosphate buffer over the pH range of 3-7.4 (Appendix 1) was employed as the solvent and solubility studies were carried out using the above procedure. Any pH adjustment required was performed using 1 M orthophosphoric acid or 1 M sodium hydroxide.

All solubility determinations were performed in triplicate.

2.3.2 Partition Coefficient

2.3.2.1 n-Octanol/Water Partition Coefficient

The octanol-water partition coefficient of chlorhexidine hydrochloride was determined at room temperature ($22\pm1^{\circ}\text{C}$). Organic phase was pre-saturated with water by shaking over night in a thermostatic water bath at 25°C . The water was pre-saturated with octanol in the same manner. Stock solution of CHX with 4×10^{-5} g/ml concentration was prepared in pre-saturated water. Equal volumes of each phase (5ml) were placed into tubes. Tubes were shaken for 3 days at room temperature. Samples of the aqueous phase were removed, centrifuged to remove any residual organic phase. 1 ml of the centrifuged solution was then diluted 50-fold with distilled water and 100 μl of it was analysed by HPLC as discussed in section 2.2.1. The concentration in the organic phase was obtained by the difference between the initial and final drug concentration ($C_{\text{org}} = C_{\text{initial}} - C_{\text{aq}}$).

Estimating the partition coefficient in advance of the determination is helpful as it allows a better estimation of solvents to be employed. For instance, if large volumes of nonpolar solvent are used with very lipophilic molecules, there will not be sufficient material left in the aqueous phase for analysis. Therefore, to overcome this problem, as there was not any reported partition coefficient value for CHX, solutions with different concentrations of CHX were used by diluting the stock solution (4×10^{-5} to 4×10^{-6} g/ml). Furthermore, since there was the possibility that adsorption to glass may occur, controls (same procedure except no organic phase, octanol, was present) for each experiment were performed and all the determination were run in triplicate.

The partition coefficient (P) between two phases was calculated using the following equation:

$$P = \frac{C_{org}}{C_{aq}} \quad (2.6)$$

Where C_{org} and C_{aq} are the solute concentration in organic and aqueous phases after partitioning respectively.

2.3.2.2 Vehicle/Membrane Partition Coefficient

The membrane-vehicle partition studies were conducted with intact human epidermis. Human skin was obtained as described in section 2.1. Epidermal membranes were prepared by the heat separation technique as discussed in section 1.6. Segments of skin were then blotted dry on filter paper and accurately weighed (150-300 mg) into screw-cap glass vials. Known volumes (3 ml) of aqueous solutions of chlorhexidine dihydrochloride (concentration range of 1×10^{-3} - 4×10^{-1} mg/ml) were placed in the same vials. The contents were gently mixed and maintained at 37°C in a water-bath for equilibration (3 days). Samples of the aqueous layer were then analysed for CHX concentration by HPLC. The membrane-vehicle partition coefficient of CHX was calculated using the following equation:

$$\frac{(C_1 - C_2) \cdot W_{aq}}{C_2 \cdot W_{skin}} \quad (2.7)$$

where C_1 is the initial CHX concentration, C_2 is CHX concentration after 3 days, W_{aq} is the weight of the aqueous phase (mg), and W_{skin} is the weight of the skin (mg).

All determinations were performed in triplicate and controls (without skin) included to assess any CHX loss due to glass uptake.

2.3.3 Preparation of Chlorhexidine Salts

2.3.3.1 Dihalide Salts of Chlorhexidine

Solutions of 10% v/v Chlorhexidine digluconate (CHD) were prepared by adding 10 ml of CHD to 100 ml distilled water in a volumetric flask. 10 ml of this solution was placed in glass vials. 0.3724 g of potassium iodide and 0.23079 g sodium bromide were then dissolved in 1 ml distilled water separately and added to each vial. The vials were shaken gently to ensure the complete mixing and left to stand for few minutes. The formed precipitates were transferred to centrifuge tubes and spun at 2500 rpm in the centrifuge for 30 minutes. The supernatants were then discarded and the precipitates were placed on the watch glass and dried overnight in a vacuum oven at 37°C. The dried precipitates were collected and stored in a desicator for the future experiments.

Solubility of the chlorhexidine di-halide salts was determined using the procedure described in section 2.3.1.

2.3.3.2 Benzoyl and Caprylate Salts of Chlorhexidine

Benzoyl and caprylate salts of chlorhexidine were prepared employing the same procedure described in section 2.3.3.1 except, sodium caprylate (0.3728 g) and sodium

benzoate (0.27386 g) were dissolved in 2 ml distilled water respectively and then added to glass vials containing 10 ml of 10% CHD solutions.

Solubility of the above chlorhexidine salts was also determined as described in section 2.3.1.

2.3.4 Permeation Studies

The diffusion studies were carried out using Franz-type diffusion cells. The membrane, (human epidermis) was mounted between the two halves of the diffusion cells and white soft paraffin and parafilm were used to help produce a seal between the membrane and the two compartments. Distilled water was used as the receptor phase and was heated to expel dissolved gases and then cooled prior to use. In order to prevent evaporation of the receptor solution, the sampling port was sealed. The membrane was allowed to equilibrate with the receptor solution for 1 hour. Air bubbles were carefully removed by tipping the cell. A teflon-coated stirring bar was used in the receptor chamber to provide efficient mixing. Each cell was then placed over a magnetic stirrer. The receptor cell was maintained at 37°C by a thermostatic water-pump which circulated water through a jacket surrounding the cell body. This resulted in donor phase and the membrane surface temperature being held at 32±1°C. Sufficient time was also allowed for temperature equilibration. Then 1 ml of drug solutions were pipetted into the donor chamber and, at appropriate intervals, samples of the receptor phase (1 ml) were removed and assayed by HPLC as discussed in section 2.2.1 (no dilution was performed as the CHX concentrations were low). The receptor solution was replenished with an aliquot of distilled water after removing each sample which resulted in dilution of the receptor phase. Therefore, the successive sample concentration was corrected to represent cumulative drug amount permeated for each sampling time by applying the following factor:

$$C_t = C_{mt} + V_s \frac{\sum_{t=1}^{t=n-1} C_m}{V_d}$$

where C_t is the true concentration of drug in the receiver phase at time t , C_{mt} is the current measured concentration of the drug in the receiver chamber, V_s is the volume of the sample removed for analysis, V_d is the volume of the receiver chamber, and ΣC_m is the summed total of the previous measured concentrations ($t = 1$ to $t = n-1$). This correction factor was applied to experimental data using a BASIC computer program written by Irwin W.J.

The permeation studies were performed over 60 minutes or less. The preliminary permeation studies of CHX across the epidermis performed over 8 hours showed no difference to the ones performed over shorter periods of time (60 min or less). The concentration of CHX in the receptor phase did not increase after 60 minutes (up to 8 h), therefore it seemed appropriate to carry the studies only for 1 hour. Moreover, the shorter experiment time reflected the *in vivo* situation more realistically as in practice; antimicrobial agents are left on the patients' skin only for few minutes. The cumulative amount of solute penetrated was then plotted versus time and the pseudo-steady-state flux was determined from the linear regression of the straight-line portion of this plot. The permeability coefficient was calculated by dividing psseudo-steady-state flux by initial donor concentration.

2.4 Results and Discussion

2.4.1 Solubility Studies

2.4.1.1 Solubility of Chlorhexidine dihydrochloride in different vehicles

Solubility determinations were performed for those solvent systems employed during the penetration experiments. The solubility of CHX was determined in (a) distilled water, (b) 10% ethanol and (c) 20% ethanol. The selection of ethanol as a cosolvent was due to the fact that ethanol itself has antibacterial properties (Maki *et al.*, 1991; Moeton, 1983) therefore combined use of ethanol and CHX can show a better antimicrobial activity (Rotter and Koller, 1990). Furthermore, in practice the alcoholic solutions of chlorhexidine are being used for the purpose of disinfecting the skin. Therefore, it seemed appropriate to choose ethanol as cosolvent.

Solubility studies of CHX in the higher concentrations of ethanol as the solvent were not performed since topical application of higher concentrations of ethanol have been reported to have a drying effect on skin (Larson *et al.*, 1991).

The mean saturated solubility values of CHX in these systems are summarised in table 2.2. A value of 0.78 ± 0.038 mg/ml was obtained for CHX solubility in distilled water which is higher than the reported value in literatures (0.06% w/v at 20°C). This can be due to the higher temperature at which the experiment was carried out ($22 \pm 1^\circ\text{C}$). As the dissolution process of CHX is endomethric (Chauhan, 1995), which means heat is absorbed when dissolution occurs, a rise in temperature will lead to an increase in the solubility.

Solubility of CHX was greater in ethanol than distilled water and an increase in CHX solubility was seen with increasing the concentration of ethanol from 10% to 20%. Heard and Ashworth (1968) also studied the effect of ethanol on the solubility of another salt of chlorhexidine, acetate, and found that increasing the percentage of ethanol in the solvent would result in an increase in the solubility of chlorhexidine.

	Distilled Water	10% Ethanol	20% Ethanol
CHX (mg/ml)	0.78±0.038	0.88±0.049	1.23±0.085

Table 2.2 Saturated solubility of CHX in different solvents at 22±1°C

2.4.1.2 Solubility of Chlorhexidine Salts

Hydrochloride salt of chlorhexidine has a poor solubility in distilled water. Although a 1.6-fold increase in CHX solubility was observed upon introducing ethanol (10-20%) as a cosolvent to the system, the solubility enhancement was not great enough. Therefore, other salts of chlorhexidine such as iodide, bromide, benzoate and caprylate were prepared and their solubilities were determined as discussed in sections 2.3.1.

The mean saturated solubility values of different salts of chlorhexidine are shown in table 2.3. Bromide, benzoate and caprylate salts showed similar solubilities, which were higher than the hydrochloride salt. The iodide salt had the highest solubility (7.20±0.056 mg/ml) among all the other prepared salts. Solubilities of commercially available Chlorhexidine digluconate has been reported as >70% w/v at 20°C in literature (Senior, 1973). From the results, it can be concluded that the dihydrochloride is the least hydrophilic (more lipophilic) salt. Hence, using this salt in our studies would be more appropriate due to the fact that lipophilicity can affect the percutaneous permeation of compounds.

Salt	Hydrochloride	Bromide	Iodide	Benzoate	Caprylate
Solubility (mg/ml)	0.78 ±0.038	2.90±0.037	7.20±0.056	2.64±0.029	2.90±0.022

Table 2.3 Saturated solubility of chlorhexidine salts in distilled water at 22±1°C

2.4.1.3 Effect of pH on the solubility of Chlorhexidine Dihydrochloride

Depending on the pH of the vehicle, a molecule can exist as an equilibrium mixture of ionised and unionised species in the system. Since it is generally believed that only the unionised molecules partition into lipid membranes, the effect of vehicle pH on the ionisation of CHX was studied. The relationship between solubility and pH can be derived from the Henderson-Hasselbach equation and for a weak base such as CHX this can be defined as:

$$pH = pK_a + \log\left(\frac{S_0}{S - S_0}\right) \quad (2.8)$$

Where S is the overall solubility of the chemical and S_0 is the solubility of the unionized species. This equation can be rearranged to:

$$S = S_0 \left(\frac{(H_3O^+) + K_a}{K_a} \right) \quad (2.9)$$

$$S = S_0 \left(\frac{(H_3O^+)}{K_a} + 1 \right) \quad (2.10)$$

Multiplying out gives:

$$S = S_0 + \frac{S_0(H_3O^+)}{K_a} \quad (2.11)$$

Therefore, the plot of solubility (S) versus (H_3O^+) should give a straight line with a gradient of S_0/K_a and an intercept of S_0 .

Saturated solubilities of CHX in aqueous buffer at $22\pm 1^\circ\text{C}$ are given in table 2.4. The degree of dissolution in this case will be determined by the fraction of ionised species in the system, with the anionic form being more soluble. The results of the solubility studies show a decrease in CHX solubility upon increasing the vehicle pH. This effect is unlikely to be related to the percentage of CHX ionisation as for a basic molecule such as CHX with pK_a values of 2.2 and 10.3, a rise in pH (up to the value of 7) does not affect the percentage of ionisation (table 2.5). The decrease in CHX solubility upon increasing the vehicle can be explained by the fact that in phosphate buffer systems, depending on the concentration of phosphate, another salt of chlorhexidine (chlorhexidine phosphate) which is less soluble will be formed (Martindale, 1999). That is why at higher pH, where more phosphate is present in the system, a lower solubility was observed. Furthermore, for the same reason, in our study, the plot of solubility versus (H_3O^+) concentration did not give a straight line as the solubility of CHX was decreased by increasing the amount of phosphate.

pH	Solubility (mg/ml)
3.0	0.741 ± 0.034
4.9	0.525 ± 0.069
5.8	0.299 ± 0.008
6.4	0.106 ± 0.008
7.0	0.054 ± 0.002

Table 2.4 Saturated solubility of chlorhexidine dihydrochloride in phosphate buffer pH 3-7

pH	Percentage of ionisation (%)	
	CHX ²⁺ (pK ₁ =10.3)	CHX ⁴⁺ (pK ₂ =2.2)
3	87.44	
4.9	99.75	0.00
5.8	99.97	0.00
6.4	99.98	0.01
7	99.95	0.05

Table 2.5 Percentage of ionisation of chlorhexidine dihydrochloride at different pH

2.4.2 Partition coefficient

Partition coefficient of the permeant is one of the main characteristics which influences its absorption. Due to the existence of lipid in the SC, which is believed to be the main barrier to the permeation process of chemicals, a penetrant must be able to partition into lipid regions. Hence, determination of partition coefficient of a chemical can give an estimate of its transport across the skin.

Partition coefficients for CHX were determined between octanol/water at room temperature (22±1°C) and between human epidermis and vehicle (distilled water) at 37°C. No loss of CHX was observed in the control vials indicating that glass vials did not uptake any CHX. Therefore, any loss of CHX in the aqueous phase can be considered as the amount partitioned into the organic phase or into the membrane.

Using equation 2.2, an octanol/water logP value of 0.798±0.12 was calculated for CHX. From this value one would expect a poor percutaneous absorption for this molecule as Hadgraft and Walters (1995) have suggested that in order to achieve the optimal permeability, the penetrant should possess an octanol/water logP value of 2.

Using equation 2.7, the vehicle/human epidermis partition coefficient determinations showed a logP value of 0.98 ± 0.068 . However, the obtained value does not reflect the real amount partitioned into the skin. This is because the amount taken up by the skin was not measured directly and was only estimated from the loss of CHX in the aqueous phase. Hence, the drug bound to the SC components, which does not contribute to the permeation process, has also been counted in the apparent partition coefficient. Considering the cationic groups in the CHX molecule (section 1.9.4), the possibility of CHX being bound to the skin, which carries a negative charge (Sims *et al.*, 1991; Wearley and Chien, 1990), is high. Hence, the actual log P value of CHX between vehicle and membrane is somewhat lower than the value obtained.

2.4.3 Permeation Studies

2.4.3.1 Permeation of Chlorhexidine Salts through Human Epidermis

Permeation studies can provide information about the ability of a system to diffuse into or across the skin. As the aim of this study was delivering the antiseptic (CHX) to the bacterial reservoir in the different depths of the skin, permeation studies were carried out and the results were used as guides for optimising the flux of chlorhexidine. Three different salts of chlorhexidine were used as each salt might exhibit a different permeation profile due to their different lipophilicity.

Saturated solutions of chlorhexidine salts were prepared using the procedure described in section 2.3.1 and were used as the donor solutions (pH=6.). The *in vitro* permeation of saturated solutions of hydrochloride, bromide and iodide chlorhexidine salts across the skin were studied and the results are depicted in figure 2.5. An appearance time of about 10 minutes was observed for the hydrochloride salt. This indicates that, if this model relates to the clinical situation, in order to eliminate the bacteria in the deeper layers of the skin, this antiseptic should probably be left on the skin for a minimum of 10 minutes. However, even after this period, the concentration of CHX may not be high enough to eradicate all the bacteria in the underlying layers of the skin. The appearance time was reduced by using iodide and bromide salts of chlorhexidine. Pseudo-steady-state fluxes and permeability coefficients derived from the linear sections of the permeation profiles

are summarised in table 2.6. Although the concentration of saturated solution of chlorhexidine iodide was higher than the bromide salt (7.20 ± 0.056 mg/ml and 2.90 ± 0.037 mg/ml, respectively), these two salts showed similar fluxes (value of $1.13 \times 10^{-4} \pm 7.86 \times 10^{-6}$ mg cm⁻² min⁻¹ for the iodide salt and $1.03 \times 10^{-4} \pm 6.08 \times 10^{-6}$ mg cm⁻² min⁻¹ for the bromide salt). The hydrochloride salt showed the lowest flux ($5.98 \times 10^{-5} \pm 9.38 \times 10^{-6}$ mg cm⁻² min⁻¹). This might be due to the different lipophilicity of these salts.

The permeation coefficient for each salt was calculated by dividing the flux by the donor concentration. The iodide salt had a lower permeability coefficient than the bromide salt, which itself was lower than the hydrochloride salt.

The permeation profiles tend to plateau within 30 minutes, indicating that equilibrium has been reached. Comparing the donor concentration with the amount penetrated across the skin within 30 minutes, one would expect that the equilibrium condition to be reached in a later time. However, HPLC analysis of the donor samples taken at 30 minutes showed a lower concentration of chlorhexidine than expected in the donor phase (value of 0.297 ± 0.087 mg/ml for the hydrochloride salt, 1.9 ± 0.187 mg/ml for the bromide salt and 3.71 ± 0.125 mg/ml for the iodide salt). This could be due to the binding of chlorhexidine to the skin (Ranganathan, 1996; Larson, 1995). Donor solutions had a pH of 6, and chlorhexidine with pK_a of 2.2 and 10.3 exists as a dicationic species in this pH. Moreover, human skin carries a negative charge at biological pH (5.5-7). Therefore, chlorhexidine can bind to the negative sites of the skin and, consequently, reduce the amount of free drug available for the permeation process.

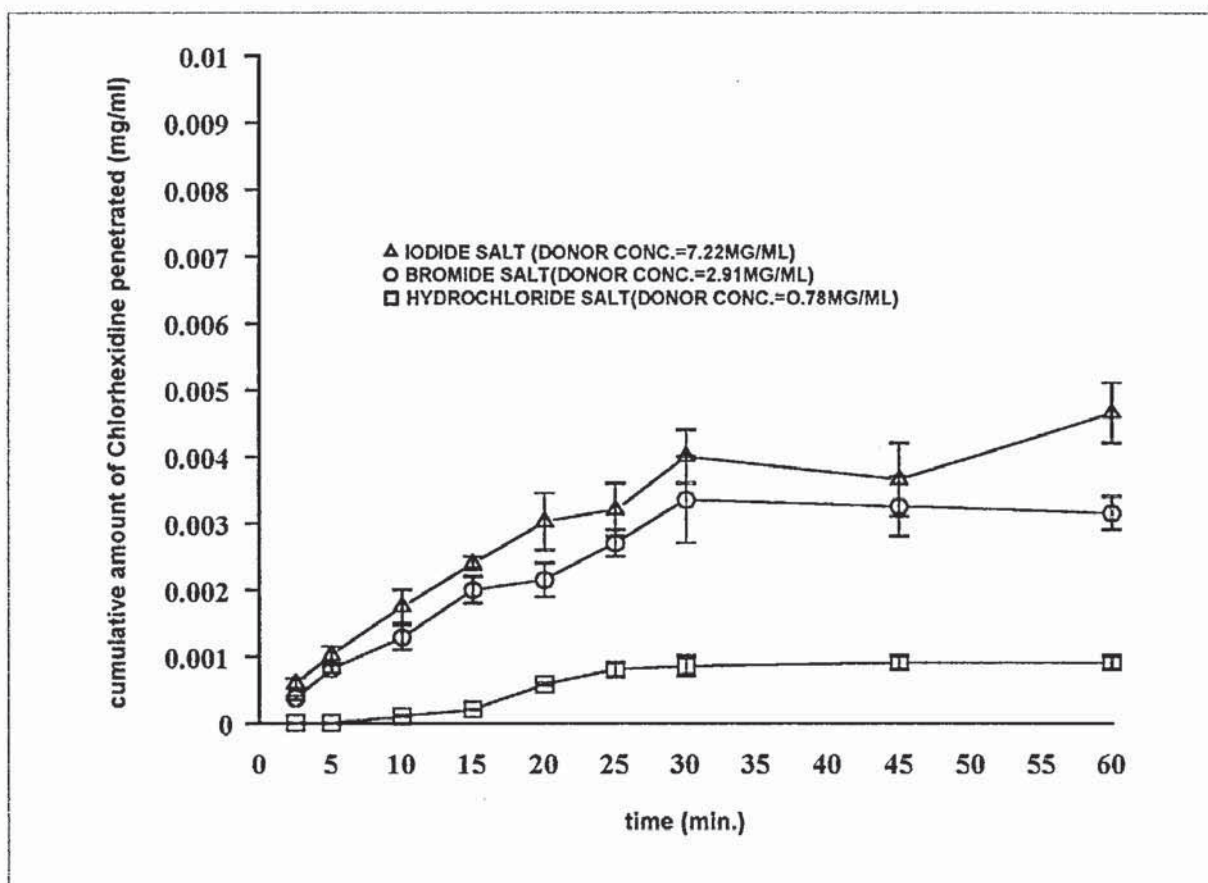


Figure 2.5 Permeation profile of chlorhexidine salts through arm epidermis
(Each point is the mean \pm sem of six determinations, n=6)

Chlorhexidine salts	Flux ($\text{mgcm}^{-2}\text{min}^{-1}$)	Permeability coefficient (cmmin^{-1})
Hydrochloride	5.98×10^{-5} (9.38×10^{-6})	7.67×10^{-5} (0.126×10^{-6})
Bromide	1.03×10^{-4} (6.08×10^{-6})	3.54×10^{-5} (2.14×10^{-6})
Iodide	1.13×10^{-4} (7.86×10^{-6})	1.57×10^{-5} (1.10×10^{-6})

Table 2.6 Permeation data for different salts of chlorhexidine across arm epidermis
(values in brackets represent the standard error, n=6)

2.4.3.2 Effect of Vehicle on the Permeation of CHX through Human Epidermis

A general approach to influence permeation rate is to incorporate solvents that may have two modes of action. One is to favour a high drug concentration in the stratum corneum which leads to an increase in the thermodynamic activity of the drug in the vehicle and thereby promotes the interfacial drug transfer into the SC (Higuchi, 1960,1978). The other is to include solvents that penetrate the SC themselves and in this way alter the barrier properties of the SC (Chandrasekaran and Shaw, 1978). Ethanol is a solvent that has been used extensively for this purpose. Since the permeation studies (section 2.4.3.1) showed a poor diffusion rate for CHX across the skin, it seemed appropriate to incorporate this solvent in the system to enhance the permeation of the CHX. Hence, saturated solutions of CHX in 10% and 20% ethanol were prepared in the same manner as discussed in section 2.3.1 and their permeation across the human epidermis were assessed over 60 minutes. The pH of the donor solutions was determined using a pH-meter which showed a value of 6. CHX saturated solutions in distilled water were used as the donor solutions in control diffusion cells.

The permeation profiles of saturated solutions of CHX in distilled water, 10% and 20% ethanol are illustrated in figure 2.6. Introducing 10% ethanol to the system resulted in 2.4-fold increase in CHX flux across the skin compared to the system containing no ethanol. The flux was further enhanced to $2.42 \times 10^{-4} \pm 2.55 \times 10^{-5} \text{ mg cm}^{-2}\text{min}^{-1}$ upon increasing the concentration of ethanol to 20% v/v. The permeation data are summarised in table 2.7.

Although, in ideal conditions, the flux from a saturated solution should be constant irrespective of the vehicle used (Iervolino *et al.*, 2000), an enhanced CHX flux which was proportional to the concentration of ethanol was observed in systems containing ethanol. Variations from this ideal behaviour are attributed to vehicle-membrane interactions (Pellet *et al.*, 1994; Twist and Zatz, 1986).

The higher flux seen with ethanolic solutions of CHX could be due to the higher concentration of CHX in ethanol which causes an increase in the concentration gradient across the skin. According to Fick's first law (equation 1.1), the flux is proportional to the

concentration gradient therefore, by increasing the concentration gradient an enhanced diffusion would be expected.

Another explanation for the increased flux of CHX could be the effect of vehicle (ethanol) on the barrier properties of the SC. Several researchers have shown the ability of ethanol to increase the penetration of chemicals across the skin (Yum *et al.*, 1994; Pershing *et al.*, 1990). The proposed mechanism of action was the ability of ethanol to alter the structure of the skin, such as extracting SC lipids and osmotic expansions, in ways that appreciably increase solute diffusivity. This effect of ethanol will be discussed in more detail in chapter 3 where ethanol is used as a penetration enhancer.

A third explanation for the enhanced flux of chemical upon employing the ethanol as the cosolvent has been suggested by several published studies (Pershing *et al.*, 1990; Berner *et al.*, 1989). They suggest that the enhanced partition of solutes into the SC is the primary mode of action of dilute ethanol. Yum *et al.* (1994) studied the contributions of increased drug solubility and diffusivity for enhancing flux of estradiol, propranolol and fentanyl across the skin by employing ethanol as a cosolvent. They resulted that the solubilities and the cumulative amounts of drugs penetrated through the skin were enhanced by increasing the concentration of ethanol.

Like permeation studies of different salts of chlorhexidine (section 2.4.3.1), the permeation profiles plateau within 30 minutes indicating the attainment of equilibrium condition. The same explanation as in section 2.4.3.1 may also be appropriate here. No samples from the donor solutions were analysed here as the strong affinity of CHX for binding to negative sites of the skin has been well documented (Ranganathan, 1996; Larson, 1995).

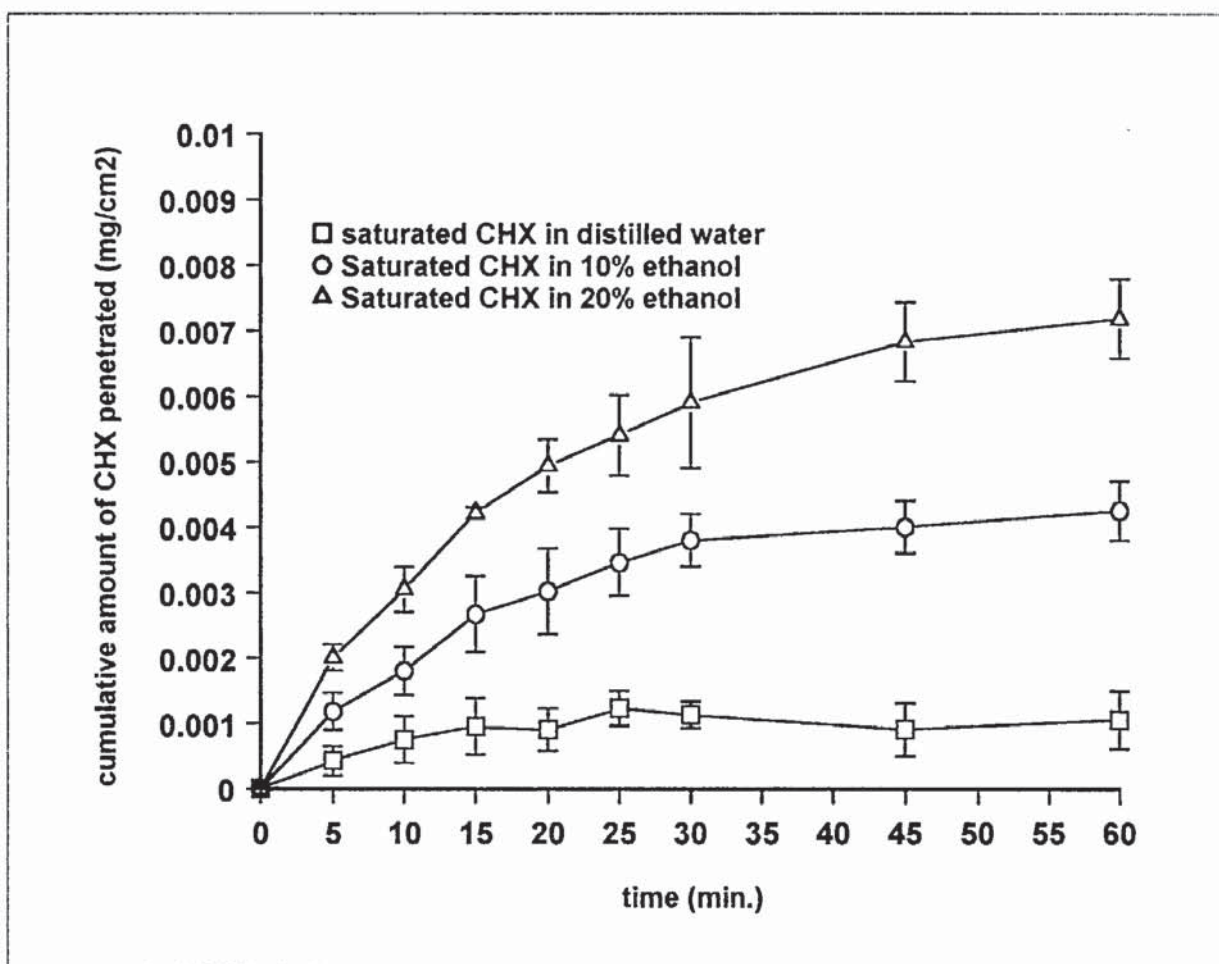


Figure 2.6 Effect of vehicle on the permeation of CHX through breast epidermis
(Each point is the mean \pm sem of six determinations, n=6)

Vehicle	Flux ($\text{mg cm}^{-2} \text{min}^{-1}$)	Permeability coefficient (cmmin^{-1})
Distilled Water	6.35×10^{-5} (7.12×10^{-6})	8.14×10^{-5} (9.96×10^{-6})
10% Ethanol	1.51×10^{-4} (1.54×10^{-5})	1.72×10^{-4} (1.99×10^{-5})
20% Ethanol	2.42×10^{-4} (2.55×10^{-5})	1.97×10^{-4} (2.5×10^{-5})

Table 2.7 Permeation data for effect of vehicle on CHX flux across breast epidermis (Values in brackets represent the standard error, n=6)

2.4.3.3 Effect of pH on Permeation of Saturated CHX through Human Epidermis

The permeation profiles demonstrating the effect of pH on the penetration of CHX across human epidermis are recorded in figure 2.8. CHX transport was accelerated by increasing the pH. At pH=4.9, a flux of $2.94 \times 10^{-5} \pm 2.97 \times 10^{-6} \text{ mgcm}^{-2}\text{min}^{-1}$ was observed for CHX which was further enhanced to $8.03 \times 10^{-5} \pm 2.98 \times 10^{-6} \text{ mgcm}^{-2}\text{min}^{-1}$, $3.92 \times 10^{-4} \pm 4.29 \times 10^{-5} \text{ mgcm}^{-2}\text{min}^{-1}$ and $7.80 \times 10^{-4} \pm 7.55 \times 10^{-5} \text{ mgcm}^{-2}\text{min}^{-1}$ by increasing the pH to 5.8, 6.4 and 7, respectively. Permeability coefficients (k_p) were also enhanced as the vehicle pH increased. At pH=4.9, k_p was $3.77 \times 10^{-5} \pm 2.98 \times 10^{-6} \text{ cm min}^{-1}$ which was promoted to $10^{-3} \pm 1.11 \times 10^{-4} \text{ cm min}^{-1}$ by increasing the vehicle pH to 7, indicating a 26.5-fold increase. Steady-state fluxes and permeability coefficients derived from the linear part of these plots are summarised in table 2.8.

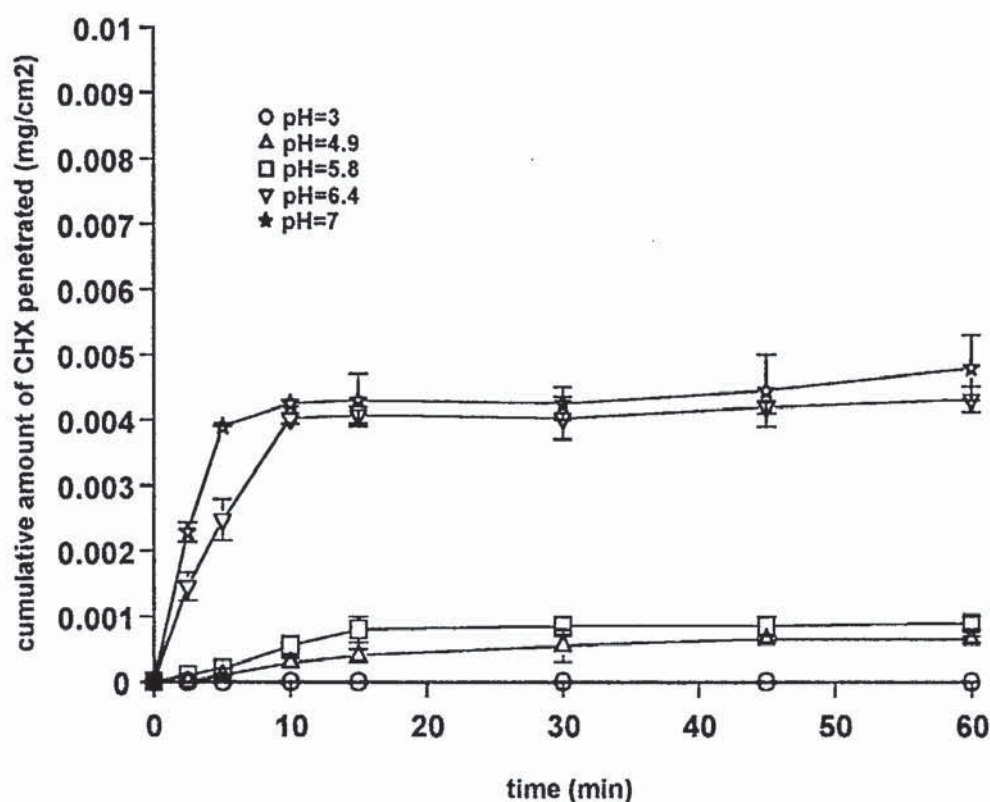


Figure 2.7 The effect of pH upon the permeation of CHX through human epidermis.
(Each points is the mean \pm sem of six determinations)

pH	Flux (mg cm ⁻² min ⁻¹)	k _p (cm min ⁻¹)
3	*	*
4.9	2.94×10 ⁻⁵ (2.97×10 ⁻⁶)	3.77×10 ⁻⁵ (4.23×10 ⁻⁶)
5.8	8.03×10 ⁻⁵ (2.98×10 ⁻⁶)	1.03×10 ⁻⁴ (6.31×10 ⁻⁶)
6.4	3.92×10 ⁻⁴ (4.29×10 ⁻⁵)	5.03×10 ⁻⁴ (6.02×10 ⁻⁵)
7	7.80×10 ⁻⁴ (7.75×10 ⁻⁵)	1.00×10 ⁻³ (1.11×10 ⁻⁴)

Table 2.8 Permeation data for chlorhexidine dihydrochloride across human epidermis from 0.78 mg/ml solutions. Effect of vehicle pH.* The flux of CHX at pH=3 was below the level of detection (Values in brackets represent the standard error, n=6)

The enhanced flux is probably not due to the effect of pH on the ionisation of CHX as the percentage of ionisation of CHX does not change over the pH range employed in this study (section 2.4.1.3). One explanation for the enhanced flux could be the effect of pH on the hydration of the skin. It has been shown that drug transport can be accelerated upon the hydration of the skin (Harris *et al.*, 1974).

Another variable which may impact on the observed trend in the rates of penetration is the varying thermodynamic activity of each vehicle. As this study was performed using solutions with the same concentration, and increasing pH decreases the solubility of CHX

(table 2.4), the thermodynamic activity of the permeant will be enhanced with increasing pH. The higher the pH, the higher thermodynamic activities of donor solutions. The flux of each vehicle is proportional to the degree of saturation. This effect is demonstrated by plotting the flux against the degree of saturation (figure 2.8). A linear relationship is evident:

$$J = 6 \times 10^{-5} x - 6 \times 10^{-5} \quad (r^2=0.9986) \quad (2.12)$$

This effect can explain the high flux of the donor solution with the pH value of 7 (CHX flux= $7.80 \times 10^{-4} \pm 7.55 \times 10^{-5} \text{ mg cm}^{-2} \text{ min}^{-1}$).

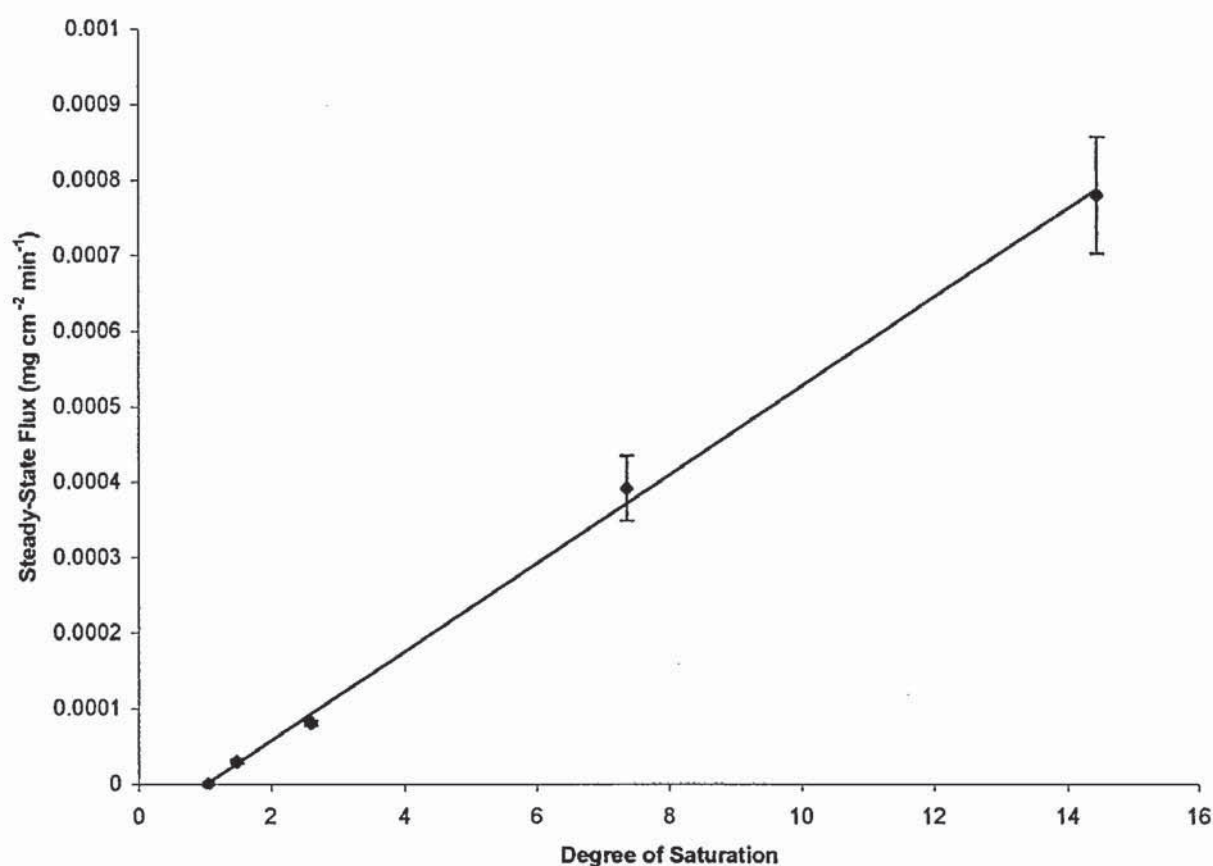


Figure 2.8 Plot of the steady-state flux of CHX across human skin from solutions of differing pH, as a function of the degree of saturation.

2.4.4 DSC Results

DSC has been extensively used to investigate the thermal transitions evident in the outer layer of mammalian skin (Naik and Guy, 1997; Golden *et al.*, 1986,1987). In this study, a four-peak thermogram was obtained for the human SC (figure 2.9), which showed one minor transition at around 35°C and three major transitions at around 68, 80, and 95°C.

To find correlations between the endothermic transitions and the nature of the components of the SC, many investigators combined thermal analysis with X-ray studies. Using wide-angle X-ray diffraction, Bouwstra *et al.* (1992) ascribed the thermal transition observed at 35°C to an orthorhombic to hexagonal phase transition in the lateral lipid packings. Furthermore, from small-angle X-ray diffraction data they found that the thermal transition between 65 and 70°C is due to the transformation of the lamellar lipid structure to a disordered structure (Bouwstra *et al.*, 1991). This supported the assignment of this transition to lipids by Golden *et al.* (1986) after using DSC and IR spectroscopy.

The transition at 80°C and 95°C may arise from gel to liquid transitions of lipids, which are associated with proteins, probably in the form of a liquid-protein complex and the denaturalization of proteins respectively (Knutson *et al.*, 1985; Golden *et al.*, 1986; Bouwstra *et al.*, 1991).

Another investigator, Barry (1991), suggested that, at physiological temperature, the lipids present in the SC are predominantly in the *trans* state, and exist as a semi-solid phase due to the large amount of unsaturated lipid present and the lipid transition evident at 35°C may reflect the increased motion of sebaceous lipids and the side chains of cholesterol molecules present in the SC. The endotherm which occurs at approximately 65-70°C has been assigned to the melting of the lipid chains of non-polar lipids present within this region.

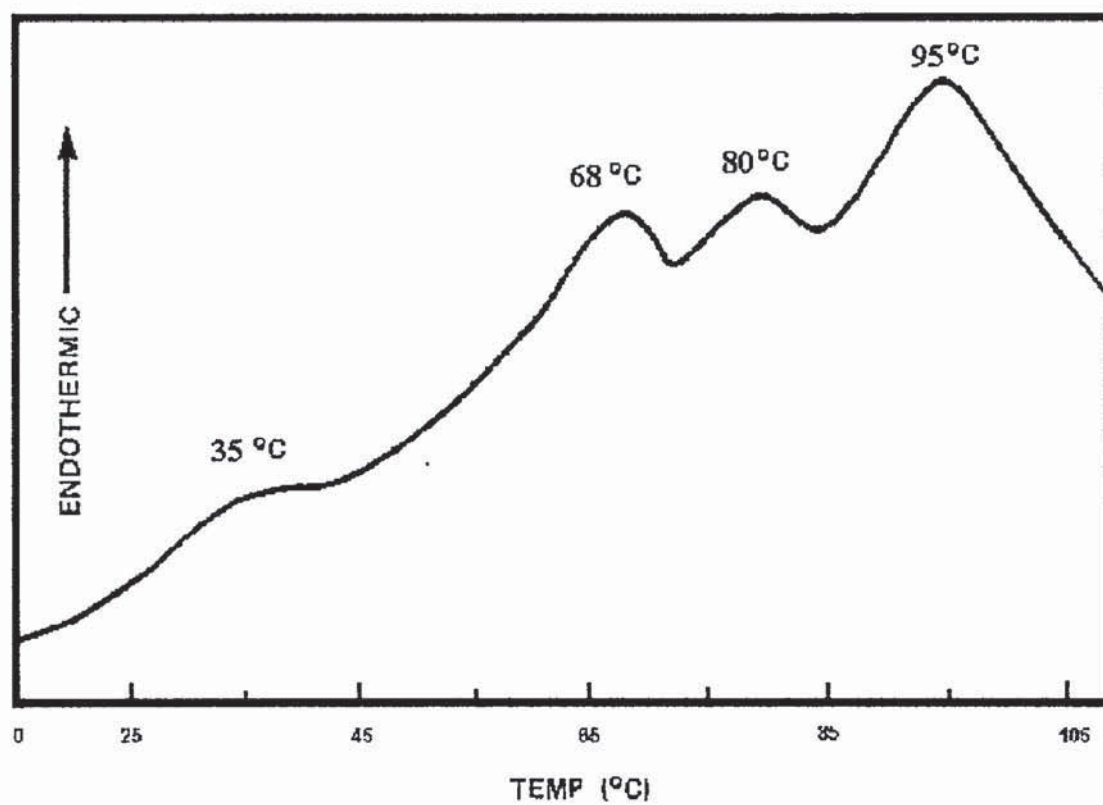


Figure 2.9 DSC thermal profiles of human stratum corneum

2.5 Summary

Permeation studies showed a poor penetration for CHX molecule across the skin. To enhance the CHX flux, ethanol was incorporated as the cosolvent in the systems. The addition of 10% ethanol resulted in 2.4-fold increase in CHX flux which was further promoted by increasing the concentration of ethanol (3.8-fold increase compared to the systems with no ethanol).

The effect of vehicle pH was also studied on the permeation of CHX across the skin. The CHX flux was enhanced with increasing the vehicle pH despite the fact that increasing the vehicle pH from 3 to 7 did not affect the percentage of ionisation of CHX. The suggested explanations were the effect of pH on the hydration state of the skin and enhanced thermodynamic activity of the donor solutions.

Although the above systems showed an increase in the transport of CHX, the amount of this antimicrobial agent penetrated across the skin was still low; hence, the total removal of the bacteria in the skin might not be achieved. As the purpose of this study was the full eradication of bacteria in the skin, different techniques such as chemical enhancers, supersaturated systems and iontophoresis were adapted in this study to enhance the diffusion of CHX into the skin and thus sterilising the skin. These techniques are described in the following chapters.

CHAPTER 3

CHEMICAL ENHANCERS

3. Chemical Enhancers

3.1 Introduction

The impervious nature of the stratum corneum presents major difficulties in delivering the antimicrobial agents to the bacterial sites in the skin. As discussed earlier (section 1.8), skin microflora are not confined to the surface of the skin but they also reside in the deeper layers of the skin. Hence, in order to sterilise the skin before any invasive treatment, the antibacterial agents should diffuse throughout the skin and reach the bacteria reservoir in the underlying layers. For many years, chemical enhancers have been used to enhance the permeation rate of many chemicals across the skin as most drugs are insufficiently skin-permeable to allow formulation into topical delivery systems. The permeation-enhancing effect of the chemical enhancers and their effect on the SC barrier properties have been shown by several researchers. Although chemical enhancers have been mostly used to increase the transdermal delivery of chemicals, due to their effect on the SC barrier properties, we anticipated that these chemicals would also assist us to deliver the antimicrobial agent to the bacteria sites in the skin and thus sterilise the skin.

Penetration enhancers are chemicals that reduce the barrier resistance of the stratum corneum and allow the drugs to reach their site of action (Barry, 1983). An ideal penetration enhancer should be:

a) non-toxic, non-allergenic, and non-irritant b) odourless, colourless, tasteless, and inexpensive c) chemically and physically stable and compatible with all the components of the formulation d) pharmacologically inert e) specific in its action f) cosmetically acceptable and it also should offer an immediate, predictable, and reversible action (Turunen *et al.*, 1994). However, it is difficult to find a chemical possessing all the desirable attributes and therefore compromises have to be made.

3.2 Mechanism of Action

Chemical penetration enhancers can increase skin permeability by several mechanisms. In 1994, Shah outlined the general effects of various enhancers on the skin, formulation, and the drug (Shah, 1994). According to Shah, enhancers:

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

Increased partitioning of drug can occur by modifying polar, non-polar, and a combination of polar and non-polar pathways (Walters, 1989; Rolf, 1988). By changing the protein conformation or by solvent swelling, the polar pathways may be altered chemically. It is proposed that many absorption enhancers interact with the polar head groups of the intercellular lipids of the structured bilayer lamellae within the SC (section 1.1.1). These interactions modify hydrogen-bonding and ionic interactive forces between polar heads disturbing the ordered packing. Modelling of the electrostatic field around the headgroups of the ceramides which make up the lipid bilayers (section 1.1.1), show that electronegative and positive regions exist on opposite sides of the molecule. The interaction of these forces has been suggested to hold the molecules together. Hence, disruption of these interactive electrostatic forces makes the domain more fluid and thus enhances the diffusion of chemicals across the SC. This disruption may allow more aqueous liquid to enter the tissue and swell the aqueous regions between the lipid bilayers. This swelling can provide a larger fractional volume of free water as distinct from structured water and hence increase the cross-sectional area available for polar diffusion (Barry, 1991a). However, using X-ray analysis, Bouwstra *et al.* (1991) have shown that lipid lamellar spacings are independent of tissue water content and increased hydration may not expand this domain.

Modification of the lipid packing integrity at the polar plane is also likely to alter the packing of the lipid chains. Hence, the lipid hydrophobic route becomes disordered and the permeation of non-polar chemicals is also enhanced. William and Barry (1992) suggested that the non-polar pathway may be altered by modifying rigidity of the lipid structure and fluidisation of the crystalline matrix of the stratum corneum.

In addition to affecting the lipid polar heads, enhancers may directly affect the composition of the aqueous region. Solvents, like enhancers, can partition into this region and alter its solubilising ability. This will alter the partition characteristics of lipophilic molecules such as steroids, which will have a more favourable partition coefficient from the donor vehicle into the skin. When the solvent diffuses out of the SC into the viable epidermis, the drug follows at a relatively high flux as it diffuses down a new, raised chemical potential gradient (Smith, 1997; Shah, 1994).

Due to their structure, absorption enhancers such as Azone and oleic acid will insert between the acyl chains of the intercellular lipid bilayers of the SC. This will disrupt packing, increase the fluidity of the bilayer and thus permit enhanced diffusion of penetrants. Just as interaction at polar head also affected the fluidity of the lipid domain, the interaction at the lipid domain will disrupt the packing of the polar heads and promote chemical penetration *via* the polar route.

Increased drug diffusivity in the stratum corneum can be achieved by dissolving the skin lipids or denaturing the α -keratin protein molecules in the skin (Zatz, 1993).

Within the intracellular route, the keratin fibrils are the primary target for penetration enhancer activity. The interaction of both polar aprotic solvents and surfactants with the protein constituent of the SC has been well characterised (Barry, 1991). It is proposed that the enhancer may displace bound protein water, thereby relaxing the binding forces and conformational restraints of the helices, substituting a looser structure in which permeants can move freely. Additionally, the enhancer may occupy polar sites on the protein leaving less available to bind and hinder molecule diffusion within the cells.

The outcome of enhancer action is usually a result of one or more of the mechanisms outlined.

3.3 Classification of Chemical Enhancers

Absorption enhancers can be broadly categorised into: solvents, surfactants, fatty acids and alcohols and other miscellaneous chemicals.

3.3.1 Solvents

Water

The state of hydration of the stratum corneum (SC) is one of the most important factors in determining the rate of percutaneous absorption of a compound. The normal water content of SC is 5% to 15% which can be increased up to 50% by occlusion. Roberts (1991) reported that the permeability coefficient of several phenols is directly related to SC water content. The mechanism by which hydration facilitate the transport of some chemicals is still the subject of debate. An increase in hydration of the skin is associated with a swelling of the SC and a softening of its texture. Harris *et al.*(1974) demonstrated that upon occlusion, SC not only swells, but also develops multiple folds resulting in a 37% increase in surface area. One proposed mechanism is, since the presence of water is associated with the polar head groups within the intercellular spaces, the binding of water to these groups may lead to an expansion of the lateral packing, relative to that of the hydrocarbon chain, and an increase in the surface area that lipid molecule occupies within the bilayer (Guy and Hadgraft, 1988). Walters *et al* (1981) demonstrated that hydration was less marked in the nail plate, in which the lipid content is less than 1% compared to 10% lipid in SC. Therefore, hydration causes an increased permeability by aqueous solvation of the intercellular lipids, in particular the glycosphingolipids and ceramides. An alternative mechanism can also be proposed based on the fact that much of the water binds to the keratin in the cells. Upon occlusion, a general swelling of the bricks in a bricks-and-mortar model will cause a loosening of the mortar and an increased movement through the intercellular keratin route. This theory suggests that hydration may be a

general plasticisation of the SC, increasing the diffusion of water by whatever route the solute permeates through the SC (Roberts and Walker, 1993).

In addition to affecting the diffusivity of solutes, hydration will affect the partitioning and solubility of solutes in the SC. The observation by Wurster and Kramer (1961) that hydration aided the absorption of pure glycol salicylate to a greater extent than methyl or ethyl salicylate would be consistent with effects of hydration on solubility.

However, occlusion does not necessarily enhance the percutaneous absorption of a chemical. Hydrocortisone absorption under occluded conditions was not increased in single-dose or multiple-dose application studies (Guy *et al.*, 1987). Hence, it can be concluded that the degree of enhancement is compound specific.

Alkanols and Alkyl Esters

Alcohols have been shown to possess the ability to enhance permeation across skin (DeNoble *et al.*, 1987). Coadministration of ethanol with estradiol caused an enhanced absorption of estradiol showing the usefulness of ethanol as an absorption enhancer (Good *et al.*, 1985). Upon application to biological membranes, they would appear to accumulate in the SC, which results in an increased partitioning of drugs into the membrane barrier (Berner *et al.*, 1989). The actual ratio of ethanol in an aqueous solvent can influence the penetration rate of compounds (Kurihara-Bergstorm *et al.*, 1990). Williams and Barry (1992) suggested that at high concentrations, ethanol and other small alcohols can extract SC lipids. Ghanem *et al.* (1987) suggested that low ethanol concentrations alter the lipoidal pathways while higher concentrations affect the polar pathways. However, using thermal perturbation and Fourier transform infrared techniques, Krill *et al.* (1988) showed that ethanol does not induce any changes in lipid alkyl chain structure at or below 37°C. Therefore, they suggested that the flux enhancement induced by dilute ethanol is not necessarily a result of lipid fluidisation but is caused by the increased partition of solutes into the stratum corneum. Levang *et al.* (1999) examined the effect of ethanol/propylene glycol solvent systems on the peak height and area of asymmetric and symmetric absorbance by FTIR studies of porcine skin. They observed that increasing concentrations of ethanol in the solvent systems

decreased peak height and area of absorbances until 80% ethanol/20% propylene glycol suggesting an overall extraction of SC lipids.

Regarding the highly concentrated solutions (>50 vol %) or neat ethanol, Higuchi *et al.* (1987) proposed that the creation of new or activated latent pores is responsible for the penetration enhancement properties of these solutions. Lipid extraction and osmotic expansions may be the cause of these pores.

Ethyl acetate (EtAc) and butyl acetate (BtAc) are examples of alkyl esters with the skin penetration enhancer properties. The use of EtAc and BtAc as solvents for Levonorgestrone, a lipophilic compound with a log octanol/water partition coefficient of 3.7, resulted in 80-fold and 40-fold increase in the drug flux, respectively (Friend and Heller, 1993).

Propylene Glycol

Propylene glycol (PG) is a solvent widely used in topical formulations, and it is valuable as a cosolvent for penetration enhancers. It has been suggested that PG may work by a direct action on epidermal structural proteins which increases the solubility of proteins and also denatures proteins causing them to lose their secondary, tertiary, and quaternary structures, thereby increasing the partitioning of compounds between the vehicle and the SC (Barry, 1987; Goldsmith, 1978). Bouwstra *et al.* (1992) employed X-ray diffraction techniques to study the influence of enhancers on the human SC. They concluded that, as in the case of water, PG causes no swelling of the intercellular domains.

Gabia *et al.* (2000) investigated the effect of penetration enhancers on isosorbide dinitrate penetration through rat skin and concluded that PG, compared to oleic acid, polyethelene glycol 400, and isopropyl myristate, had a higher enhancement factor.

Propylene glycol is often used as a cosolvent for more lipophilic enhancers, such as oleic acid, providing the opportunity for synergy and amplification of effect.

Acetone

Acetone is widely used as a solvent during permeation studies. It is a lipid solubiliser and can cause an enhancement in permeation (Behl *et al.*, 1980) by optimising the thermodynamic activity of the penetrant in the vehicle. However, it is unlikely that acetone finds a use in transdermal drug delivery despite having been used in preformulation stage of product development.

Alkyl Methyl Sulphoxide

The use of alkyl methyl sulphoxides as penetration enhancers stems from early observations on the effect of dimethyl sulphoxide (DMSO) on the skin. DMSO is a clear, dipolar solvent which is miscible with water and common organic solvents. Its structure is shown in figure 3.2. The concentration-dependence of this molecule is well known; solutions of 60-70% and over are usually necessary to promote steady-state drug permeation. By using IR, it has been shown that DMSO converts some of the α -helical portion of the intercellular keratin filaments of human SC to an anti-parallel β -sheet structure, by competing with water for the protein-binding sites. It has also been suggested that DMSO solutions of 20-60% enhance drug permeability by displacing bound water in proteins and substituting a looser structure. However, solutions of this concentration do not markedly enhance permeability and thus an alternative explanation of its action is needed. Scientists have proved that solutions of higher concentration than 60% show lipid interaction (Foley *et al.*, 1992; Kurihara-Bergstrom *et al.*, 1987). Most likely this involves competition with water for polar head-groups, disrupting the lipid structure through hydrogen-bonding rearrangement and, therefore, increasing lipid fluidity. The creation of this solvation shell around the head groups would loosen the lipid packing. DMSO is known to swell the SC because of the high osmotic activity, which may distort the laminated structure of the SC and induce channels within the matrix. Such channels enhance the penetration of some compounds. Because DMSO may be present in both protein and lipid regions, it may enhance the partitioning of polar and relatively non-polar drugs into the skin. However, its application has limitations owing to its irritating effect.

Longer chain alkyl homologues of methyl sulphoxide have been evaluated as absorption enhancers. Decylmethyl sulphoxide has been reported as optimal (Sekura and Scala, 1972). It is effective at lower concentrations and has the advantage that the degradation products are less odorous than dimethyl sulphoxide (Touitou and Abed, 1985). Decylmethyl sulphoxide can enhance the permeation of polar and ionic compounds, suggesting that it affects the polar route. Activity can be due to its characteristic as a non-ionic surfactant (critical micelle concentration of 0.002M) (Cooper, 1982). Kim *et al.* (1999) made some modifications to the DMSO molecule where the oxygen atom of the DMSO was replaced by a nitrogen atom, which in turn was substituted with an arylsulphonyl, aroyl or aryl group. These compounds could be used at lower concentration compared to DMSO (below 10%), which may result in less skin irritation. These cyclic derivatives were tested for their enhancement activities using hairless mouse skin and hydrocortisone as a model drug *in vitro*. They showed that the 4-bromobenzoyl derivative produced the highest activity of all the enhancers tested and 2-methoxycarbonylbenzenesulphonyl derivative not only had no enhancement effect but it also retarded the permeation of hydrocortisone.

Pyrrolidones

Pyrrolidones, present in natural moisturizing factor, have been evaluated as potential penetration enhancers. Pyrrolidone carboxylic acid has been shown to increase the water-binding capacity of the stratum corneum (Barry, 1983). The most widely studied derivatives of the naturally occurring pyrrolidone carboxylic acid, 2-pyrrolidone and N-methyl-2-pyrrolidone, have been shown to increase the transport of steroids (Bennett *et al.*, 1985), ibuprofen and flurbiprofen (Akhtar and Barry, 1985). Pyrrolidone derivatives have a wide range in their hydrophilic/lipophilic nature depending on their substituted functional groups. The lipophilicity of these enhancers affects both their behaviour and localization within the SC. Sasaki and co-workers conducted a series of studies with full-thickness rat skin and various pyrrolidone compounds. One study found that increasing the length of alkyl chain from methyl to lauryl increased both the penetration and skin retention of hydrophilic drug phenol red (Sasaki *et al.*, 1988, 1990). In another study, Sasaki *et al.* (1991) found that the same increase in chain length increases the drug penetration of a number of both hydrophilic and lipophilic model drugs such as 5-

fluorouracil, triamcinolone acetonide, and flurbiprofen. The enhancers tested include 1-methyl, 1-hexyl, and 1-lauryl-2-pyrrolidone. It was shown that the enhancers increase the penetration of all drugs tested. In addition, the lag time decreases with increasing chain length of the derivatives (Sasaki *et al.*, 1991). Additional evidence of dependence on the chain-length and enhancing ability is shown in a study by Godwin *et al.* (1997). They examined the penetration-enhancing abilities of a wide range of pyrrolidone compounds, including those with differing chain lengths and functional groups on hydrocortisone using *in vitro* hairless mouse skin model. They showed that N-dodecyl-2-pyrrolidone and 2-pyrrolidone-1-acetic acid dodecyl ester produce a higher enhancement compare to 1-methyl, 1-ethyl, and 1-hexyl-2-pyrrolidone derivatives. Furthermore, Sasaki *et al.* (1991) showed that these enhancers also greatly increase the solubility of the drugs in the vehicle, isopropyl myristate. These results suggest that enhancers may increase the flux of these model drugs by increasing their solubility within the SC.

It has also been suggested that the primary site of action for this group is the polar route and also hydration of the skin, owing to their intrinsic humectant activity, is a significant factor in their effectiveness (Southwell and Barry, 1983). In addition, using differential scanning calorimetry, Barry and Bennett (1987) proposed that lipophilic pyrrolidone enhancers exert their effects by entering the lipophilic domains of the SC and disrupting their structure, thereby increasing drug permeability.

Laurocapram (Azone)

Azone (1-dodecylazacycloheptan-2-one) is a clear, colourless, liquid, with a relative molecular mass of 281.49 Da, a melting point of -7°C , and a boiling point of 160°C at 0.05 mmHg. It is a molecule with a log P octanol/water of 6.21. Although extremely lipophilic, its structure (figure 3.2) involves a long alkyl chain bounded by a polar azacycloheptan-2-one ring, which is soluble in water as well as organic solvents, thereby rendering it amphiphilic. Hence, the polar group would be expected to interact with the hydrophilic head groups regions of the lipid lamellae, while the carbon side chain inserts into the structured alkyl chains of the lipid matrix, therefore perturbing the lipid structure. Using monolayers of dipalmitoyl phosphatidylcholine (DPPC) spread at an air-water interface, it is possible to study the Azone-lipid interaction in more detail. The

interactions caused large surface expansions, measured using a Langmuir film-balance, which, in conjunction with molecular graphics, indicates that the headgroup is in a bent conformation relative to the alkyl chain and that the ring structure lies in the plane of the polar headgroups of the phospholipids (Lewis and Hadgraft, 1990). This head group arrangement of Azone has an electronegative site at the carbonyl moiety, but no complementary positive site. This is in contrast to the natural polar head of the ceramide lipids which have complementary electropositive and negative groups on opposite sides of the molecule. When Azone inserts into the lipid structure, it will leave an unbalanced electronegative site on the ceramide, which could give rise to a permeability defect.

The generally accepted mechanism of action for Azone is that it disrupts the ordered lipid packing within the bilayers and increases the fluidity of this layer. This increased fluidity will allow easier diffusion of hydrophobic penetrants. The structure of the Azone hydrocarbon chain is linear, which implies that the hydrophobic tail is not responsible for the lipid structure disruption. The disruption created by the lactam ring at the polar head region will promote permeation of hydrophilic compounds via the polar route (Hadgraft *et al.*, 1993). Another mechanism of action has been proposed for Azone by Hadgraft *et al.* (1985). They indicated that an ion-pairing type mechanism might be operative for promoting the partitioning of some ionised permeants into the lipid phase. This would then alter the thermodynamic activity of the anionic drug and, thereby, affect its bioavailability within the tissue.

Several authors have investigated Azone analogs and have shown that they increase penetration of various drugs such as hydrocortisone 21-acetate (Fuhrman *et al.*, 1997; Michniak *et al.*, 1994), ibuprofen, and estradiol (Sasaki *et al.*, 1991, 1988).

Azone is at its most effective when used in conjunction with a polar material such as ethanol (Stoughton and McClure, 1983) or propylene glycol (Bennett *et al.*, 1985). The concentration of Azone that maximally enhances drug penetration varies with different compounds, composition of topical vehicle, and biological species. However, it seems that low concentrations, usually between 1 and 10% are more effective.

3.3.2 Surfactants

Surfactants are major components of many pharmaceutical formulations and cosmetic products. These are used as emulsifier, suspending, wetting, solubilising, and stabilizing agents. These substances are characterized by the presence of both polar and non-polar groups on the same molecule and they owe their properties to their ability to adsorb at interface of two immiscible substances and lower interfacial tension. It has been shown that there is a concentration-dependent biphasic action on the rate of drug permeation through membranes (Attwood and Florence, 1983). At low concentrations, the enhancement effect of surfactants can be seen which is due to the ability of the surfactant molecule to penetrate the membrane and increase its permeability. However, above the critical micelle concentration (CMC) of the surfactant if there is any interaction between micelle and the drug, a decrease in the permeation can occur. This is due to solubilisation of active chemical by surfactant micelles which cause a decrease in the thermodynamic activity of the drug, therefore inhibiting further absorption enhancement.

Surfactants are broadly categorized into three groups, anionic, cationic and nonionic.

Anionic Surfactants

Anionic surfactants can penetrate and interact strongly with skin. The structure of surfactant, mainly the alkyl length chain, highly influences the penetrating amount of the surfactant in the skin (Howes, 1975). The hydrophilic head group can also affect the penetration rate (Prottey and Ferguson, 1975). Most anionic surfactants can induce swelling of the SC and the viable epidermis. The most widely studied anionic surfactants are the alkyl sulphates. Rhein *et al* (1986) examined the relation between swelling of the SC and the structure of various surfactants. They found that the swelling was concentration-dependent and increased with extended exposure times. A possible mechanism of action is that the hydrophobic interaction of the alkyl chains with the substrate leaves the negative end group of the surfactant exposed, creating additional anionic sites on the membrane. This results in the development of repulsive forces that separate the protein matrix, uncoil the filaments, and expose more water-binding sites, possibly increasing the hydration level of the tissue. Separation of the protein matrix could also result in a disruption of the long-range order within the corneocytes, leading to

increased intracellular diffusivity and enhanced penetration. Barry (1988) suggested that sodium lauryl sulphate could also affect SC lipid components since the anionic surfactant disrupted the entire SC membranes. He proposed that the swelling of the corneum by water uptake allows easier chemical penetration. The most widely studied anionic surfactants are the alkyl sulphates. The increase in the volume of the intercellular spaces facilitates entry of the sodium lauryl sulphate molecules into the intercellular lamellar lipids, causing further disruption of molecular structure of the SC. It has been shown that sodium dodecyl sulphate can increase the permeation of chloramphenicol (Aguiar and Weiner, 1969) and naproxen (Chowhan and Pritchard, 1978).

Although this group of surfactants can reduce the barrier properties of the skin, their ability to induce an irritation of the skin may limit their application in delivery systems.

Cationic Surfactants

This group of surfactants has not been widely investigated because they are the most topically irritant of the all. Alkyl amines and alkyl ammonium bromides, such as cetylmethyl ammonium bromide, are among this group and they have been shown to enhance the permeation of several chemicals, such as sodium and potassium ions (Bettley, 1965) and naloxone (Aungst *et al.*, 1986). The suggested mode of action for this group is ion-pairing (Baker and Hadgraft, 1981) however, Hadgraft *et al* (1986) showed that the ion-pair facilitation could not be the sole means of transport. They showed that N,N-bis[2-hydroxyethyl] oleylamine, a cationic enhancer, did not enhance the penetration of caffeine, a weakly basic drug, across the artificial membrane while it caused penetration enhancement of the drug across the skin. Kushla and Zatz (1991) reported that the cationic surfactants could extract the SC lipids and penetrate into the intercellular lipid matrix of cornified layer of SC.

Non-ionic Surfactants

The hydrophobic portion of non-ionic surfactants usually consists of alkyl or acyl chains that are attached to a polar head group. Of the many different types of non-ionic surfactants, studies of skin enhancers are limited to four main groups. These are the polysorbates (ethoxylated esters or partial esters of sorbitol); polyethoxylated alkyl ethers

and esters in which the alkyl chain can be either saturated, unsaturated, branched or linear; polyoxyethylene alkylphenols in which the hydrophobic group is normally octyl or nonylphenol; and poloxamers (polyoxyethylene-polyoxypropylene block copolymers) in which the polyoxypropylene chain acts as the hydrophobic moiety. The general structure of these surfactants are shown in figure 3.1.

Among the surfactants, the non-ionic group has the least potential for irritancy. It has been suggested that the mode of action on the skin is related to their ability to partition into the intercellular lipid phases of the stratum corneum. This results in increased fluidity in this region which reduces diffusional resistance. Shahi and Zatz (1978) showed that polysorbates 20, 40, 60 and 80 at concentrations below 0.5% can increase the flux of hydrocortisone through hairless mouse skin. Sarpotdar and Zata (1986) reported the same effect with lidocaine. Furthermore, surfactants are known to penetrate into the intracellular matrix and both interact with and affect the keratin of the stratum corneum (Breuer, 1979; Dominguez *et al.*, 1977). This mechanism seems optimal for dodecyl-based surfactants.

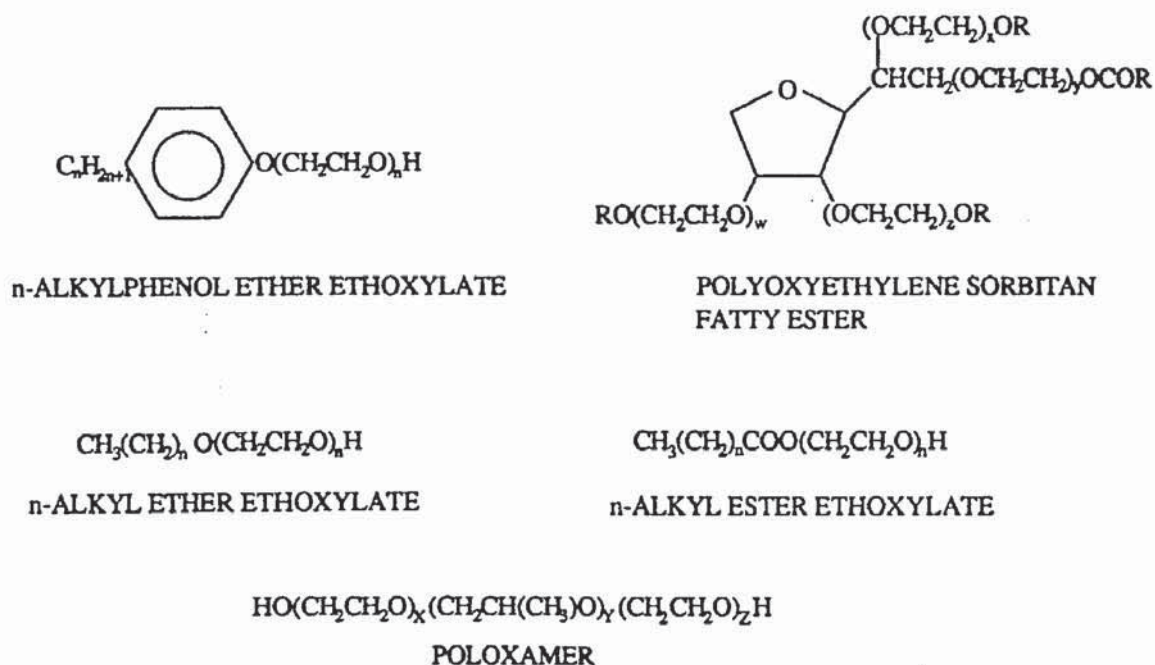


Figure 3.1 Structure of various classes of non-ionic surfactants (French *et al.*, 1993)

3.3.3 Fatty Acids and Alcohols

Long-chain fatty acids have been shown to be effective penetration enhancers for a variety of drugs (Aungst *et al.*, 1986; Cooper *et al.*, 1985). Fatty acids are also a major component of SC lipids, but these are largely saturated. In general, the penetration-enhancing effects of saturated fatty acids are greatest for C10 and C12 fatty acids (Aungst *et al.*, 1986; Ogiso and Shintani, 1990), while unsaturated long-chain fatty acids are more effective compared to their saturated counter parts (Cooper, 1984).

Oleic acid is an unsaturated fatty acid ($\log K_{o/w}$ value of 4.5) with a double bond in the *cis* configuration; this includes a kink structure which affects its mode of packing into lipid matrices. Studies have shown that SC lipids can be fluidised upon oleic acid treatment (Naik *et al.*, 1995; Menon and Elias, 1997). It has also been proposed further that the predominant effect of the fatty acid is the formation of phase-separated domains, thereby forming permeation ability defects at liquid-solid interfaces (Pechtold *et al.*, 1996; Ongpipattanakul *et al.*, 1991). Goodman and Barry (1989) suggested that oleic acid operates by inserting into the lipid structure, with its polar end close to the lipid polar head. Because of its kinked *cis* chain, it then disrupts and increases the fluidity of the lipid packing. The bent structure of the acid may substantially inhibit the hydrocarbon chains from packing tightly; some of the chains have so much rotational freedom that they are effectively solubilised.

Various authors have reported the use of fatty acids, notably oleic acid in promoting the penetration of lipophilic compounds (Touitou and Fabin, 1988; Green *et al.*, 1988) and the dependence of oleic acid uptake into the SC, on the duration of application time (Jiang *et al.*, 2000). By using electron microscopy, they showed that application of oleic acid for 1 hour did not induce notable alterations in SC lipid lamellar structure. However, by 2-hours application, membrane structure and their lamellar organization were drastically altered. Evidence from thermal analysis and freeze-fracture electron microscopy of SC pretreated with oleic acid (0.16 M in PG), indicates that oleic acid builds up a new type of structure within the skin lipids, which differs from the normal lipid structure and from the structure of oleic acid in PG itself. It is suggested that this new structure is created by eutectic mixing of oleic acid with the skin lipids and this has a reduced barrier capacity in comparison to untreated skin (Tanojo *et al.*, 1994).

Oleic acid shows a better effect when used with a polar cosolvent such as propylene glycol (Tanojo *et al.*, 1998; Takeuchi *et al.*, 1998).

3.3.4 Miscellaneous chemicals

Terpenes

Terpenes, naturally occurring volatile oils, appear to be promising candidates for clinically acceptable enhancers (William and Barry, 1991). Terpenes were reported to have good toxicological profiles, high percutaneous enhancement abilities, and low cutaneous irritancy at low concentrations (1-5%), (Obata *et al.*, 1991; Okabe *et al.*, 1990). A variety of terpenes have been shown to increase the permeation of both hydrophilic and lipophilic drugs (Gao and Singh, 1998; Moghimi *et al.*, 1997). X-ray diffraction and differential scanning calorimetric studies suggest that terpenes increase the drug percutaneous permeation mainly by disrupting the intercellular packing of the SC lipids (Cornwall and Barry, 1994; Williams and Barry, 1991). Furthermore, this interaction with the SC lipids has been reported to be reversible (Okabe *et al.*, 1990). El-Kattan *et al* (2000) studied the effect of terpenes on the permeation of hydrocortisone and reported that terpenes can enhance the flux of hydrocortisone. Another study carried out by Bhatia and Singh (1998) investigated the effect of terpenes (5%) in combination with ethanol on the permeation of luteinizing hormone releasing hormone (LHRH). They concluded that limonene and then carvone enhanced LHRH flux significantly. Cineole and thymol had similar enhancement effect but less than limonene.

Urea

Urea is a colourless, odourless, slightly hygroscopic crystalline powder, which is very soluble in water (1g in 1.5ml water). It is used most commonly as a hydrating agent and is employed in hyperkeratotic skin conditions. It has been suggested that urea increased the water content of the skin (Barry, 1991) and also acts as a mild keratolytic agent which could affect the SC corneocytes (Williams and Barry, 1989). However, it has been proposed that urea may alter the integrity of the lipids of the epidermal barrier. Beastall (1986) suggested that urea may lower the phase transition temperature of the lipids of the

SC so that they become fluidized at the ambient temperature of the skin. Finally, due to the fact that urea decreases the lag time required to reach steady-state diffusion of drugs such as 5-fluorouracil, one study has suggested that urea may interact with the proteins of the SC thereby decreasing drug binding (Williams and Barry, 1989). Urea is also a component of the natural moisturizing factor present in the skin. A study examining the effect of urea on the penetration of ketoprofen through rat skin showed that urea elevated permeation 8-50-fold depending on the vehicle used. They also revealed that urea formed large, hydrophilic channels through the excised skin (Kim *et al*, 1993).

In addition to urea, there have been a few studies done on analogs of urea. Godwin *et al* (1998) studied urea, 1-dodecylurea, 1-dodecyl-3-methylurea, 1,3-didodecylurea, 1-dodecyl-3-phenylurea, and their thio-analogs for their transdermal penetration enhancing properties on hydrocortisone using hairless mouse skin. They found 1-dodecyl-3-thiourea the most effective enhancer of the series.

Biodegradable Enhancers

In recent years, there has been a considerable interest in the development of biodegradable penetration enhancers which make use of the enzyme activity in the skin to fragment the enhancer into smaller innocuous compounds. This approach has the distinct advantage that the toxicity of the penetration enhancer is reduced. The enhancing activity dodecyl 2-[N,N-dimethylamino]propanoate (Buyuktimkin *et al*, 1993), and aminocaproic acids esters (Dolezal *et al.*, 1993), which may be cleaved by the esterases present in the skin into alcohols and substituted amino acids, have been examined. The data presented in these reports reveal that low toxicity enhancers facilitate enhanced skin permeation of acidic, basic, and neutral drugs. Dolezal *et al.* (1993) report that aminocaproic esters do not induce discernible skin irritation *in vivo* in mice and that these chemicals may be non-toxic. However, it should perhaps be emphasized that to date studies involving the use of biodegradable chemicals have not examined the nature of the interaction of the enhancer with the structural components of the SC. Buyuktimkin *et al.* (1993) proposed that the alcohol degradation product of dodecyl 2-[N,N-dimethylamino]propanoate can modify the structural integrity of the intercellular lipids present in the SC.

The structures of a variety of penetration enhancers are shown in figure 3.2. Figure 3.3 illustrates the suggested mechanisms of action of skin penetration enhancers by Barry (1989).

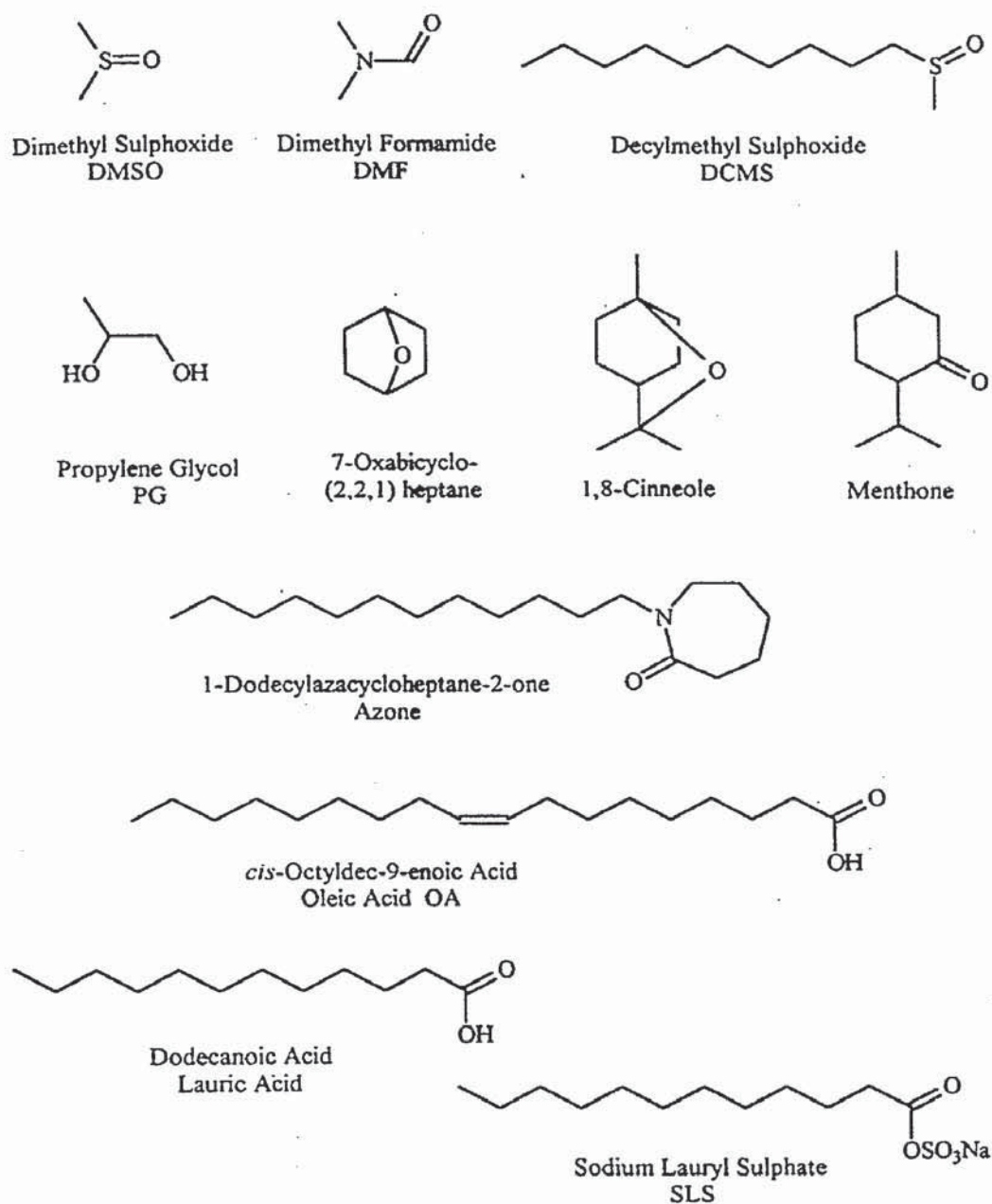


Figure 3.2 Structures of various penetration enhancers



Figure 3.3 Suggested mechanisms of action of skin penetration enhancers (Barry, 1989)

3.4 Aims and Objectives

The main objective was to enhance the flux of CHX through the skin in order to deliver a sufficient amount of antimicrobial agent to the bacterial sites in the skin for the purpose of skin sterilisation. Permeation studies carried out on CHX (section 2.4.3) showed a poor penetration rate for this chemical. This poor permeation rate is believed to be due to the impervious nature of the SC. Hence, any disruption in the structure of this barrier can result in an accelerated penetration of CHX across the skin. Therefore, the membrane was pretreated with chemical enhancers prior to the permeation studies. Various enhancers with different mechanisms of action were used in this study to overcome the barrier properties of the SC. The employed enhancers were ethanol, DMSO, propylene glycol, oleic acid, azone and surfactants. As discussed earlier in this chapter, chemical enhancers can reduce the barrier properties of the SC by disrupting the lipid, or protein or both phases of the SC. Ethanol, oleic acid, azone affect the lipid sites while propylene glycol, and anionic surfactants affect the SC structural proteins. DMSO induces its effects by affecting both lipid and protein sites. Furthermore, ethanol, propylene glycol, azone and cationic surfactants can also promote the partitioning of the drugs into the SC. Ethanol can increase the partitioning of drugs into the SC by accumulating in the membrane while Azone and cationic surfactants use an ion-pairing mechanism for their enhanced partitioning properties. This ion-pairing mechanism could be of benefit as CHX is a cationic drug and thus pretreatment of the skin with Azone or cationic surfactant can result in an enhanced penetration of CHX in the skin.

The next objective was to study the effect of co-solvent vehicle on the permeation enhancement abilities of oleic acid and azone. Many researchers have reported that these enhancers are more effective when used in conjunction with propylene glycol (Tanojo *et al.*, 1998; Bennett *et al.*, 1985).

Furthermore, differential scanning calorimetry (DSC) was employed to establish the mechanism of action of enhancers.

3.5 Material and Methods

3.5.1 Materials

Ethanol, dimethyl sulphoxide (DMSO), oleic acid, Propylene glycol (PG), dodecyl trimethyl ammonium bromide (DTAB), and sodium dodecyl sulphate (SDS) were purchased from Sigma chemicals. Azone was a gift from Whitby Research, USA.

3.5.2 Assay Procedure

CHX assays were carried out using HPLC as discussed in section 2.2.1.

3.5.3 Donor Solutions

In order to maintain a constant thermodynamic activity in the applied donor phase, saturated aqueous solutions of CHX were used as described in section 2.3.1.

3.5.4 Enhancer Solutions

Ethanol (neat and 10%), DMSO (neat and 10% v/v in ethanol), oleic acid (neat, 10% v/v in either ethanol or PG), Azone (10% in either ethanol or PG), SDS (10% w/v in PG) and DTAB (10% w/v in PG) solutions were prepared and employed for pretreatment of the skin.

3.5.5 Membrane Preparation

Intact human epidermis was prepared as described in section 2.3.2.2. The membrane was then pre-treated by placing 1 ml of enhancer solutions on the skin for 1 hour at room temperature. The epidermis was then washed thoroughly with double distilled water and gently blotted.

3.5.6 Membrane/Vehicle Partition Coefficient

The membrane-vehicle partition coefficients for the enhancer treated skin samples were determined in a similar fashion as to that described in section 2.3.2.2. However, in this experiment, skin samples were pretreated with 1 ml of either neat or 10% v/v of

enhancers in different solvents for 1 hour. After 1 h, they were washed with distilled water, gently blotted, accurately weighed and placed in each vial. All determinations were performed in triplicate and two sets of controls, one with no skin for the assessment of any CHX loss due to glass-uptake and the other one contained skin without any pretreatment, were also included.

3.5.7 Differential Scanning Calorimetry (DSC) studies

DSC was employed to examine the thermal behaviour of human epidermis as modified by enhancers. The changes in the endotherms indicate how the enhancers modify phase transitions within the epidermis. DSC studies were carried out on the hydrated and treated skin samples as described in section 2.2.3.

3.5.8 Permeation Procedure

Jacketed Franz-type diffusion cells were set up as described in section 2.2.2. Pre-treated skin was mounted between two halves of the cell. An aliquot of 1 ml of the donor phase was introduced into the donor compartment. The donor chamber and the sampling port were occluded with Parafilm to avoid any evaporation. 1 ml of receptor samples were then taken at appropriate time intervals over 60 minutes and analysed for CHX by HPLC as discussed in section 2.2.1 (no dilution was performed as the CHX concentrations were low). The receptor solution was replenished with an aliquot of distilled water after removing each sample which resulted in dilution of the receptor phase. Therefore, by applying the same factor as described in section 2.3.4, the sample concentration was corrected to represent cumulative drug amount permeated for each sampling time. Each experiment was run in six replicates and the mounted membranes were from a similar source.

3.6 Results and Discussion

3.6.1 Membrane/Vehicle Partition Coefficient

The ability of a drug to partition into the skin is dependent on a number of physicochemical properties including its partition coefficient between vehicle and skin (section 2.3.2.2). Some chemical enhancers are able to enhance the permeation of chemicals into the skin by promoting the partitioning of chemicals into the skin. To study how the different skin pre-treatment regimen affect the partitioning of CHX into the epidermis, the partition coefficients for CHX between enhancer pretreated human epidermis and vehicle (distilled water) were determined. No loss of CHX was observed in the first set of control vials (vials containing CHX solutions with no skin), therefore any loss of CHX in the aqueous phase was considered as the amount penetrated into the epidermis. A logP value of 0.98 ± 0.068 was found for the untreated epidermis (second set of control vials) by using equation 2.7. Results are shown in figure 3.4. Although the obtained log partition coefficient is close to unity, which was believed to be a requirement for a good skin penetration (Barry 1983; Marzulli *et al.*, 1965), the results of permeation studies in chapter 2 (section 2.4.3.1) did not support this belief. This could be due to the fact that experimentally determined partition coefficients include any contributions that drug binding to the SC component makes. With extensive adsorption, it is possible to get high apparent partition coefficients yet have a slow penetration rate and as mentioned before (section 2.4.2), CHX carries positive charges and tends to bind to the negative sites of the skin.

Except for 10% ethanol and 10% DMSO the employed enhancers increased CHX partition coefficient between vehicle and the epidermis with 10% Azone/PG having the greatest effect. Pretreatment of epidermis with 10% ethanol or 10% DMSO in water had no significant effect on the partitioning of CHX into the skin ($P > 0.05$) while neat ethanol and neat PG increased the partitioning by a factor of 1.35 ($\log P = 1.323 \pm 0.143$) and 1.68 ($\log P = 1.65 \pm 0.234$), respectively. 10% oleic acid/ethanol ($\log P$ value of 1.54 ± 0.153) and 10% Azone/ethanol ($\log P$ value of 1.95 ± 0.059) pre-treatment were more effective in increasing the partitioning of CHX between vehicle and the membrane compared to 10% ethanol and 10% DMSO pre-treatment regimens. However, when compared to neat ethanol, 10% oleic acid/ethanol did not increase the partitioning significantly (t-test,

$P > 0.05$), indicating that the increased partitioning observed with 10% oleic in ethanol was owed to the ethanol not oleic acid.

Several investigators have reported a synergistic enhancing effect for combined use of PG with oleic acid and azone (Takeuchi *et al.*, 1998; Bennett *et al.*, 1985). Hence, the cosolvent ethanol was substituted with PG. The partitioning of CHX into the skin upon pre-treatment of the skin with 10% oleic acid/PG was enhanced 2.4 times compared to the untreated skin and 1.54 times compared to the oleic acid/ethanol pretreatment. 10% Azone/PG pre-treatment resulted in a higher enhancement, a 2.8-fold increase, in the partitioning of CHX between the membrane and the vehicle compared to the untreated skin. Pretreatment of skin with surfactants (SDS and DTAB) also enhanced the partition coefficient of CHX between vehicle (distilled water) and the epidermis. 10% DTAB/PG showed less effect ($\log P = 2.1 \pm 0.201$) compared to 10% SDS/PG ($\log P = 2.36 \pm 0.167$), which itself was similar to the effect of 10% oleic acid/PG pre-treatment ($\log P = 2.375 \pm 0.135$). However, the possible interaction of CHX with SDS should not be neglected, as SDS is an anionic surfactant and can bind to CHX which carries a positive charge and thus reducing the antibacterial properties of CHX. This possible interaction will be studied later in chapter 6 where microbiology properties of CHX will be assessed. The employed technique was subject to inaccuracies and difficulty in interpretation. Since only the aqueous phase was monitored for CHX, the amount taken up by the membrane could only be estimated. Immersion of skin samples in a solvent for lengthy periods of time leading to alteration in the skin hydration state or dissolution of drug in vehicle entrapped in the skin do not resemble the *in vivo* situation and pose further difficulties (Barry, 1983).

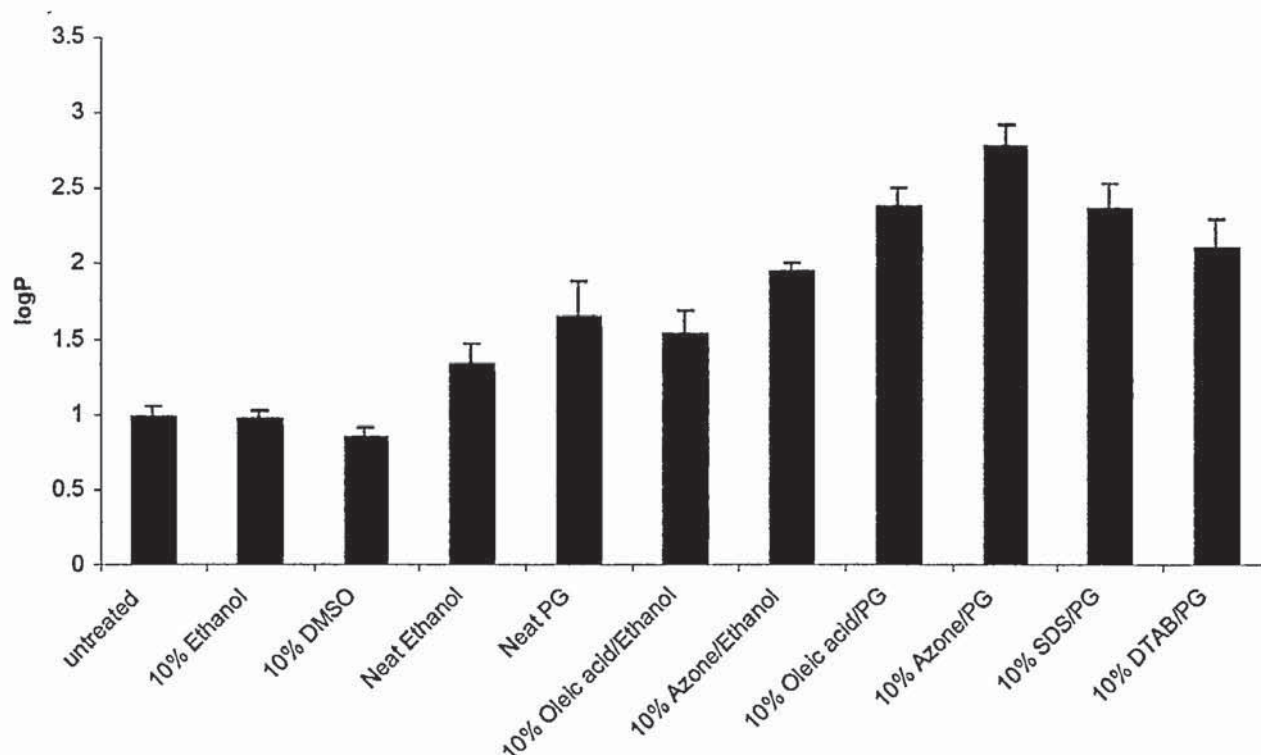


Figure 3.4 Effect of pretreatment on vehicle/membrane partition coefficients of CHX (bars represent the standard errors, n=6)

3.6.2 DSC Studies

To explore the possible mechanism of action of permeation enhancers, DSC was used to study the changes in the human skin. Six replicates for each treatment was used. The effect of each treatment has been explained below.

Ethanol Pre-treatment

DSC studies were carried out on the hydrated and ethanol pretreated skin samples. The result of thermal analysis on pretreated SC with ethanol is presented in figure. 3.5. Ethanol pretreated skin thermogram showed no apparent change in transition temperatures compared to the untreated skin thermogram ($T_1=35^\circ\text{C}$, $T_2=66^\circ\text{C}$, $T_3=80^\circ\text{C}$ and $T_4=95^\circ\text{C}$). This result indicates that 1-hour ethanol pretreatment has no effect on the

SC structure. This result was in agreement with the work of Golden *et al.* (1987) who showed that there were no changes in the IR spectra and transition temperatures in porcine stratum corneum treated with ethanol.

Although several published studies suggest increased lipid fluidity as the mode of action for ethanol, our findings did not support this suggestion. The lack of effect could be due to the short duration of skin pretreatment (1 hour).

DMSO Pre-treatment

Treatment of stratum corneum with DMSO lowered the lipid transitions T_2 and T_3 and made T_4 smaller and broader (figure 3.6). Increased lipid fluidity could be the reason behind the lowered thermal transitions. DMSO is a powerful aprotic solvent which mixes exothermically with water. Therefore, it may displace water from the lipid head groups and create a larger solvation shell around these groups. This larger shell could then loosen the lipid packing, which explains the lower transition temperatures.

Considering the polar nature of DMSO ($\log P$ [octanol/water] = -1.35) and its effect on T_2 and T_3 transitions, it is unlikely that the enhancer would partition directly into the lipid chains, at least not in great amounts as this would significantly reduce the lipid transitions.

The smaller and broader T_4 indicates that lipid interaction is not the only effect of DMSO on skin permeability and it affects the protein sites in SC as well. DMSO may displace bound protein-water, substituting a looser structure in which permeants can move more freely. Additionally, DMSO may occupy many hydrogen-bonding sites on the protein, leaving less available to bind and hinder molecule diffusion within the cells.

Propylene Glycol (PG) Pre-treatment

Figure 3.7 shows the DSC thermogram of a pretreated stratum corneum with propylene glycol. The interaction of PG with proteins is clearly shown by the disappearance of the

transition peak at 95°C (T₄). One explanation for this effect is PG might withdraw water from the protein owing to its hygroscopicity.

A less but clear effect was also noticed on the lipids in the form of decreased transition temperatures at 80°C (the transition temperature was lowered to 70°C). This result was in agreement with the work of Tanojo *et al.* (1997) who associated this transition with lipid-protein complex. They suggested that the loss of interaction with protein might decrease the transition temperature of the lipids, since less energy will be required for gel-liquid transformation without the necessity to break the lipid-protein bonding.

Oleic Acid Pre-treatment

The effect of pretreatment of skin with oleic acid was examined using DSC. This pretreatment of the stratum corneum caused the disappearance of T₁ and a shift in T₂ (from 66.5°C to 59°C) and T₃ transition temperatures (from 80°C to 69°C) (figure. 3.8). The lack of effect on the T₄ implies that oleic acid perturbs primarily the lipids of SC. Oleic acid probably operates by penetrating into the lipid structure, with its polar end close to the lipid polar heads. Because of its bent structure, it then disrupts and increases the fluidity of the lipid region.

Azone Pre-treatment

The effect of Azone on the SC was examined using DSC. As illustrated in figure 3.9, all three lipid transitions (T₁, T₂ and T₃) disappeared indicating the dramatic effect of Azone on SC lipid structure. Contrary to results obtained by Hirvonen *et al.* (1994), the T₄ transition in this study was intact indicating no protein interaction. This result suggests that Azone does not enter the cells in significant amount, at least at the concentration used (10%). This could be due to the non-polar nature of Azone molecule (log P [octanol/water] =6.6). The DSC data and Azone's non-polar nature suggested that it partitions directly into the lipid bilayer structure, disrupting it.

SDS Pre-treatment

The DSC thermogram of pretreated stratum corneum with 10% SDS in water is shown in fig. 3.10. It can be seen that SDS affected all the thermal transitions, eliminating T_1 , T_2 and T_3 transitions and reducing the size of T_4 . Hence, it can be concluded that SDS disrupted the entire membrane affecting both protein and lipid structure. The observed effect could be because SDS is an anionic surfactant which grossly swells the SC, uncoiling and extending α -keratin helices and thereby opening up the protein-controlled polar pathway. The expansion of intercellular spaces and the insertion of SDS molecules into the lipid structure also disrupt this region.

DTAB Pre-treatment

The effects of DTAB pretreatment on the SC, presented in figure 3.10, were not significantly different from those of SDS except for T_4 transition, which a more significant reduction in its size was seen. DTAB is a cationic surfactant and one of its suggested mode of action is the extraction of the SC lipids (Kushla and Zatz, 1991). The disappearance of T_1 , T_2 and T_3 transitions in the thermogram of the pretreated SC with DTAB supports this mechanism of action and shows that DTAB can penetrate into the intercellular lipid matrix of cornified layer of SC. Furthermore, it is likely that the SC structural proteins have also been affected by DTAB as the T_4 transition has been reduced in size.

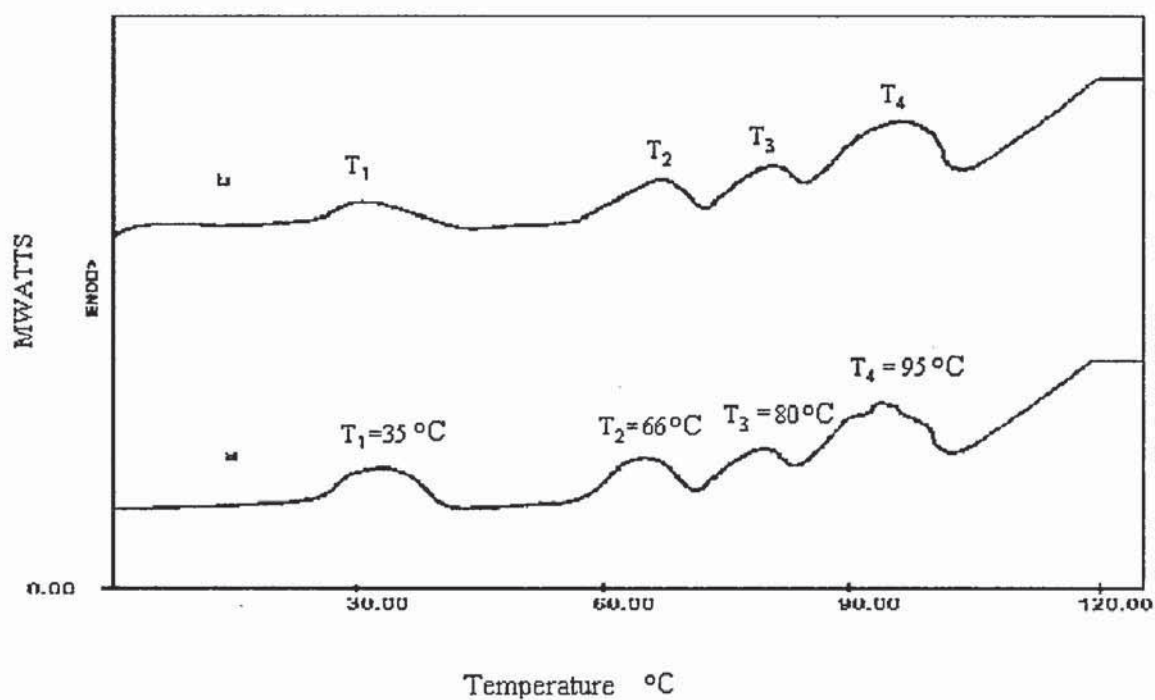


Figure 3.5 DSC thermogram of abdominal skin (a) untreated (b) ethanol pretreated

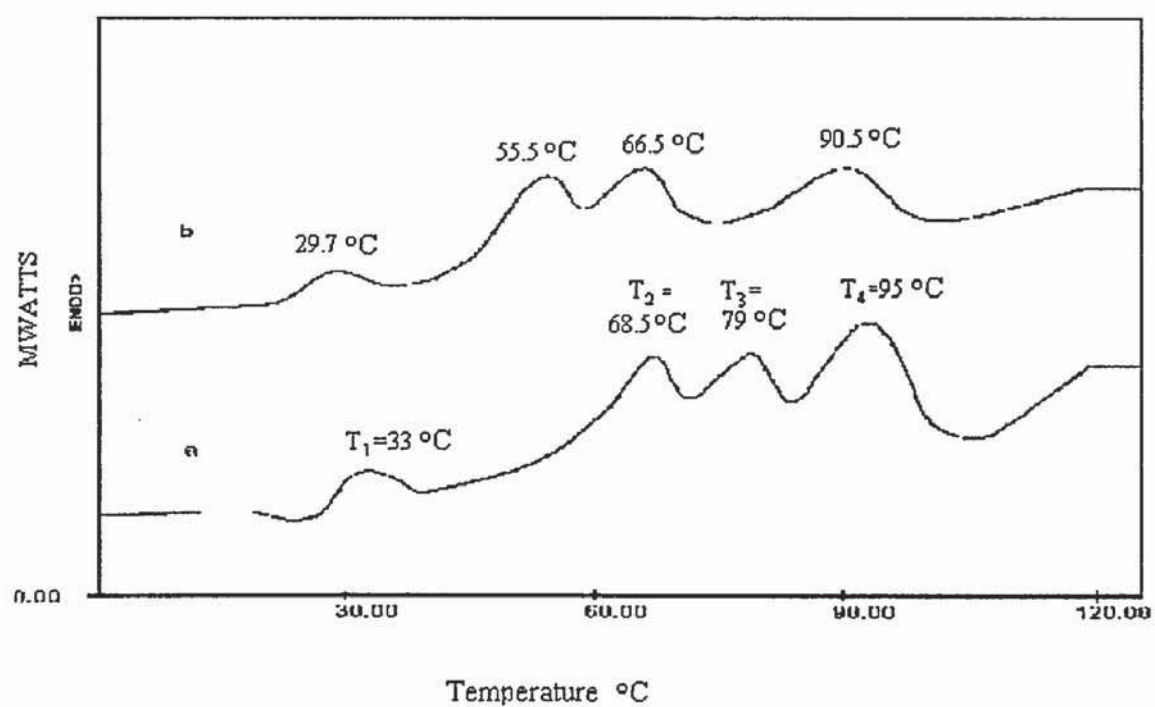


Figure 3.6 DSC thermogram of breast skin (a) untreated (b) DMSO pretreated

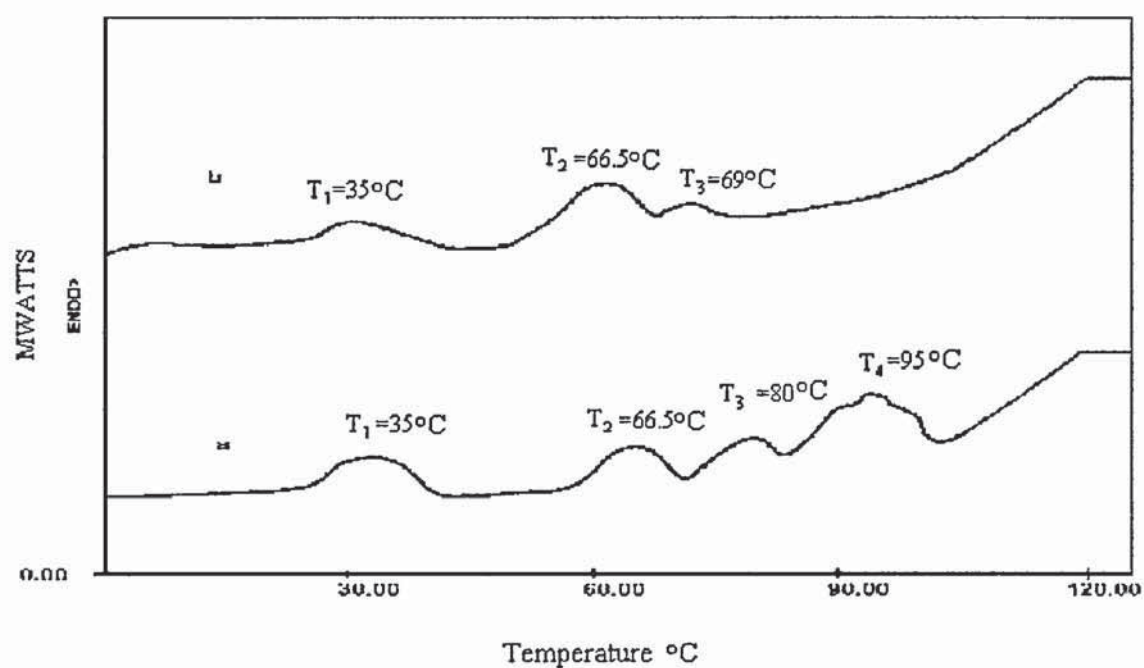


Figure 3.7 DSC thermogram of abdominal skin (a) untreated skin (b) pre-treatment with propylene glycol

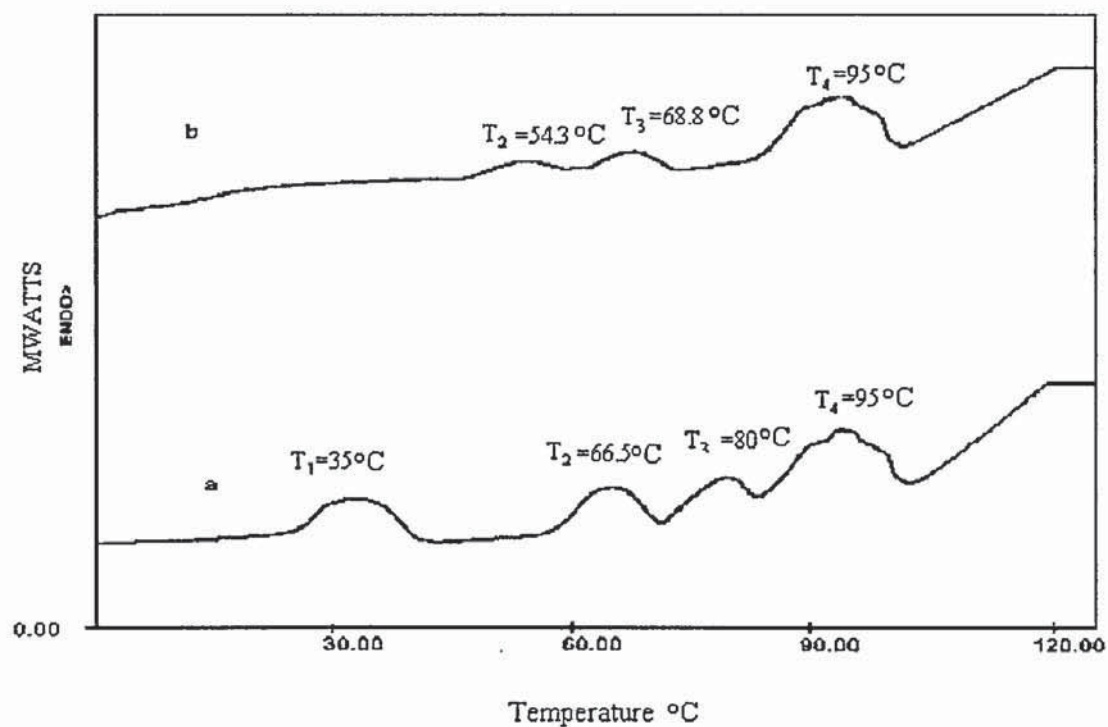


Figure 3.8 DSC thermogram of abdominal skin (a) untreated (b) oleic acid pretreated

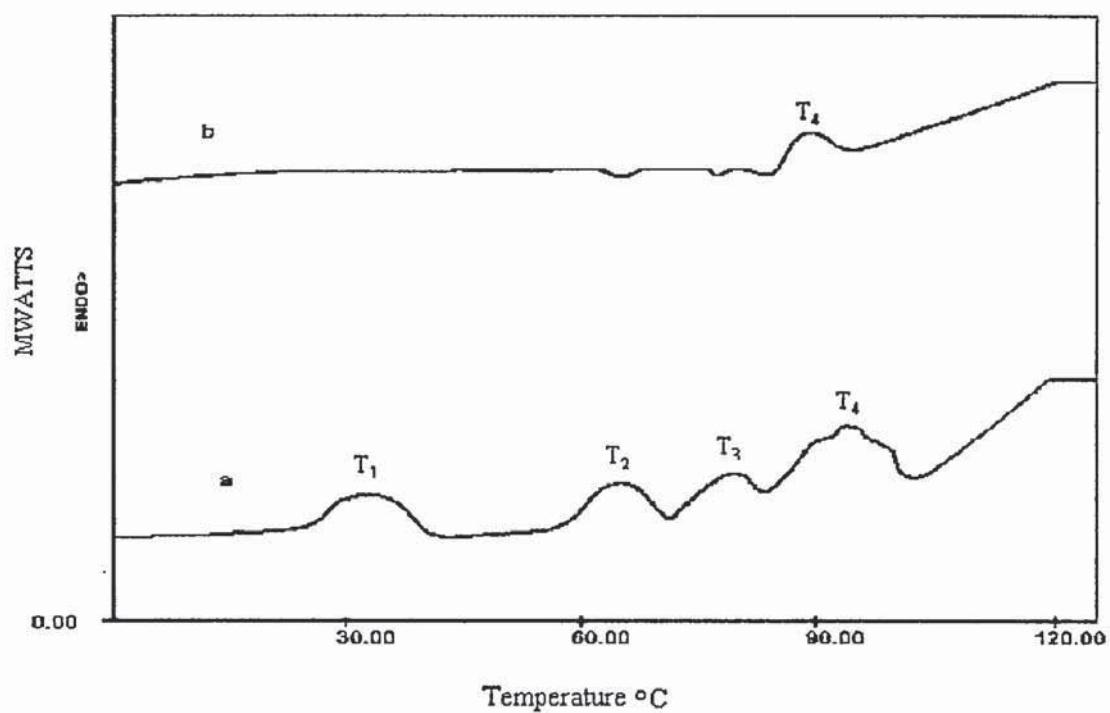


Figure 3.9 DSC thermogram of abdominal skin (a) untreated (b) Azone pretreated

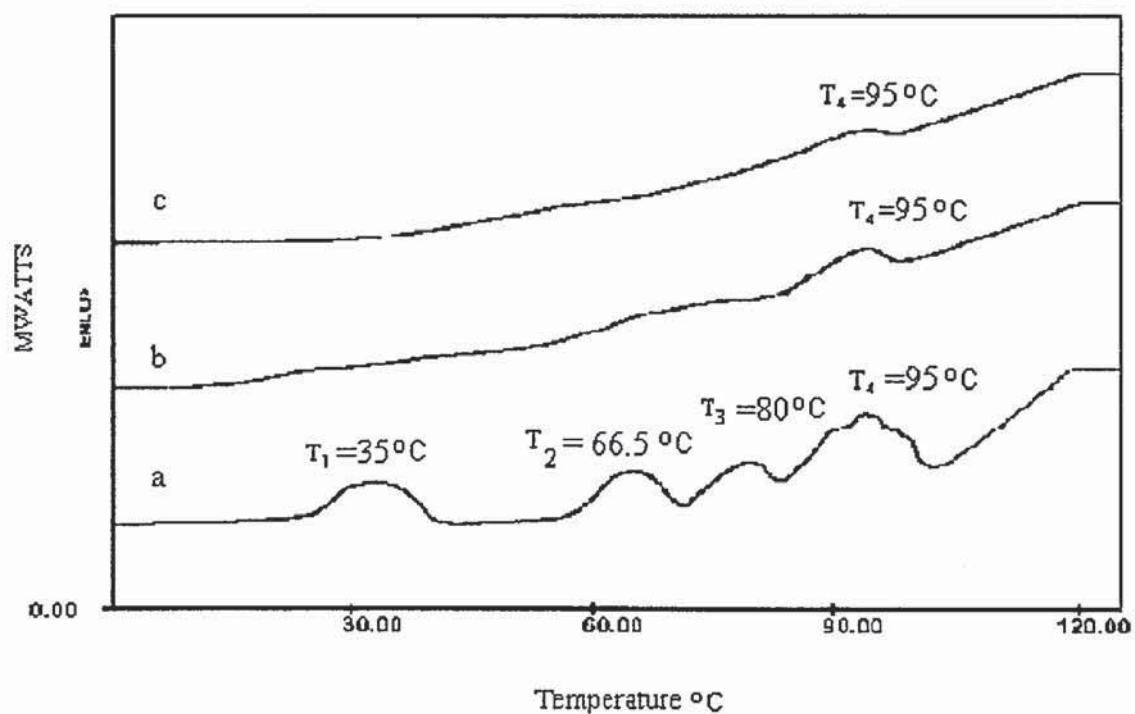


Figure 3.10 DSC thermogram of abdominal skin (a) untreated (b) 10% SDS pretreated (c) 10% DTAB pretreated

3.6.3 Permeation Studies

Results of permeation studies in Chapter 2 (section 2.4.3.1) demonstrated that the rate of flux of CHX across human epidermis was low. Hence, chemical enhancers were employed to promote the permeation of CHX across the epidermis in order to deliver the antimicrobial agent to the bacterial reservoir in the underlying layers of the skin. DSC studies showed that chemical enhancers can disrupt the SC structure and thus reduce the barrier properties of this layer. To study the effect of these enhancers on the flux of CHX across the epidermis, sheets of intact epidermis were pretreated by enhancer solutions and the permeation of CHX across this membrane was studied over 1 hour. The enhancers and co-solvents used in this study were chosen with reference to previous works investigating the effects of percutaneous absorption enhancers. Ethanol and propylene glycol were employed as a co-solvent because of their broad use in topical products. A synergistic enhancing effect has also been reported by many investigators for combined use of propylene glycol with oleic acid and Azone (Tanojo *et al.*, 1998). Ethanol has been shown to be an absorption enhancer in its own right by promoting the percutaneous absorption of aspirin (Levang *et al.*, 1999). Furthermore, ethanol and propylene glycol (PG) are both water miscible which indicates that they can be washed from the skin surface upon the pretreatment.

Saturated solutions of CHX in distilled water (with concentration of 0.78 ± 0.038 mg/ml and pH of 5.6) were used as the donor solutions in all the experiments. The pseudo-steady-state flux in each experiment was calculated from the linear part of the resulting profiles. Of particular interest is the permeability coefficient, which gives a numerical value to the rate of permeation of CHX independent of the exposure concentration or area of application. The effectiveness of penetration enhancers was determined by comparing CHX flux through pretreated skin with enhancers to CHX flux through the similar skin sample with no pretreatment:

$$\text{Enhancement factor} = \frac{\text{CHX flux following enhancer pretreatment}}{\text{CHX flux with no pre-treatment}} \quad (3.1)$$

These permeation parameters are summarised in table 3.1.

3.6.3.1 Permeation of CHX through Ethanol pre-treated Human epidermis

Permeation of saturated solution of CHX was studied across the untreated human epidermis (control) over a 60 minute period. No CHX was detected in the receptor samples for the first 8 minutes. The calculated pseudo-steady-state flux from the slope of the linear portion of the graph showed values indicating poor permeation rates.

In order to assess the effect of ethanol on the penetration of CHX across the skin, human epidermis was pretreated with neat and 10% aqueous solution of ethanol and permeation studies were once again performed over an hour period. The cumulative amounts of CHX penetrated across the skin per cm^2 were then plotted against time (figure 3.11) and the pseudo-steady-state fluxes and permeability coefficients were calculated from the initial linear portions of the resulting graph. It can be seen that neat and 10% ethanol pre-treatment can enhance the flux of CHX from $2.17 \times 10^{-4} \pm 1.34 \times 10^{-6}$ (control) to $0.001 \pm 2.87 \times 10^{-4}$ and $6.75 \times 10^{-4} \pm 2.89 \times 10^{-4} \text{ mg cm}^{-2} \text{ min}^{-1}$, respectively. The flux and permeability values are summarised in table 3.1. Although other researchers have correlated this enhancement with lipid extraction (Goaates and Knutson, 1994; Bommannan *et al.*, 1991), the DSC studies carried out here showed no such effect. Hence, another explanation is needed for the penetration enhancement effect of ethanol on CHX. Several published studies strongly suggest that the enhanced partition of solutes into the stratum corneum is the primary mode of action of ethanol as a permeation enhancer (Pershing *et al.*, 1990; Berner *et al.*, 1989). They have shown that the increased flux of solute across the epidermis is directly proportional to its concentration in the tissues, which was also increased by ethanol.

Ethanol pre-treatment of the epidermis also reduced the appearance time of CHX in the receptor (less than 1 min). Unexpectedly, the permeation graphs plateaued after 15 minutes. Plateau can be due to equilibration or the wash-out of the enhancer. Considering the CHX concentration in the donor phase ($0.78 \pm 0.038 \text{ mg/ml}$) and the amount penetrated after 15 minutes, one would expect a later equilibration time. Although the use of solutions in the donor phase (without any excess powder) results in a progressive decrease in thermodynamic activity of the drug as the source is depleted continuously and gives rise to a gradual plateauing in the rates of penetration, the early equilibration can not be explained by this fact. The possible explanation could be the binding of the CHX to the SC component which reduces the concentration of CHX in the donor phase. In the

case of enhancer being washed out, the membrane regains its barrier properties and therefore no more CHX can penetrate across the skin.

Surprisingly, neat ethanol did not induce a much higher enhancement ratio compared to 10% ethanol (4.6 and 3.1 respectively). This can be explained by the fact that at a 100% concentration of neat ethanol, the SC dehydrates (at low water concentrations, the keratins shrink) and the epidermis becomes more impermeable (Moellgaard and Hoelgaard, 1983). Hence, CHX transport does not increase as much as expected.

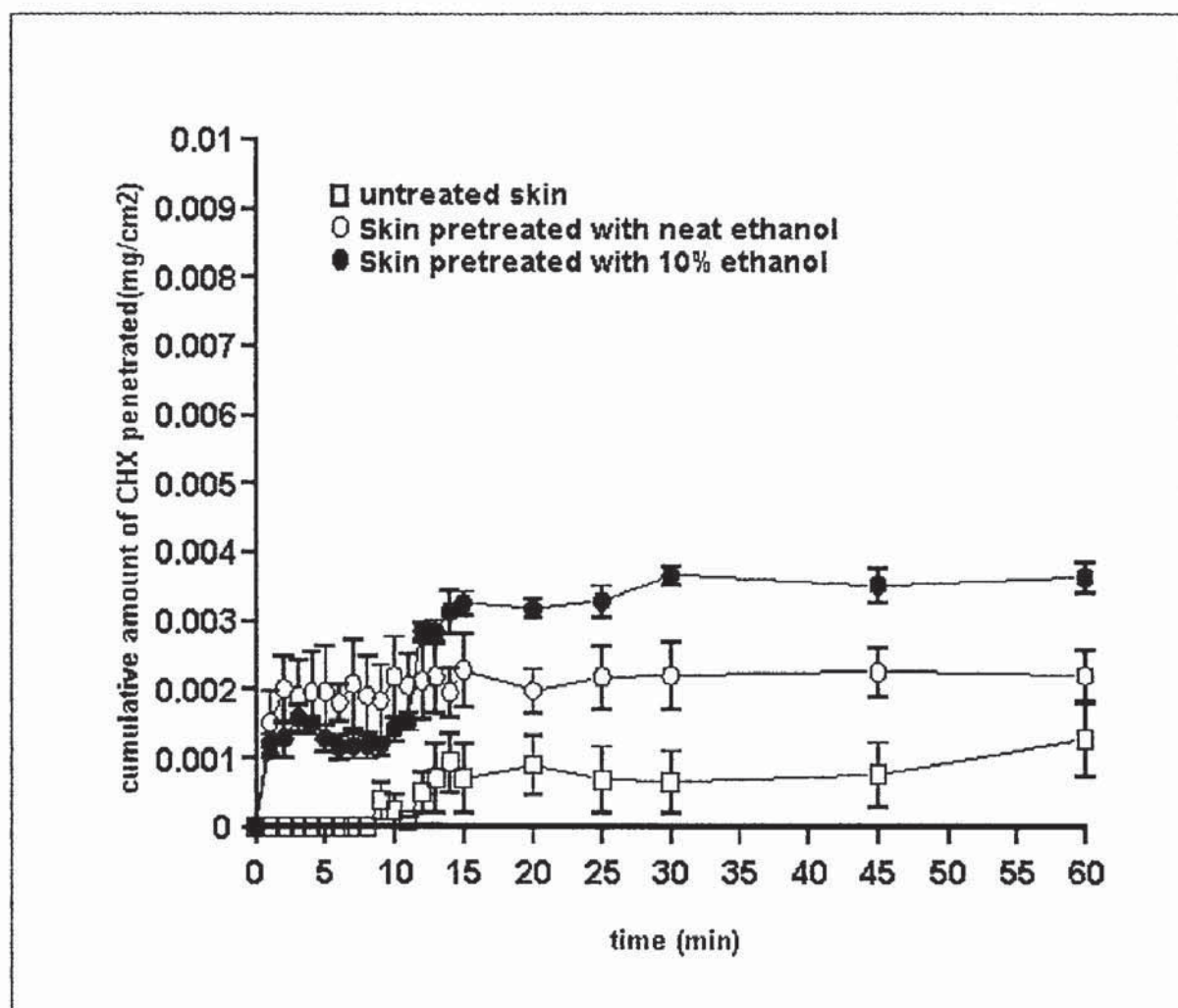


Figure 3.11 Passive permeation of a saturated solution of CHX across untreated human arm epidermis and following pretreatment with neat and 10% ethanol (each data point is the mean \pm s.d. of six determinations, $n=6$).

3.6.3.2 Permeation of CHX through DMSO pre-treated Human epidermis

The effect of DMSO on permeation of CHX across human epidermis is presented in figure 3.12. The concentration-dependence of this chemical enhancer can also be seen in this figure. Neat DMSO induced higher flux of CHX after 60 minutes ($J=3.93 \times 10^{-4} \pm 7.51 \times 10^{-5} \text{ mg cm}^{-2} \text{ min}^{-1}$) compared to 10% DMSO ($J=2.82 \times 10^{-4} \pm 3.123 \times 10^{-5} \text{ mg cm}^{-2} \text{ min}^{-1}$) in water and the control (no pretreatment, $J=2.17 \times 10^{-4} \pm 1.34 \times 10^{-6} \text{ mg cm}^{-2} \text{ min}^{-1}$). The permeability coefficients and the enhancement factors are summarized in table 3.1. Lag time was also reduced upon pretreatment of the skin as, after 1 minute CHX was present in the receptor phase. Permeation curves reach a plateau state after 15 min which can be explained by the reasons mentioned in section 3.6.3.1.

Considering the DSC thermogram of pretreated skin with DMSO (fig. 3.6) and the published results by other investigators, DMSO penetration enhancement property can be correlated to its effect on lipids and proteins of stratum corneum. By increasing lipid fluidity and solvation of the intercellular keratin, drug mobility and consequently drug transport increases.

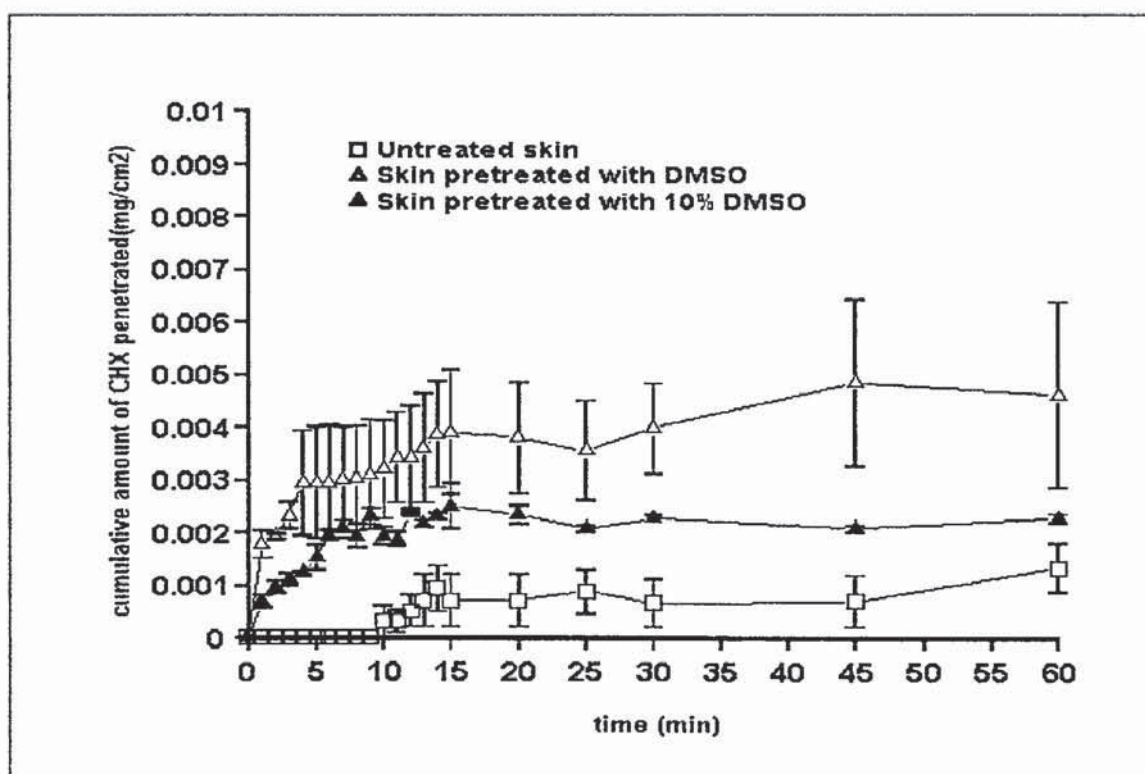


Figure 3.12 Passive permeation of a saturated solution of CHX across untreated arm epidermis and following pretreatment with neat and 10% DMSO (each data point is the mean \pm s.d. of six determinations, $n=6$).

3.6.3.3 Permeation of CHX through Propylene Glycol pre-treated Human epidermis

To investigate the effect of the employed cosolvent (PG), human skin was pretreated with neat PG for 1 hour. Results are presented in figure 3.13. Permeation of CHX was significantly increased (flux= $7.41 \times 10^{-4} \pm 3.34 \times 10^{-5} \text{ mg cm}^{-2} \text{ min}^{-1}$), indicating the effect of PG on the barrier properties of the skin. Similar results were reported by Mollgaard and Hoelgaard (1983), who studied the effect of PG on skin penetration of metronidazole and oestradiol. Similar to the previous pretreatment regimens, lag time was reduced to 1 minute upon pretreating the skin with PG.

DSC thermogram of SC pretreated with PG presented the effect of PG on the SC proteins. This could be the reason for the enhanced permeation. A further effect of PG on skin permeability arises from its solvent power for most chemicals. It promotes the drug partitioning into the skin, thus yielding higher fluxes (Mollgaard, 1993).

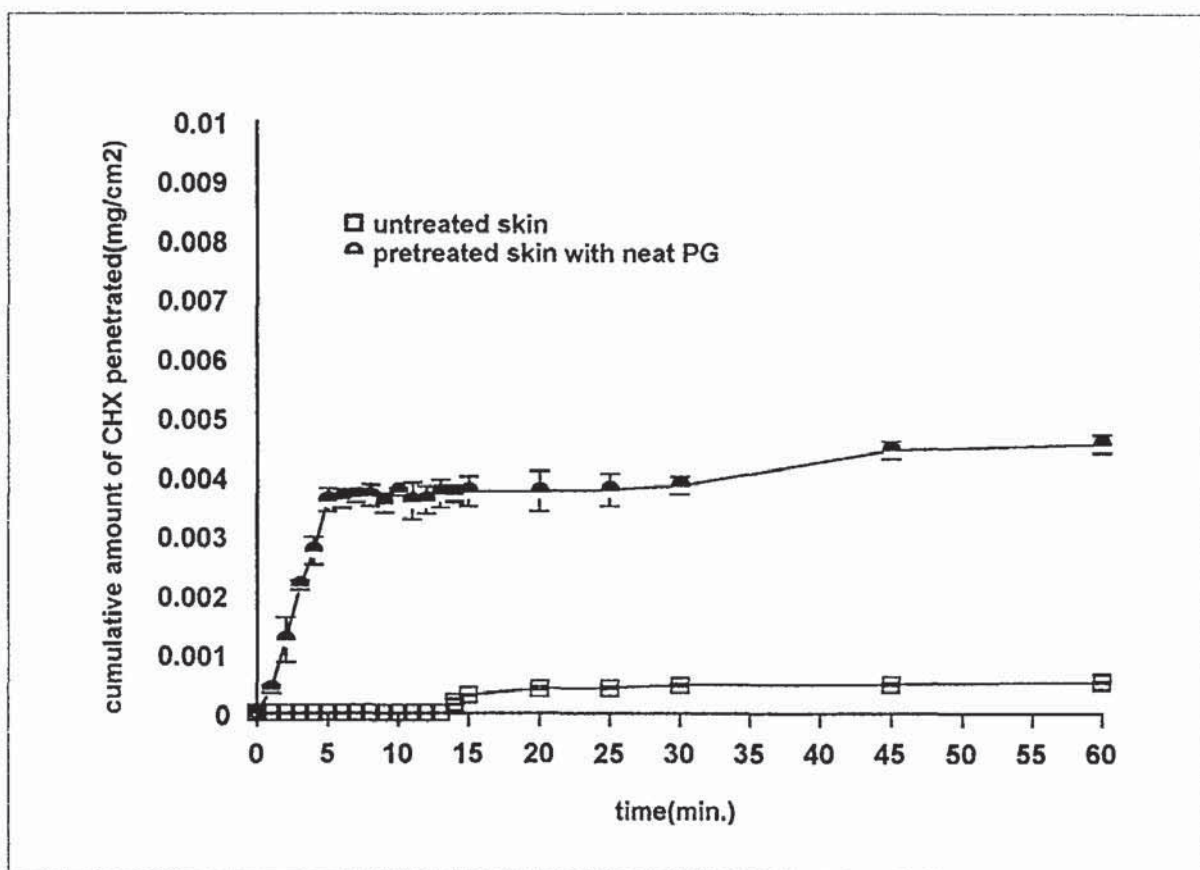


Figure 3.13 Passive permeation of a saturated solution of CHX across untreated human arm epidermis and following pretreatment with propylene glycol (each data point is the mean \pm s.d. of six determinations).

3.6.3.4 Permeation of CHX through Oleic acid pre-treated Human epidermis

Pretreatment of the skin with oleic acid was found to promote the permeation of CHX across human skin (fig. 3.14). The degree of enhancement was dependent on the employed cosolvents. When used neat, oleic acid showed only an enhancement factor of 2.94. 10% oleic acid in ethanol pretreatment was more effective compared to neat oleic acid in facilitating the penetration of CHX (about 4.8-fold increase). It has been suggested that the polar cosolvents such as ethanol can influence the enhancing potential of some lipophilic enhancers (Tanojo *et al.*, 1998; Wotton *et al.*, 1985). To investigate this effect, further studies were carried out using PG as the cosolvent. Results are depicted in figure 3.15 and shows that oleic acid in combination of PG can further increase the penetration of CHX across the skin ($\text{flux} = 0.0011 \pm 4.64 \times 10^{-4} \text{ mg cm}^{-2} \text{ min}^{-1}$). The permeation enhancement effect seen with oleic acid in ethanol or PG can not be connected to oleic acid only since the solvents themselves have permeation enhancement properties. Comparing the CHX permeation profile upon pretreatment of the epidermis with neat ethanol (figure 3.11) to the one upon 10% oleic acid/ethanol pretreatment (figure 3.14) shows no significant difference between CHX penetration rate ($P > 0.05$). Hence, we can conclude that 10% oleic acid possesses little enhancement properties on CHX transport. However, in the case of PG as the solvent, a significant difference ($P < 0.05$) can be observed in the CHX transport across pretreated skin with neat PG (figure 3.13) and 10% oleic acid/PG (figure 3.15). These results indicate a synergistic effect for oleic acid and PG when used in combination.

As discussed in section 3.2.6, oleic acid affects SC lipid either by increasing lipid fluidity or lipid-phase separation. Thus drug mobility and consequently drug diffusion in this less tightly packed arrangement can increase. Combining oleic acid with ethanol can further increase the CHX diffusion across the skin. According to Bhatia *et al.* (1997), one possible explanation is that oleic acid and ethanol treatment not only increases the average lipid acyl chain disorder of the treated stratum corneum but also leads to extensive lipid extraction. Any changes in the lipid matrix would result in the higher permeability. However, our DSC studies did not show any effect on lipid matrix upon ethanol pretreatment of SC. Hence, there should be another explanation for the observed enhanced permeability.

The highest penetration rate was observed with oleic acid/PG pretreatment. This could be due to the cosolvent's ability to reduce the polarity of the aqueous regions of the SC so increasing the solubility of the SC to solubilise oleic acid. In addition, Oleic acid affects the physical properties of the SC lipids without any influence on the proteins while PG interacts with SC proteins. Therefore, the disruption of SC structure is greater and the higher permeation is expected.

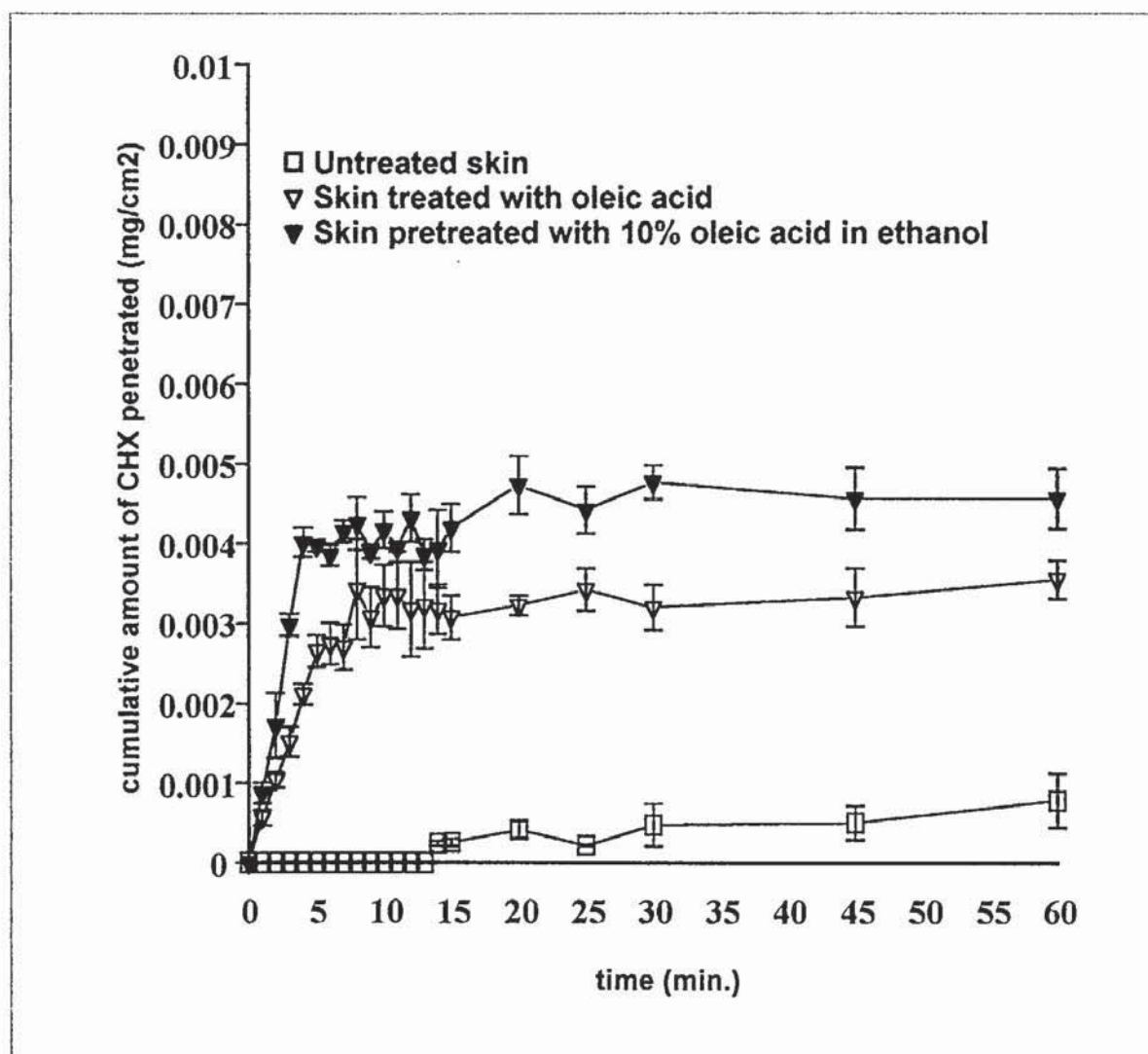


Figure 3.14 Passive permeation of a saturated solution of chlorhexidine dihydrochloride across untreated abdominal epidermis and following pretreatment with neat and 10% oleic acid in ethanol (each data point is the mean \pm s.d. of six determinations, $n=6$).

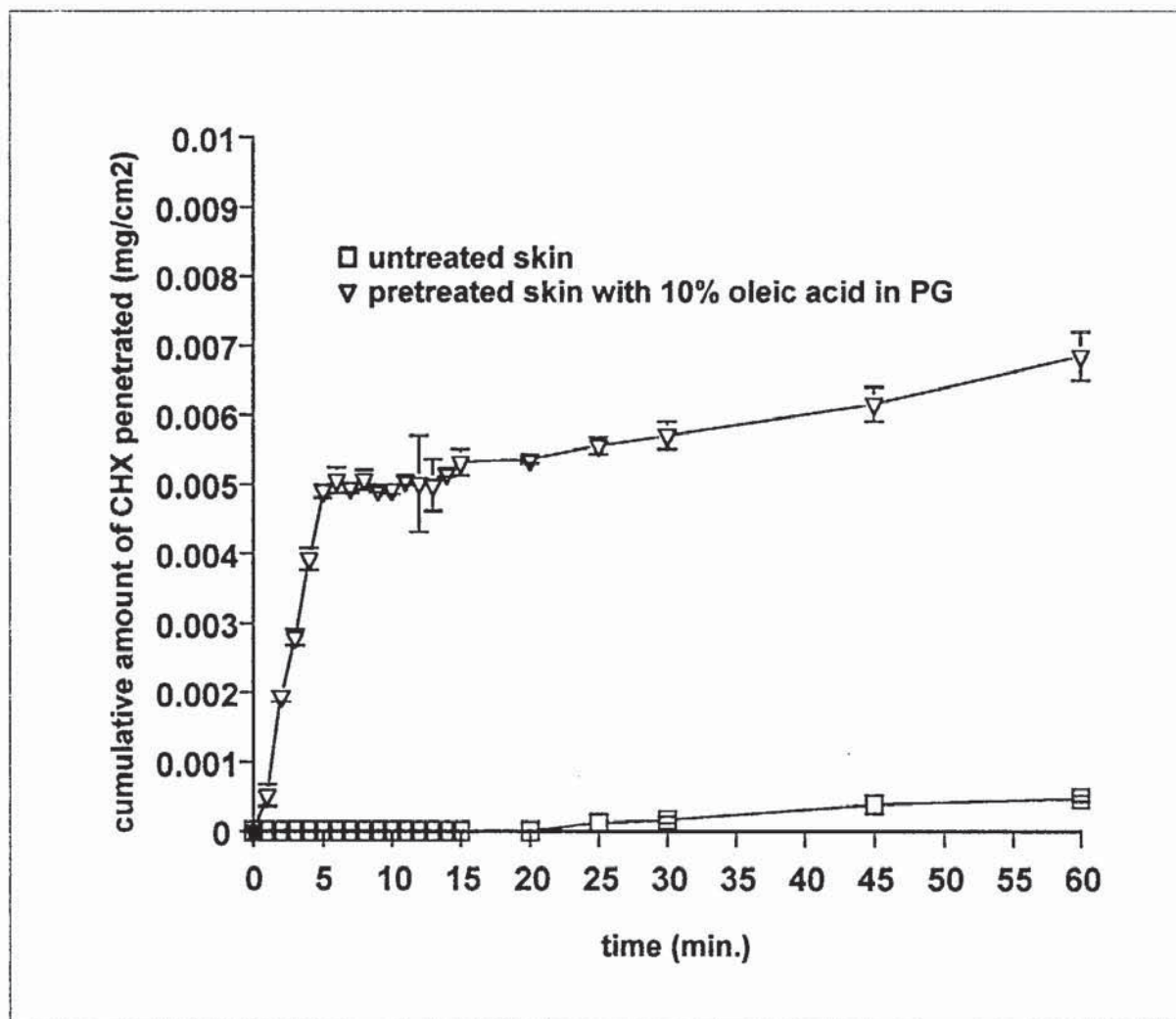


Figure 3.15 Passive permeation of a saturated solution of chlorhexidine dihydrochloride across untreated abdominal epidermis and following pretreatment with 10% oleic acid in propylene glycol (each data point is the mean \pm s.d. of six determinations).

3.6.3.5 Permeation of CHX through 10% Azone pre-treated Human epidermis

Figure 3.16 profiles CHX permeation across the human epidermis from a saturated donor solution as a function of Azone pretreatment. Azone pretreatment significantly elevated passive diffusion of CHX relative to that of untreated SC. CHX flux was enhanced from $2.1 \times 10^{-4} \pm 5.29 \times 10^{-5} \text{ mg cm}^{-2} \text{ min}^{-1}$ to $5.40 \times 10^{-4} \pm 3.79 \times 10^{-5} \text{ mg cm}^{-2} \text{ min}^{-1}$ following pretreatment of skin with 10% Azone/ethanol, indicating 2.6-fold increase. As shown in the DSC thermogram of SC pretreated with Azone (fig. 3.8), disruption of SC lipids could be the explanation for CHX enhanced permeation.

Azone was much more effective when used in conjunction with propylene glycol as the flux was elevated to $0.0013 \pm 5.19 \times 10^{-4} \text{ mg cm}^{-2} \text{ min}^{-1}$ (fig.3.17). Fluxes, permeability coefficients and the enhancement factors are summarised in table 3.1. An explanation for this behavior is that Azone enhances intercellular drug diffusion only; the intercellular protein contents, which offer considerable diffusional resistance, remain unaffected by this accelerant. However, unlike ethanol, PG affects the SC proteins, therefore enhances intracellular transport, so the Azone-PG combination is more effective. Aungst *et al.* (1986) had shown that the effects of a variety of penetration enhancers on naloxane absorption are vehicle-dependent. Wotton *et al.* (1985) demonstrated that the choice of vehicle was an important factor and that PG was necessary for promoting the effects of Azone on the permeation of metronidazole; similar effects have been reported for trifluorothymidine (Sheth *et al.*, 1986).

Azone pre-treatment also reduced the appearance time to 1 minute. Unlike other permeation profiles, the plotted graph of cumulative amount of CHX penetrated across the pretreated skin against time reached plateau after 30 minutes. The possible explanation can be the delayed washout of Azone from the skin as Azone is more lipophilic ($\log P=6.21$) compared to other enhancers. Hence, the pre-treated skin remains permeable to CHX longer. An increase in the permeability coefficient was also mediated by Azone application. The permeability coefficient is related to the partitioning of the permeant between the skin and donor medium, the diffusivity of the permeant within the skin and the thickness of the barrier. Since there was no evidence to show that skin thickness is reduced by Azone, enhanced penetration coefficient could be due to increased diffusivity within the SC and/or to enhanced partitioning tendencies.

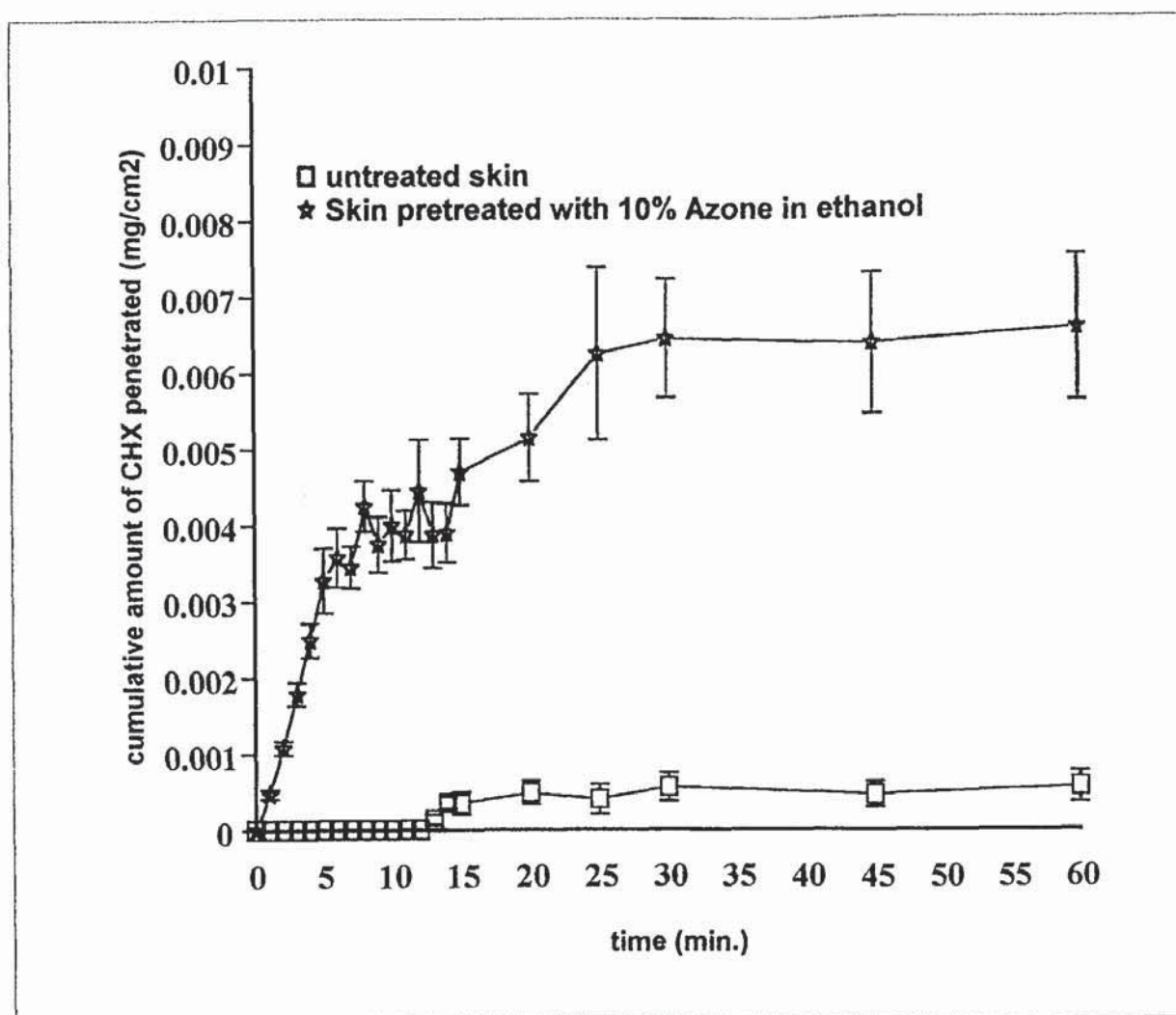


Figure 3.16 Passive permeation of a saturated solution of chlorhexidine dihydrochloride across untreated human breast epidermis and following pretreatment with 10% Azone in ethanol (each data point is the mean \pm s.d. of six determinations).

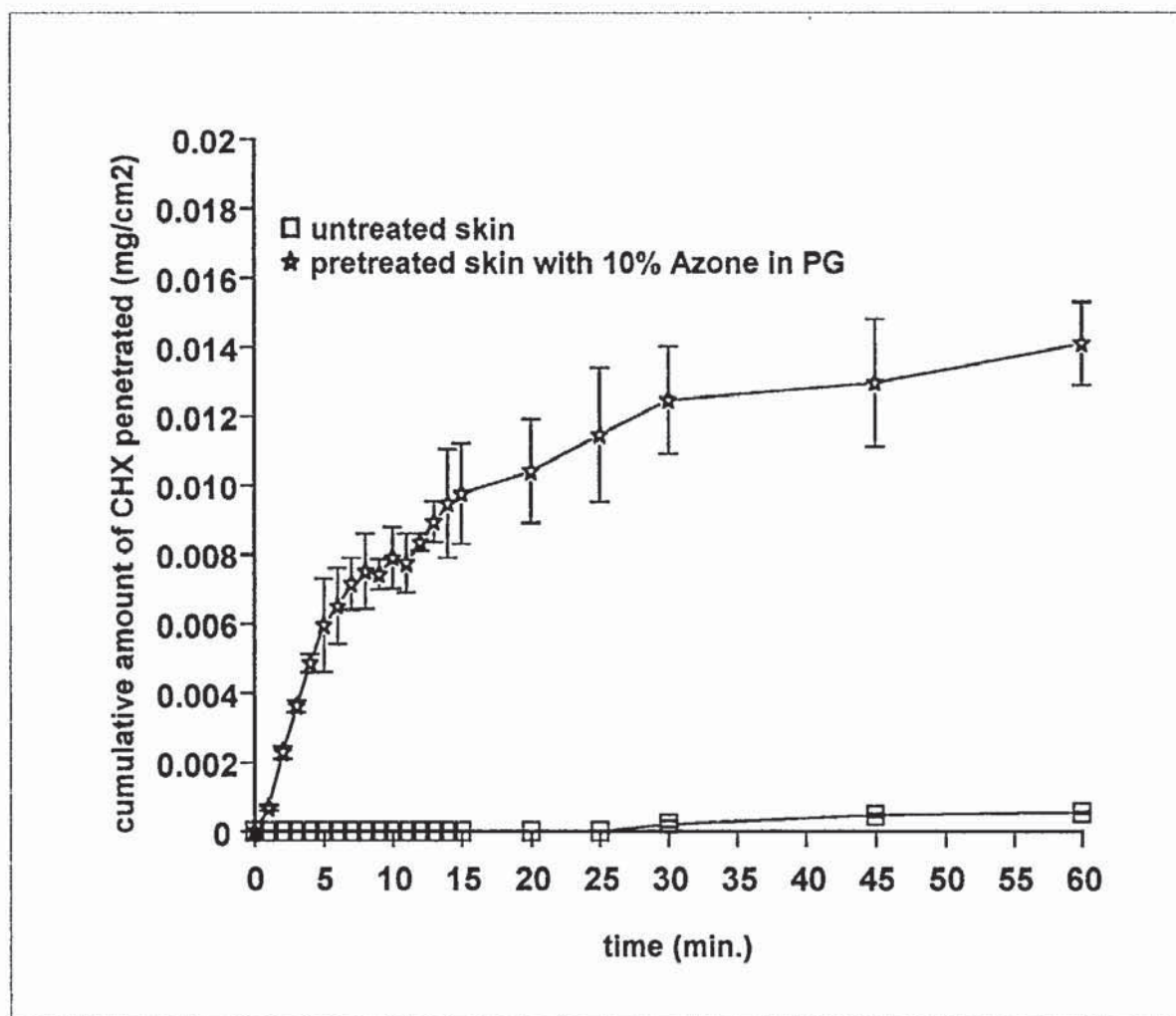
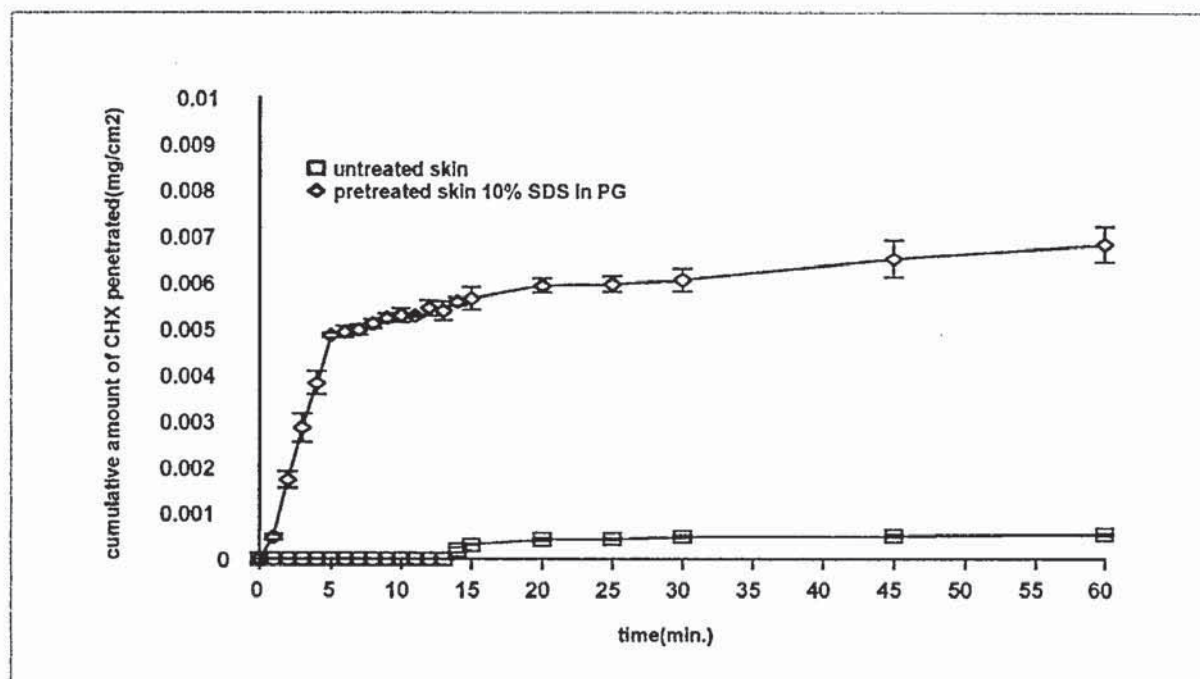


Figure 3.17 Passive permeation of a saturated solution of chlorhexidine dihydrochloride across untreated human breast epidermis and following pretreatment with 10% Azone in propylene glycol (each data point is the mean \pm s.d. of six determinations).

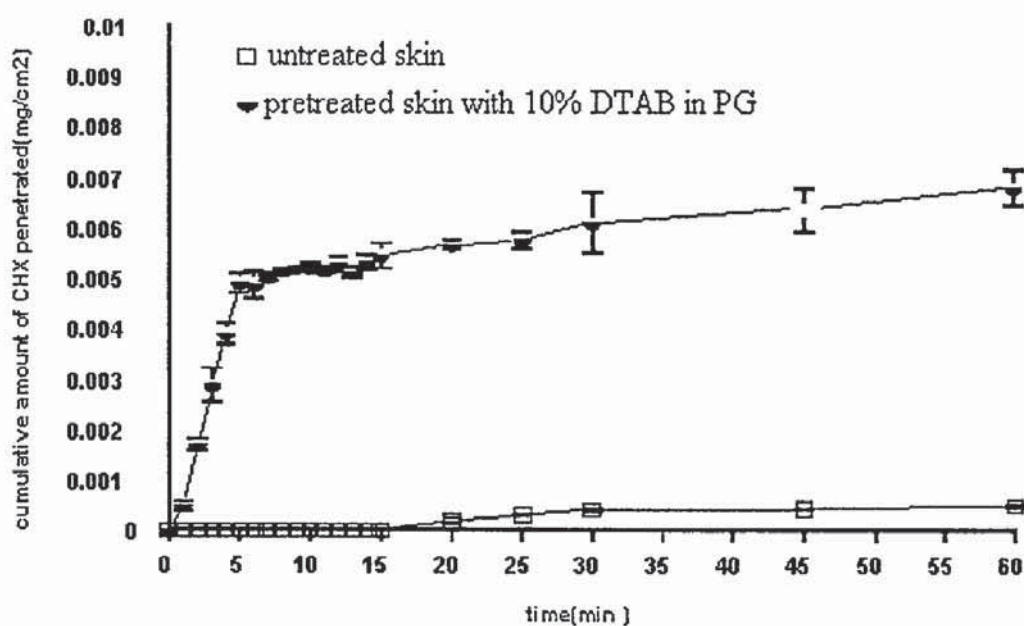
3.6.3.6 Permeation of CHX through Surfactant pre-treated Human epidermis

Passive diffusion of drugs could be enhanced by pretreating SC with surfactants (Ashton *et al.*, 1992). Pretreatment of SC with SDS (anionic surfactant) and DTAB (cationic surfactant) resulted in the increased permeation of CHX (fig. 3.18) when compared to the untreated one. The CHX flux was enhanced to $0.00101 \pm 4.74 \times 10^{-5} \text{ mg cm}^{-2} \text{ min}^{-1}$ upon SDS pretreatment and $0.00103 \pm 4.78 \times 10^{-5} \text{ mg cm}^{-2} \text{ min}^{-1}$ following DTAB pretreatment (table 3.1). Although DTAB, a cationic surfactant, is believed to result in a greater birefringence measurement when compared to its anionic counterparts (SDS), the difference between SDS- and DTAB-mediated enhancement ratio was negligible. One theory for the induced increase in penetration by SDS could be its charge, which can add to the skin negative charge, leading to enhanced attraction between membrane and permeant and thus enhancing the diffusion. It has also been shown that anionic surfactants induce swelling of the SC and the viable epidermis (Blake-Hoskins *et al.*, 1986) and destroy its integrity upon application (Rhein *et al.*, 1986).

Hadgraft *et al.* (1986) suggested ion-pairing as the mode of action for cationic surfactants. However, this can not be the sole means of transport for DTAB, as permeation of CHX, a weakly basic acid, across the human skin was enhanced by this cationic surfactant. Affecting the lipids and proteins in the SC as shown in the DSC thermograms of skin pretreated with SDS and DTAB, could be the mode of action of this enhancer.



(a)



(b)

Figure 3.18 Effect of surfactants on permeation of a saturated solution of chlorhexidine dihydrochloride across human arm epidermis (a) 10% SDS/PG (b) 10% DTAB/PG (each data point is the mean \pm s.d. of six determinations, $n=6$).

Pretreatment	Flux (mg cm⁻² min⁻¹)	Permeability Coefficient (cm min⁻¹)	ER
Neat Ethanol	0.001 (2.87×10 ⁻⁴)	1.28×10 ⁻³ (2.87×10 ⁻⁴)	4.6
10% Ethanol	6.75 ×10 ⁻⁴ (2.89×10 ⁻⁴)	8.65×10 ⁻⁴ (2.89×10 ⁻⁴)	3.1
Neat DMSO	3.93×10 ⁻⁴ (7.51 ×10 ⁻⁵)	5.04×10 ⁻⁴ (7.51 ×10 ⁻⁵)	1.87
10% DMSO	2.82×10 ⁻⁴ (3.123×10 ⁻⁵)	3.62×10 ⁻⁴ (3.123×10 ⁻⁵)	1.34
Neat PG	7.41×10 ⁻⁴ (3.34×10 ⁻⁵)	9.4×10 ⁻⁴ (4.27×10 ⁻⁵)	4.32
Neat Oleic acid	5.25×10 ⁻⁴ (9.71×10 ⁻⁶)	3.23×10 ⁻⁴ (9.71×10 ⁻⁶)	2.94
10% Oleic acid/EtOH	0.001 (4.54×10 ⁻⁴)	1.28×10 ⁻³ (4.54×10 ⁻⁴)	4.76
10% Oleic acid/PG	0.0011 (4.64×10 ⁻⁴)	1.41×10 ⁻³ (4.64×10 ⁻⁴)	5.24
10% Azone/EtOH	5.40×10 ⁻⁴ (3.79×10 ⁻⁵)	6.92×10 ⁻⁴ (3.79×10 ⁻⁵)	3.03
10% Azone/PG	0.0013 (5.19×10 ⁻⁴)	1.71×10 ⁻³ (5.19×10 ⁻⁴)	7.3
10% SDS/PG	0.00101 (4.74×10 ⁻⁵)	1.29×10 ⁻³ (3.21×10 ⁻⁵)	4.81
10% DTAB/PG	0.00103 (4.78×10 ⁻⁵)	1.32×10 ⁻³ (3.23×10 ⁻⁵)	4.76

Table 3.1 CHX Permeation data across the pretreated human epidermis, ER is the enhancement ratio, (values in brackets are standard error)

3.7 Summary

The results presented in this chapter demonstrate the ability of chemical enhancers to facilitate the passive diffusion of CHX across the skin with 10% Azone in PG showing the highest effect. Pretreatment of the epidermis with Azone/PG compared to other pre-treatment regimens increased the partition coefficient of CHX between skin and the vehicle (distilled water) to a higher extent. Separate application of ethanol or PG and oleic acid all improved the permeation of CHX across the skin, however, the efficiency of the combined application of oleic acid and ethanol or PG appeared to be higher. Cationic (DTAB) and anionic (SDS) surfactants had a similar but less effect on the flux of CHX compared to Azone/PG and oleic acid/PG pre-treatment. This enhanced CHX permeation can be useful in delivering the antimicrobial agent into the skin and thus eliminating the residing bacteria in the underlying layers.

CHAPTER FOUR

SUPERSATURATED SYSTEMS

4.1 Introduction

The importance of chemical potential or thermodynamic activity, in formulations was first recognised by Higuchi (1960) and later tested by Coldman *et al.* (1969). It is well established that the principal driving force for diffusion across the skin is thermodynamic activity of the permeant in the donor vehicle. This activity is reflected by the concentration of the permeant in the donor vehicle as a function of its saturation solubility within that medium. Figure 4.1, after Higuchi, shows a model of percutaneous absorption when the stratum corneum is the rate-limiting barrier and there are no interactions between the skin and the vehicle.

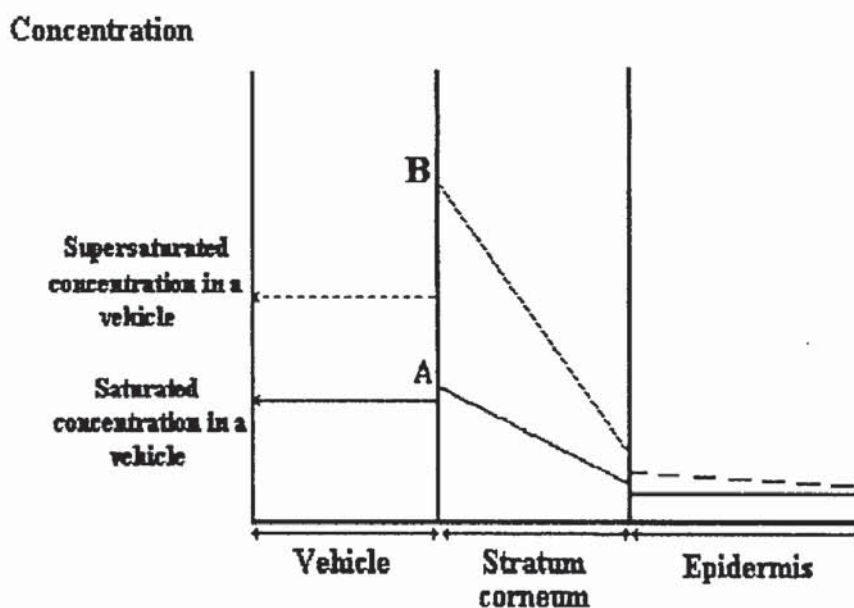


Figure 4.1 Model of percutaneous absorption (Higuchi, 1960)

The concentration of the solute within the stratum corneum is the driving force for the diffusional process and is dependant on the concentration of the solute in the vehicle (C_v) and the partition coefficient (P) of the solute between the vehicle and the stratum corneum.

$$C_{\text{skin}} = C_{\text{vehicle}} \times P \quad (4.1)$$

Hence, at equilibrium conditions, the flux will be dependent on concentration of solute in the vehicle. The closer to saturation the higher is the thermodynamic activity and the greater is the escaping tendency of the permeant from the vehicle.

According to the equation 4.1, there is an inverse relation between the saturated solubility of the solute in the vehicle and the partition coefficient between that vehicle and the skin, therefore, for all saturated systems, their product is a constant which is the saturated solubility of the solute in the stratum corneum (Poulsen, 1972).

Therefore, the flux of any given compound across a membrane from a saturated solution, irrespective of its concentration, is constant, provided that there are no interactions between the membrane and the components of the formulation (Twist and Zatz, 1986). Hence, under normal circumstances, the flux of a drug is limited by its solubility and can be expressed in terms of the thermodynamic activity of the penetrating agent the following equation:

$$J = \frac{a \cdot D \cdot A}{h \cdot \gamma} \quad (4.2)$$

where a is the thermodynamic activity, D is the diffusion coefficient, A is the cross-sectional area, h is the thickness of the membrane, and γ is the effective activity coefficient of the drug in the membrane.

From equation 4.2, it is apparent that the absorption of drugs can be accelerated by increasing the thermodynamic activity of the drug in the vehicle above unity and this can be achieved through the use of supersaturated systems, which contain concentrations of solute in excess of the saturated solubility.

Many authors have published experimental data to support the theoretical model. Poulsen *et al.* (1968) showed that partition coefficient and *in vitro* release of fluocinolone acetonide and its acetate ester were dependent on the thermodynamic activity of the drug in the vehicle and were at a maximum from saturated solutions. Other studies which show the importance of thermodynamic activity of the drug in the vehicle are the works of Dugard and Scott (1986), and Woodford and Barry (1982).

Based on the above reports regarding the dependence of flux of chemicals on their thermodynamic activities in the applied vehicles, supersaturated systems were employed in this study to assess their ability in increasing the permeation of chlorhexidine across the skin. The poorly soluble salt of chlorhexidine (hydrochloride salt) was chosen for this study to facilitate the study and to see if high activity can be obtained from this system. Moreover, using the hydrochloride salt would allow us to investigate the potential for a low concentration CHX product with an improved skin antiseptis.

4.2 Supersaturation

Supersaturation is the state where drug concentration in a vehicle is greater than the saturated solubility. Therefore, supersaturated solutions increase the activity of a drug, so that it has a greater leaving tendency, producing an increased flux (Pellet *et al.*, 1997). However, these systems, by nature, are physically unstable and, in many instances, the drug tends to crystallise upon preparation of the solution. This is because a solution that contains concentrations of solute in excess of the saturated solubility of a lower-energy solid state will undergo a phase change to form a suspension of the lower-energy solid in equilibrium with its saturated solution.

4.3 Nucleation and Antinucleation

Crystallisation in addition to supersaturation requires formation of a nucleus of critical size and then crystal growth around the nucleus. The formation of a critical nucleus happens by a process of random collision, the critical size depending upon the degree of

supersaturation. The growth of crystals may create undesirable changes in many pharmaceutical preparations, such as undissolved drug particles in the topical dosage forms which will cause a reduction in skin permeation. Crystal growth may also play an important role in polymorphic reversion. When polymorphism is present, the various crystal modifications of solid drugs can exhibit different physical characteristics such as different solubilities and dissolution rates (Davis and Hadgraft, 1993).

This problem can be overcome by the use of anti-nucleant polymers. Their mechanism of action is not yet known. They might act as a protective colloid, coating the incipient nucleus and preventing access of new molecules which would lead to crystal growth. Hydroxypropyl methyl cellulose (HPMC) and polyvinylpyrrolidone (PVP) have been shown to inhibit the crystal growth of paracetamol from saturated solution (Femi *et al.*, 1994). Simonelli *et al.* (1970) investigated the effect of PVP with different molecular weights on the inhibition of sulfathiazole crystals and suggested that PVP formed a net-like structure over a growing crystal which allows the sulfathiazole to grow out in finger-like crystals between the pores of the PVP network. Pellett *et al.* (1997) suggested that HPMC prevented the formation of a hydrate form of piroxicam, which was less soluble than its anhydrous form in aqueous based solvent systems and therefore solutions are supersaturated with respect to the hydrate form. Various reports regarding the inhibition of polymorphic transitions of drugs to more stable forms in the presence of polymers have been published. Miyazaki *et al.* (1976) showed the effect of additives on the polymorphic transformation of chlortetracycline hydrochloride crystals.

Other methods of crystal growth inhibition have also been investigated, such as the inhibitory effect of viscous systems. It was shown that hydroxypropylcyclodextrins have been able to supersaturate pancratistatin, an anti-cancer drug (Torres *et al.*, 1990) as well as inhibiting the crystal growth of isosorbide 5-mononitrate from tablets (Uekama *et al.*, 1985).

4.4 Preparation of Supersaturated Systems

In percutaneous drug delivery, there are several procedures by which supersaturated systems can be prepared.

4.4.1 Changes in Vehicle Composition

The potential of supersaturation in topical drug delivery was first demonstrated by Coldman *et al.* (1969). Using volatile:non-volatile systems, an increased flux of hydrocortisone alcohol across ethylene-vinyl-acetate membranes by evaporation of the volatile solvent from an acetone/water solution, has been reported (Theeuwes *et al.*, 1976). Kondo *et al.* (1987) showed an increase in flux through the synthetic membrane and rat skin by using ethanol and diethyl sebacate as volatile and non-volatile solvents in the study of supersaturated solutions of nifedipine. Also, evaporation of ethanol from an ethanol/propylene glycol/water gel resulted in supersaturation of hydrocortisone butyrate propionate with a corresponding increase in flux across polydimethylsiloxane membrane (Tanaka *et al.*, 1985). Loss of ethanol from an ethanol/propylene glycol/water solution led to supersaturation of minoxidil in the solution and a subsequent increase in percutaneous flux through human skin *in vitro* (Chiang *et al.*, 1989).

Other changes to vehicle composition, such as absorption of solvents may also lead to supersaturated systems and an increase in flux. Thus, Kondo *et al.* (1988) have suggested that the enhancing effect of N,N-diethyl-m-toluamide on the percutaneous absorption of nifedipine is caused by the formation of a supersaturated solution of nifedipine owing to loss of this solvent from the vehicle into the skin, rather than to direct effects on the skin barrier (Kondo *et al.*, 1988).

4.2.2 Mixed Cosolvent Systems

Saturated solubility plots will often show an exponential increase with solvent composition, depending on the relative polarities of the solute and the components of the cosolvent system (Figure 4.2). Curve BB' in figure 4.2 shows the saturated solubility of a solute in the mixed cosolvent system (ab). To produce such a curve, different ratios of solvent (a) and (b) should be added together followed by the addition of excess solute to

each system. Saturated solubilities in each system can be determined by the same procedure as described in section 2.3.1 and then plotted against the mixed solvent composition. By adding solvent (a) to saturated solute in solvent (b) in different ratios, determining the concentration of solute in the resultant mixed-solvent systems and plotting the determined concentrations against the mixed solvent composition, line AB can be produced. If the solute concentration in a mixed solvent system (e.g. C on line AB) is lower than the saturated solute concentration in the same system (C' on curve BB'), the produced solution is subsaturated. And, if the solute concentration in a mixed solvent system is higher (e.g. E on the line AB) than the saturated solute concentration in the same system (E'), the produced solution is supersaturated. The degree of supersaturation can then be calculated by dividing the value on the line AB by the value on BB' at each solvent composition.

Pellett *et al.* (1997) have shown an increased flux (about 9-fold) of piroxicam across stratum corneum by using mixed cosolvent systems of propylene glycol and water. Also, this procedure has been used successfully to prepare supersaturated solutions of oestradiol (Megrab *et al.*, 1995) and hydrocortisone acetate (Davis and Hadgraft, 1991) which caused an increased flux through human skin and silastic membrane. Schwarb and co-workers (1999) studied the transport of fluocinonide, a topical corticosteroid, in a mixed cosolvent system (propylene glycol, glycerol, ethanol and water) through silicone membrane. They concluded that supersaturated solutions prepared by the mixed cosolvent system, could enhance skin penetration of the drug. Moreover, the membrane transport of fluocinonide was proportional to the degree of saturation.

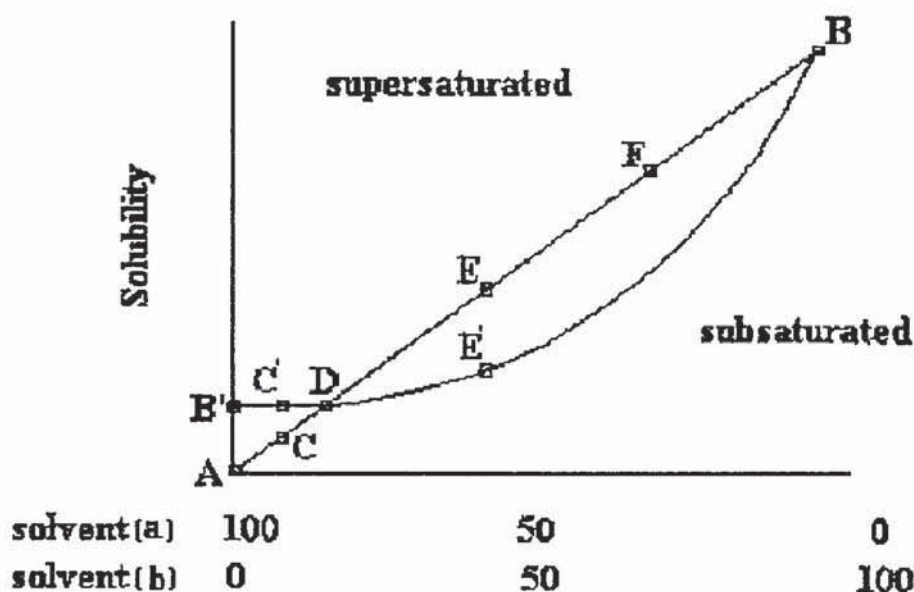


Figure 4.2 Saturated solubility of a solute in a binary cosolvent system. Effects of mixing: BB' is the saturated solubility curve of solute in binary cosolvent system ab. System B (saturated solute in solvent b) is mixed with system A (no solute in solvent a) to produce subsaturated, saturated, or supersaturated solutions exemplified by C, D and E, F respectively (Davis and Hadgraft, 1993)

4.4.3 Use of Amorphous States

Solid solutions or molecular dispersion systems were first produced by Chiou and Riegelman (1971) in an attempt to increase the dissolution rate of poorly water-soluble drugs. Dissolution of a solid dispersion will often result in generation of supersaturated systems depending on the conditions of dissolution system used (Hasegawa *et al.*, 1988). Similarly, amorphous states prepared by grinding with carrier or by deposition on a carrier have also been studied.

DSC studies of a mixture of tolinaftate with β -cyclodextrin showed a more amorphous state for this mixture compared to the original crystalline material (Szeman *et al.*, 1987). This mixture showed typical supersaturation behaviour on dissolution. When applied to

mouse skin, the tolnaftate and β -cyclodextrin mixture gave rise to a 2 to 3-fold increase in skin and blood levels over either tolnaftate alone or simple mixture of this drug with β -cyclodextrin polymer. Morita *et al.* (1985) prepared amorphous hydrocortisone acetate by grinding with crystalline cellulose or by forming dispersions by coprecipitation from ethanol with polyvinylpyrrolidone (PVP). *In vivo* vasoconstrictor studies in volunteers showed a general trend in blanching with increasing in amorphous nature of hydrocortisone acetate from both ground mixture and the coprecipitation, and *in vitro* dissolution studies demonstrated formation of supersaturation from the coprecipitation. Norman (1977) prepared coprecipitates of hydrocortisone with PVP. Supersaturated solutions with a degree of saturation of up to 12, were prepared by dissolution of the coprecipitate in water. Permeation studies showed enhanced transport, which was proportional to the degree of saturation, across a cellulose membrane.

4.2.4 Heating and Cooling

In general, saturated solubility of chemicals has direct relation with temperature. Henmi *et al.* (1994) prepared a gel of indomethacin by heating a mixture of the drug with hydrogenated soybean phospholipid and liquid paraffin and then cooling. There was a 10-fold difference in the permeation of the heated gel when compared to a gel prepared at room temperature.

4.2.5 Other Methods

One other system is to conduct a chemical reaction under conditions in which the new compound, which is less soluble in solution than the original starting solutes, become supersaturated and usually crystallise out of the solution.

Another method for preparing supersaturated solutions is addition of a substance to a solution which reduces the solubility of the solute. Substances which have a common ion with the drug under investigation can be added to the system. Due to the common-ion effect, the drug solubility will be reduced. Having an appropriate anti-nucleating polymer in the system prior to the addition of compound can result in supersaturation. In this

study, the novel method of common-ion effect was used to reduce the solubility of CHX. The common-ion effect will be explained in detail in the following sections.

4.5 Aims and Objectives

This chapter discusses the potential of supersaturated solutions in enhancing percutaneous absorption of CHX. Any correlations between the diffusion of CHX across the skin and the degree of saturation was also investigated. Moreover, this chapter serves to highlight the essential role of polymers in stabilising supersaturated states. By using the hydrochloride salt the potential for a low concentration CHX product with an improved skin antiseptics was also assessed.

4.6 Materials and Methods

4.6.1 Materials

Chlorhexidine dihydrochloride, sodium chloride, hydroxypropyl methyl cellulose (HPMC) (2% aqueous solution had a viscosity of 200 centipose), two grades of polyvinylpyrrolidone (PVP) (K25 and K30 with the average molecular weight of 24000 and 40000, respectively) were purchased from Sigma chemicals (UK). Human skin was obtained from the Plastic Surgery department of Selly Oak Hospital, Birmingham.

4.6.2 Effect of Increasing Chloride Ion Concentration on the Solubility of CHX

Solubility of chlorhexidine dihydrochloride in the presence of an increasing chloride ion concentration was determined at the room temperature ($20 \pm 1^\circ\text{C}$) in the same manner as described in section 2.3.1. Sodium chloride (0.1 to 0.3 M) was used as the source of chloride ion. After the equilibrium was reached (48 hours), 5 ml samples were removed from the test suspensions and filtered through a pre-saturated 0.2 μm membrane filter (GA-8S, Gelman Sciences). 1 ml of the filtered solution was then diluted 20-fold in a 20 ml volumetric flask using distilled water and assayed for CHX by HPLC as discussed in section 2.2.1.

4.6.3 Preparation of Supersaturated Solutions

Chlorhexidine dihydrochloride supersaturated solutions were prepared using the common-ion method described in section 4.4.5. Different amounts of NaCl (0.1, 0.2 and 0.3 M) were added to saturated solutions of CHX in order to reduce the solubility of CHX. Concentration of supersaturated solutions were determined by taking out 1 ml samples from each solution, filtering them through a pre-saturated 0.2 μm membrane filter (GA-8S, Gelman Sciences), diluting the samples with the distilled water (samples of solutions containing 0.1 M NaCl were diluted 50 times while the solutions containing 0.2 and 0.3 M NaCl were diluted 20 times), and injecting the diluted samples to HPLC.

Since precipitation (crystallisation) from supersaturated solutions requires the presence of nuclei of critical size, whether of the same molecular structure as the drug or otherwise, followed by crystal growth around the nuclei, most care was taken not to accelerate this phenomenon. Hence, all the glassware were thoroughly cleaned and dried before use.

4.6.4 Stability of Supersaturated Solutions

The effect of different polymer additives on the stability of chlorhexidine supersaturated solutions was investigated by measuring the concentration of chlorhexidine hydrochloride in solution at different intervals up to 4 hours.

Prior to the addition of sodium chloride, different concentrations of polymers (1-10% PVP and 0.5 % HPMC) were added to the systems. At each time point samples were taken out, filtered through a presaturated 0.2 μm membrane filter (GA-8S, Gelman Sciences), diluted with distilled water, and then analysed by HPLC as discussed in section 2.2.1. Solutions free from anti-nucleating polymer were used as controls. All the glassware was thoroughly cleaned and dried prior to use in order not to accelerate the crystallisations. All the experiments were repeated three times.

4.6.5 Effect of Anti-nucleating Polymers on the Solubility of CHX in the Presence of Chloride Ion

The effect of anti-nucleating polymers, 0-20% w/v PVP (K25), 0-10% w/v PVP (K30) and 0.5% w/v HPMC, on the solubility of CHX in the presence of chloride ion was also studied. NaCl, followed by the excess amount of CHX, was added to a series of polymer-water mixtures and the saturated solubilities were determined in the same manner as described in section 2.3.1 All the solubility determinations were carried out in triplicate.

4.6.6 Permeation Studies

To assess the effect of supersaturation on the transport of CHX across human skin, permeation studies were carried out over 30 minutes using Franz-type diffusion cells. The procedure was similar to the one explained in section 2.3.4 with the exception that 1 ml of

supersaturated solutions were placed in the donor compartment. As control, saturated solutions of CHX were used and their permeations were studied across the skin from the same source. Sampling of the receptor phase was done as previously described in section 2.3.4. The CHX concentration was determined by injecting 200 μ l of undiluted samples to HPLC in a similar manner to that described in section 2.2.1. All the experiments were carried out in six replicates.

4.7 Results and Discussion

The efficiency of topical formulation is dependent on the ability of drugs they contain to be released from their vehicle and diffuse onto and through the skin. As a passive diffusion process, the driving force for diffusion is the gradient of the chemical potential and therefore, the flux of a given drug through the skin is a function of the thermodynamic activity of the drug in the vehicle. Hence, by increasing the thermodynamic activity of the drug in the vehicle above the unity, we can accelerate the permeation of drugs into and through the skin. Enhanced thermodynamic activity can be achieved through the use of supersaturated systems, which contain concentrations of solute in excess of the saturated solubility. In this study, supersaturated solutions of CHX were prepared by using the common-ion effect method in order to promote the diffusion of CHX into the skin. Using sodium chloride as the chloride source (common ion), supersaturated solutions were prepared. To explain how the addition of chloride ions can result in the supersaturation state, we should first understand the solubility product (K_{sp}).

The solubility of chlorhexidine dihydrochloride (CHX.2HCl) in water may be presented in two steps, as



Since there is an equilibrium between the solute and saturated solution phases, the Law of Mass Action defines an equilibrium constant, K_{eq}

$$K_{eq} = \frac{\alpha \text{CHX.2H}^+_{(\text{solution})} \cdot \alpha \text{Cl}^-_{(\text{solution})}}{\alpha \text{CHX.2HCl}_{(\text{solid})}} \quad (4.2)$$

where α is the activity of each entity.

In a very dilute solution (e.g., for a slightly soluble salt), concentrations may be substituted for activities. In this situation K_{eq} has a special name, the solubility product (K_{sp}), thus the equation 4.2 can be rewritten as:

$$K_{sp} = [\text{CHX.2H}^+] [\text{Cl}]^2 \quad (4.3)$$

If K_{sp} is exceeded by the product of the concentration of the ions, then the equilibrium shown in equation 4.1 moves towards the left to restore equilibrium, and CHX will be precipitated. Thus, if the $[\text{Cl}]^2$ is increased, the concentration of $[\text{CHX.2H}^+]$ is expected to decrease in order to maintain a constant solubility product.

The decreased concentration of $[\text{CHX.2H}^+]$ would result in the precipitation of CHX out of the system. The CHX precipitation can be inhibited or retarded by anti-nucleating polymers and thus, the system will contain the concentration of CHX in excess of its solubility and therefore, it is supersaturated.

4.7.1 Solubility Determination

Using sodium chloride as the chloride source, the solubility of CHX in the presence of increasing concentrations of chloride was determined. As the chloride concentration was increased from 0.1 M to 0.3 M, the solubility of CHX decreased from $1.49 \times 10^{-1} \pm 2.12 \times 10^{-4}$ mg/ml to 0 respectively (zero value could be due to the limit of the detection) (figure 4.1). The reduced solubility of CHX with increasing chloride concentration is due to the common ion effect. The addition of a common ion reduces the solubility of a slightly soluble salt. This salting out occurs because the ions of the added electrolyte interact with water molecules and, thus, reduce the amount of water available for solution of CHX.

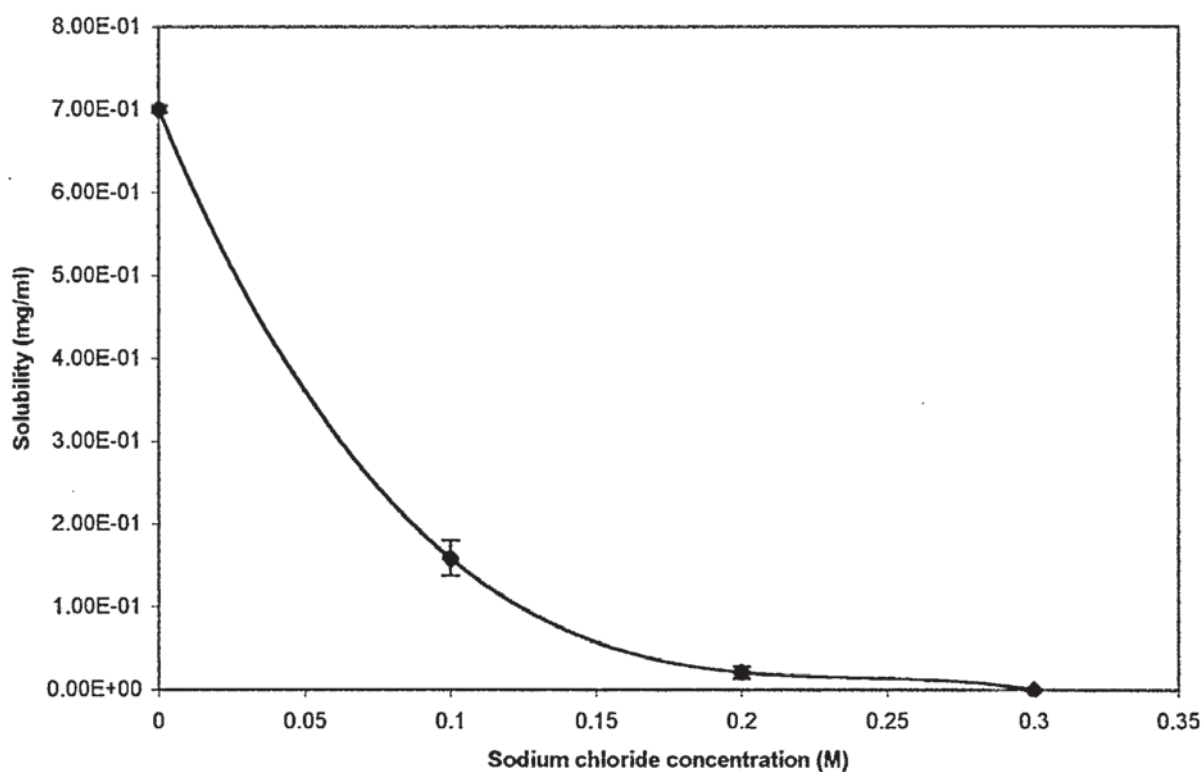


Figure 4.3 Solubility of CHX in the presence of increasing sodium chloride concentration (each point represents mean \pm s.d, n=3)

According to equation 4.3, if the chloride ions concentration increases, the concentration of dissolved chlorhexidine $[\text{CHX} \cdot 2\text{H}^+]$ will decrease in order to maintain a constant solubility product. Using the solubility data of CHX at each chloride concentration and equation 4.3, the K_{sp} was calculated to determine whether it was a constant value. The values obtained for K_{sp} are summarised in table 4.1.

Concentration of sodium chloride (M)	Solubility of CHX (mg/ml)	Solubility of CHX (M)	K_{sp} ($\text{mol}^3 \text{l}^{-3}$)
0	7.01×10^{-1} (4.22×10^{-3})	1.21×10^{-3} (7.30×10^{-6})	1.21×10^{-3} (7.30×10^{-6})
0.1	1.59×10^{-1} (2.12×10^{-2})	2.58×10^{-4} (3.66×10^{-5})	2.58×10^{-6} (3.66×10^{-7})
0.2	8.30×10^{-2} (7.21×10^{-3})	1.44×10^{-4} (1.25×10^{-5})	5.76×10^{-6} (5.00×10^{-7})
0.3	*	-	-

Table 4.1 Solubility product values using solubility of CHX in the presence of increasing sodium chloride concentration. *The value was possibly below the limit of detection. (Values in brackets represent standard deviations).

Although the CHX solubility was reduced with increasing chloride ion concentration, the solubility product of CHX did not remain constant but increased. This indicates that the common-ion effect is less pronounced than predicted from equation 4.3, and thus CHX is not truly a sparingly soluble salt.

4.7.2 Supersaturated Solutions of CHX with Different Degree of Saturation

Supersaturated solutions of CHX with the same concentration but different degrees of saturation were prepared. For this purpose, sodium chloride (0.1-0.3 M) was added to the saturated solutions of CHX in water. The initial CHX concentration of all systems was 0.701mg/ml. However, on addition of sodium chloride, CHX saturated solutions became cloudy over the time indicating the recrystallisation of CHX and hence, the reduction of the degree of saturation. To determine the degree of saturation, the CHX concentration was measured at each time point. 1 ml samples were taken out of the systems at the

appropriate intervals, filtered through a presaturated 0.2 μm membrane filter (GA-8S, Gelman Sciences), diluted with distilled water and assayed for CHX. The concentration of CHX was decreased over the time upon addition of sodium chloride and consequently, the degree of saturation was also decreased (figure 4.2). To simplify the results, $t_{1/2}$ (time that the initial CHX concentration is halved) for each system was calculated in order to compare the effect of different concentration of chloride ions on the CHX precipitation rate. Addition of 0.1 M sodium chloride reduced the initial CHX concentration ($0.701 \pm 4.22 \times 10^{-3}$ mg/ml) by half within 97 minutes ($t_{1/2}$) while 0.2 M sodium chloride reduced this time to 10.5 minutes. $t_{1/2}$ of the system containing 0.3 M sodium chloride was further reduced to 3 minutes indicating the increased rate of precipitation with increased chloride ion concentration.

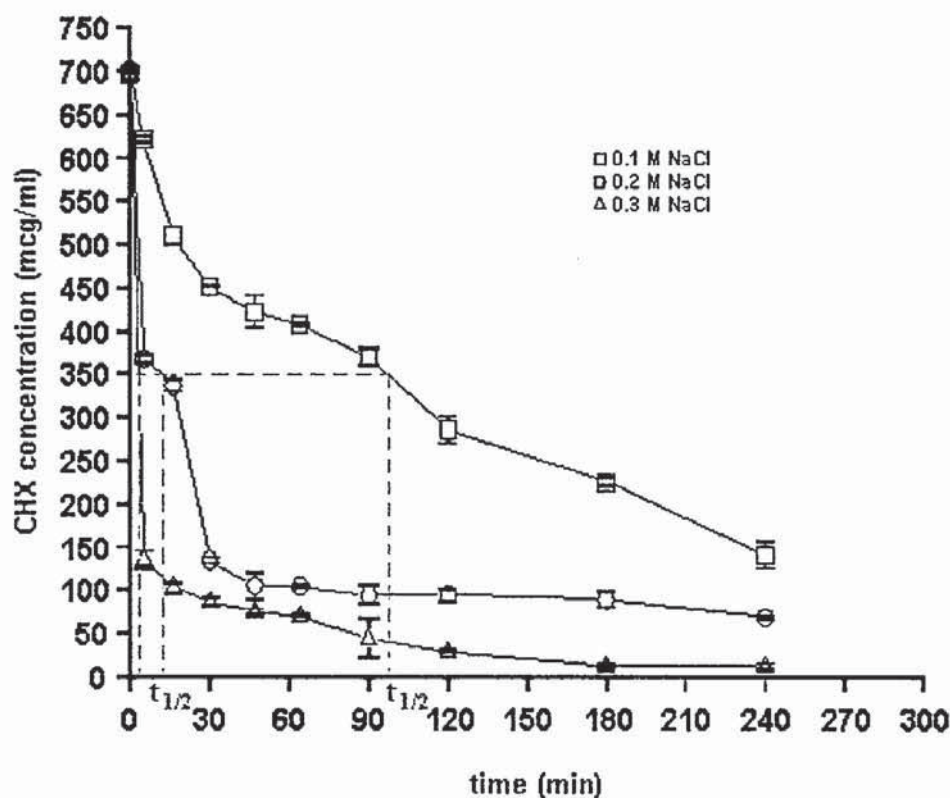


Figure 4.4 Reduction of solubility of CHX on addition of various concentration of NaCl (Points represents the mean \pm s.d., $n=3$)

The degree of saturation, DS, of each system can be calculated as:

$$DS = \frac{\text{Concentration of the drug}}{\text{Saturated solubility of the drug in the vehicle}} \quad (4.4)$$

Using the above equation, the degree of saturation for each supersaturated solution at each time point can be calculated. The obtained values are presented in table 4.2. At time zero, supersaturated solutions possessed the highest degree of saturation, however, as CHX started to precipitate out of the system the degree of saturation was lowered. At time zero, the degree of saturation for the system containing 0.1 M sodium chloride was 4.4 which was lowered to 2.8 after 30 minutes. The reduction of degree of saturation was accelerated as the concentration of chloride ions were increased. The initial degree of saturation for systems containing 0.2 M sodium chloride was 8.4 which was lowered to 1.4 after 60 minutes. The degree of saturation of each system at different time is presented in table 4.2. Due to the zero value obtained for the solubility of CHX in 0.3 M NaCl, the degree of saturation for these supersaturated systems could not be calculated.

Time (min)	Degree of Saturation	
	System A	System B
0	4.4	8.4
5	3.9	7.3
15	3.2	4.4
30	2.8	2.6
45	2.7	1.5
60	2.6	1.4

Table 4.2 Degree of saturation of supersaturated systems at each time point, (System A = supersaturated system containing 0.1 M NaCl, System B = supersaturated system containing 0.2 M NaCl)

4.7.3 Effects of Anti-Nucleants on the Stability of Supersaturated Solutions

It has been demonstrated that drug permeation across the skin increases with drug thermodynamic activity beyond saturation to a supersaturated level (Davis and Hadgraft, 1991). However, such systems are thermodynamically unstable and the supersaturated drug has a tendency to form crystals, which may cause a reduction in skin permeation. Therefore, the control of drug crystallization is essential for the efficiency and quality of the topical supersaturated systems.

Some polymers have been shown to inhibit nucleation and sustain supersaturation for prolonged periods of time (Raghavan *et al.*, 2000; Schwarb *et al.*, 1999; Pellett *et al.*, 1997). Polyvinylpyrrolidone (PVP) and hydroxypropyl methyl cellulose (HPMC) are two polymers which have been used extensively for this purpose. Femi-Oyewo and Spring (1994) have shown the ability of HPMC and PVP to inhibit the crystal growth of paracetamol from supersaturated solutions. Raghavan *et al.* (2000) have also shown the ability of HPMC to stabilise the supersaturated solutions of hydrocortisone acetate.

Based on these reports and the fact that the prepared CHX supersaturated solutions were unstable (section 4.7.2), PVP and HPMC were used to stabilise the CHX supersaturated systems.

PVP is a synthetic polymer consisting essentially of linear 1-vinyl-2-pyrrolidinone groups, the degree of polymerisation of which results in polymers of various molecular weights. It is characterised by its viscosity in aqueous solution, relative to that of water, expressed as a K-value, ranging from 10-120. The K-value is related to viscosity by the equation shown below:

$$\log z = c \left(\frac{75 k^2}{1 + 1.5 kc} \right) + k \quad (4.5)$$

where z is the relative viscosity of the solution of concentration c , k is the K-value $\times 10^{-3}$, and c is the concentration in % w/v.

PVP is used as a suspending, stabilising or viscosity-increasing agent in a number of topical solutions and suspensions. The solubility of a number of poorly soluble active drugs can also be enhanced by mixing with PVP. The structure of PVP is illustrated in figure 4.5.

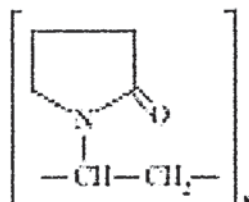


Figure 4.5 Structure of PVP

Two different molecular weights of PVP, 24000 (K25) and 40000 (K30), were used in this study and their ability to inhibit or retard the recrystallisation of CHX in supersaturated systems were assessed. PVP K25 at the concentration range of 1-10% w/v was added to the saturated solutions of CHX followed by sodium chloride (0.1-0.3 M). Systems with no polymer were used as control. (Due to increasing viscosity which may hinder the permeation process, concentrations of up to 10% w/v of the polymers were only used).

The concentration of CHX at each time point was determined in the same manner as that described in section 4.7.2. Crystallisation of CHX in supersaturated solutions free from anti-nucleating polymer proceeded quickly as the chloride ion concentration increased. However, crystallisation was retarded when the polymers were added. At a constant chloride ion, increasing the amount of polymer in the system resulted in a more stable supersaturated system. Results are depicted in figure 4.6-4.7. $t_{1/2}$ values (time point at which the CHX concentration was reduced by half) of each system were calculated and used to study the effect of different concentrations of PVP K25 on the supersaturated systems. The system with the highest value of $t_{1/2}$ was considered to be the most stable system. In the absence of PVP, supersaturated solution containing 0.1 M sodium chloride showed a $t_{1/2}$ of 97 minutes however, when PVP (K25) was introduced to the mixtures at

the concentration of only 1% w/v, $t_{1/2}$ was prolonged to 118 min. The prolonged $t_{1/2}$ indicates the ability of PVP (K25) to retard the recrystallisation of CHX in the supersaturated system. Moreover, $t_{1/2}$ was further prolonged to 191 min. and >240 min. by increasing the concentration of the anti-nucleant polymer to 5% and 10% respectively. $t_{1/2}$ values are summarized in table 4.2.

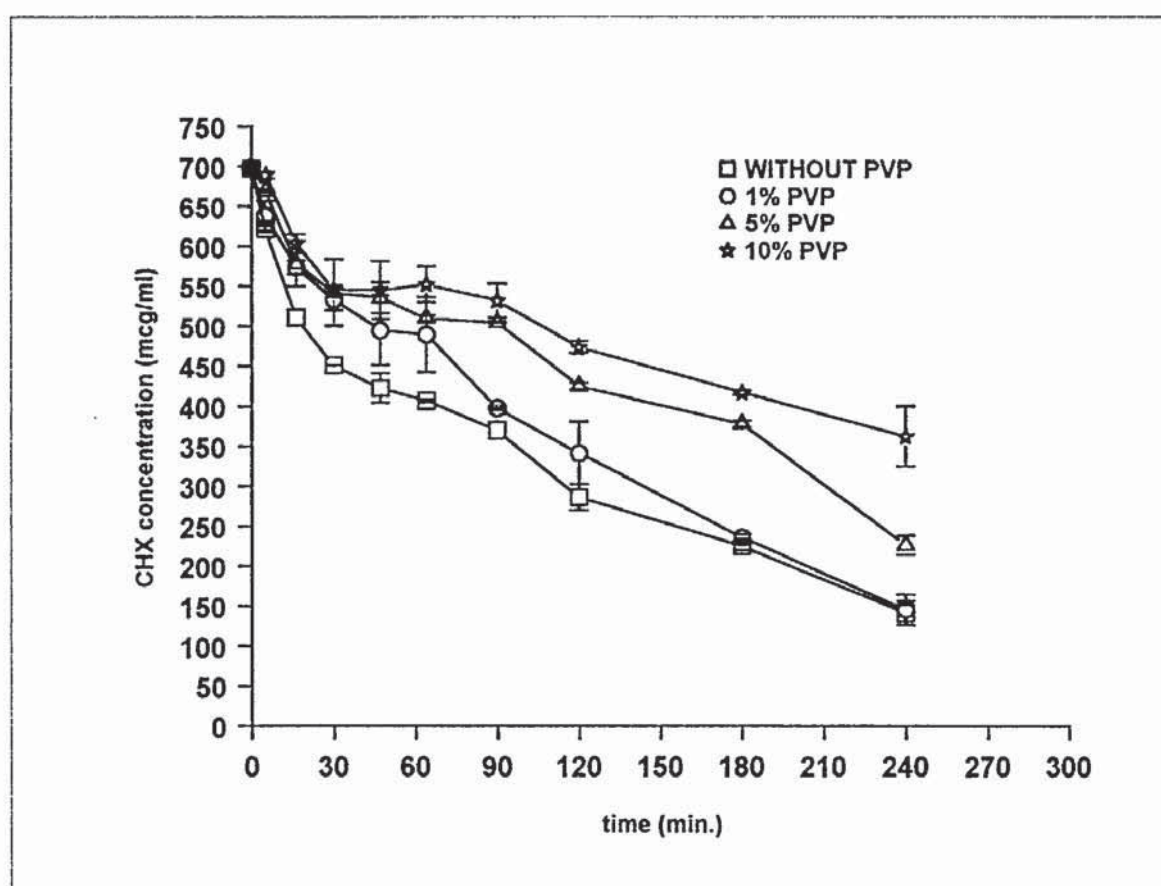
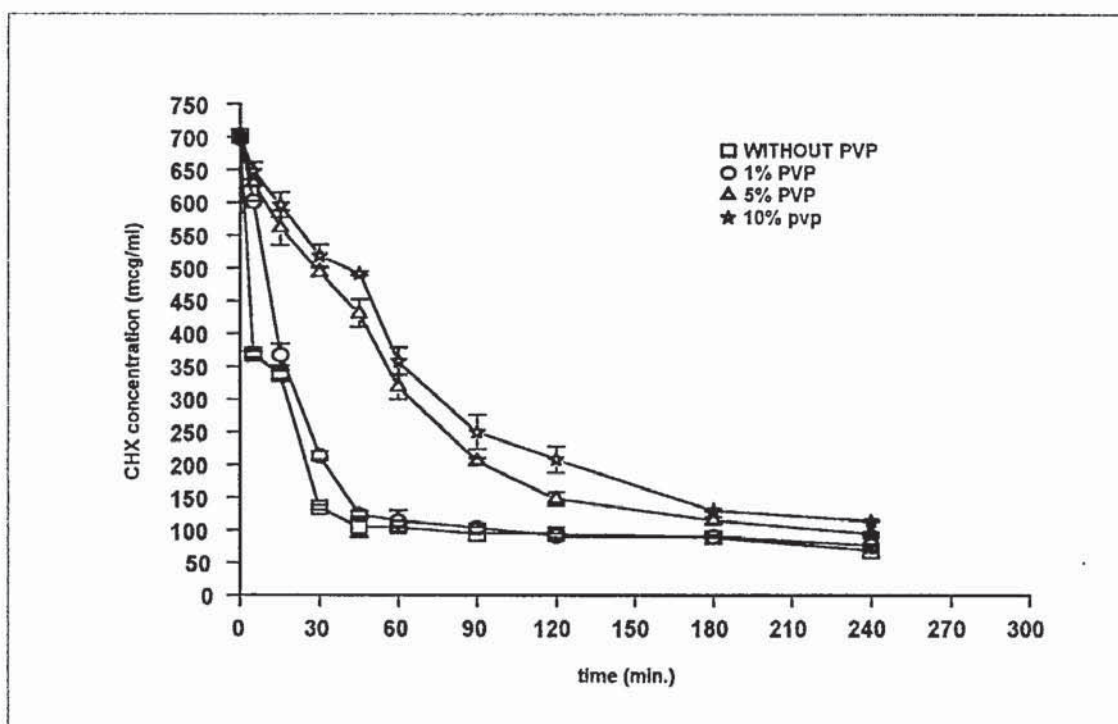
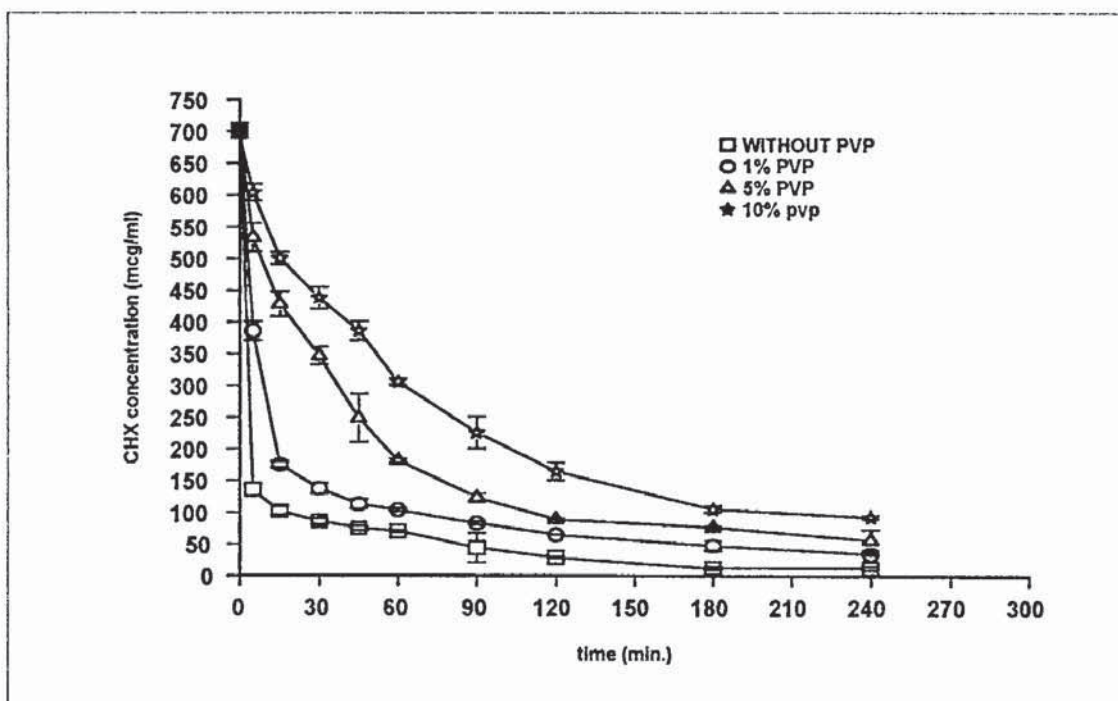


Figure 4.6 Reduction of solubility of CHX on addition of 0.1 M NaCl in the presence of PVP (K25) (Points represent the mean \pm s.d., $n=3$)



(A)



(B)

Figure 4.7 Reduction of solubility of chlorhexidine hydrochloride on addition of (A) 0.2 M NaCl (B) 0.3 M in the presence of PVP (K25) (Points represent the mean \pm s.d., $n=3$)

To investigate whether a more stable supersaturated solution of CHX can be achieved, PVP K25 was replaced with its higher molecular weight counterpart (PVP K30) and the ability of this polymer to stabilise the supersaturated systems was assessed. 1-10% PVP (K30) was added to the saturated solutions of CHX followed by different concentration of NaCl (0.1-0.3 M). The concentration of CHX at each time point was determined in the same manner as described in section 4.7.2. CHX concentration at each time point was plotted against time and then $t_{1/2}$ of each graph was calculated (Figure 4.8-4.9). PVP (K30) was shown to be more effective compared to its lower molecular weight counterpart. There was also an increase in efficiency when the polymer concentration increased from 1% to 10% w/v. $t_{1/2}$ of the supersaturated system containing 0.1 M NaCl was prolonged from 97 min (no polymer) to 121, 198 and >240 min upon addition of 1%, 5% and 10% w/v PVP (K30) to the system, respectively (fig.4.8-4.9). The $t_{1/2}$ values for each system are summarized in table 4.3.

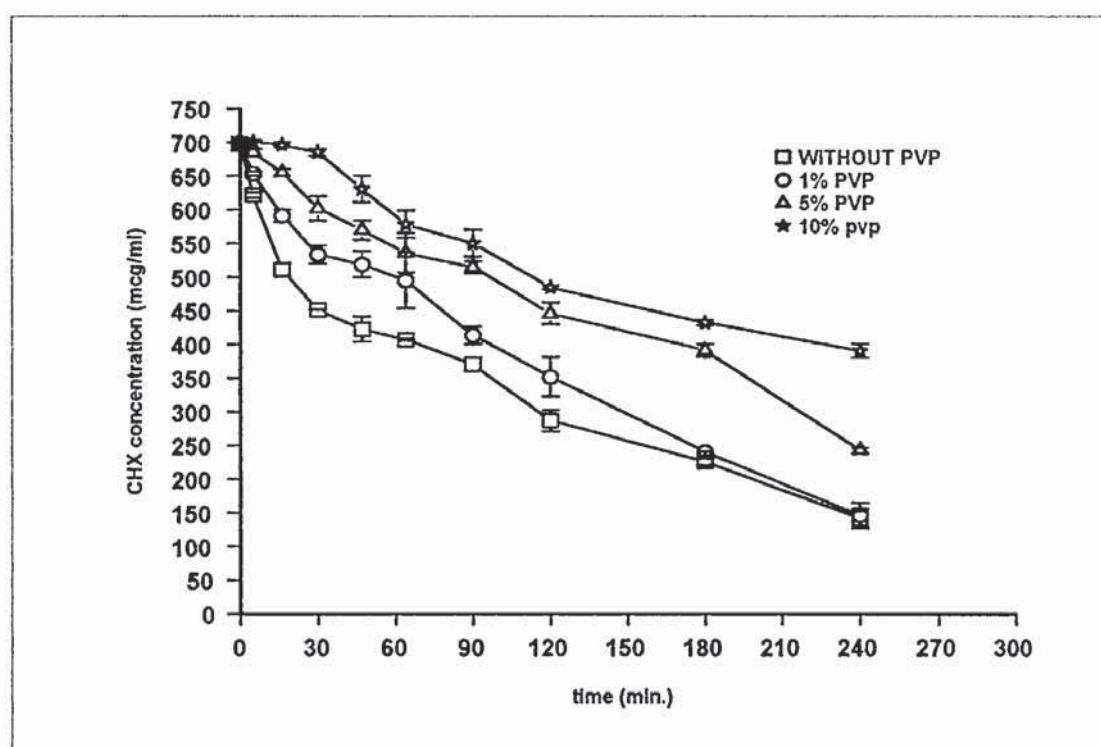
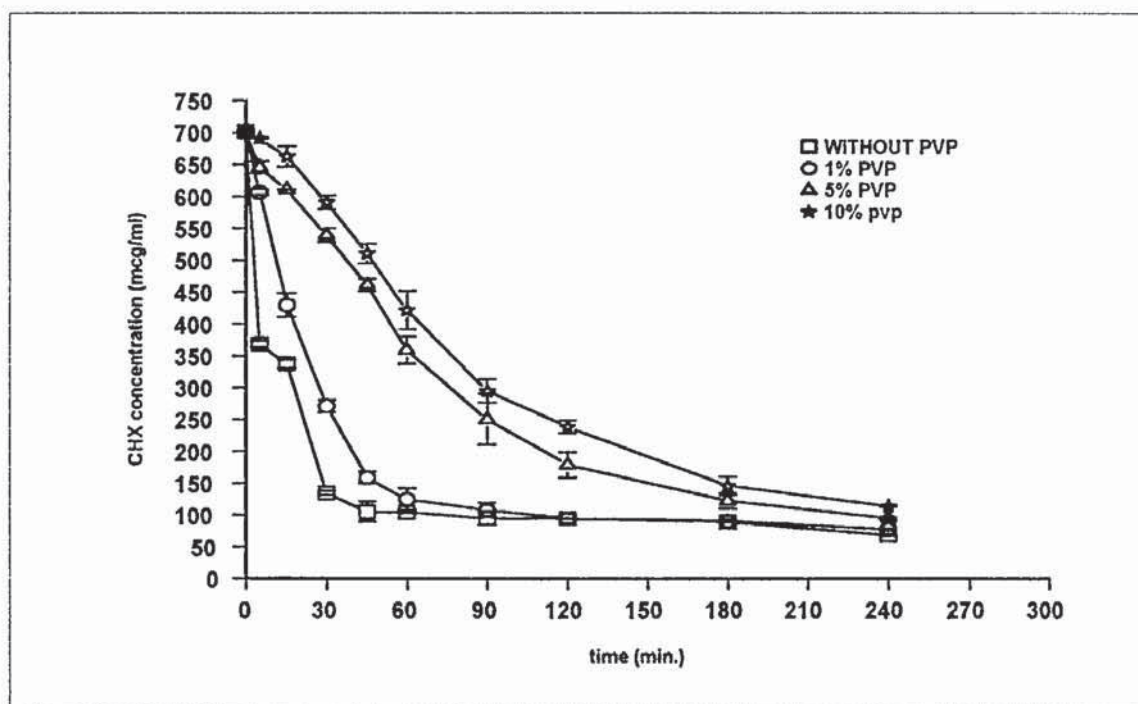
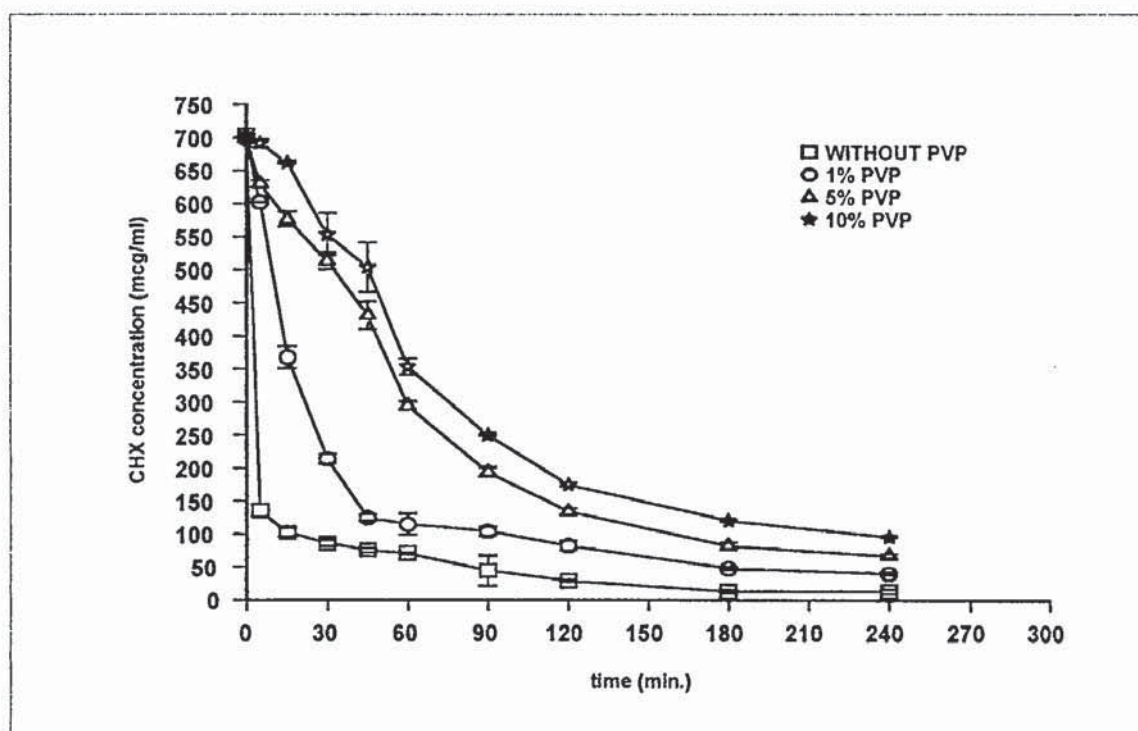


Figure 4.8 Reduction of solubility of CHX on addition of (A) 0.1 M NaCl in the presence of PVP (K30) (Points represent the mean \pm s.d., $n=3$)



(A)



(B)

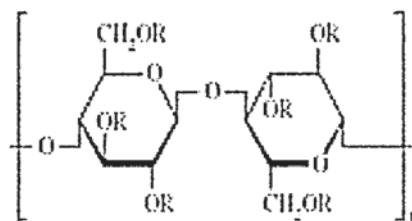
Figure 4.9 Reduction of solubility of CHX on addition of (A) 0.2 M NaCl (B) 0.3 M in the presence of PVP (K30) (Points represent the mean \pm s.d., $n=3$)

	Control (no polymer)	PVP (K 25) (% w/v)			PVP (K 30) (% w/v)		
		1	5	10	1	5	10
System A	97	118	191	>240	120	198	>240
$t_{1/2}$ (min) System B	10.5	18	56	68	24	63	78
System C	3	6	30	54	18	54	62

Table 4.3 $t_{1/2}$ of supersaturated systems ($t_{1/2}$ = time when the initial concentration of CHX (0.701mg/ml) is reduced by half; System A, B and C contain 0.1, 0.2 and 0.3 M NaCl, respectively)

Comparing the $t_{1/2}$ values, it can be seen that PVP can retard the recrystallisation of CHX in supersaturated systems. Moreover, PVP (K30) is more effective compared to its lower molecular weight counterpart in stabilizing the supersaturated solutions of CHX.

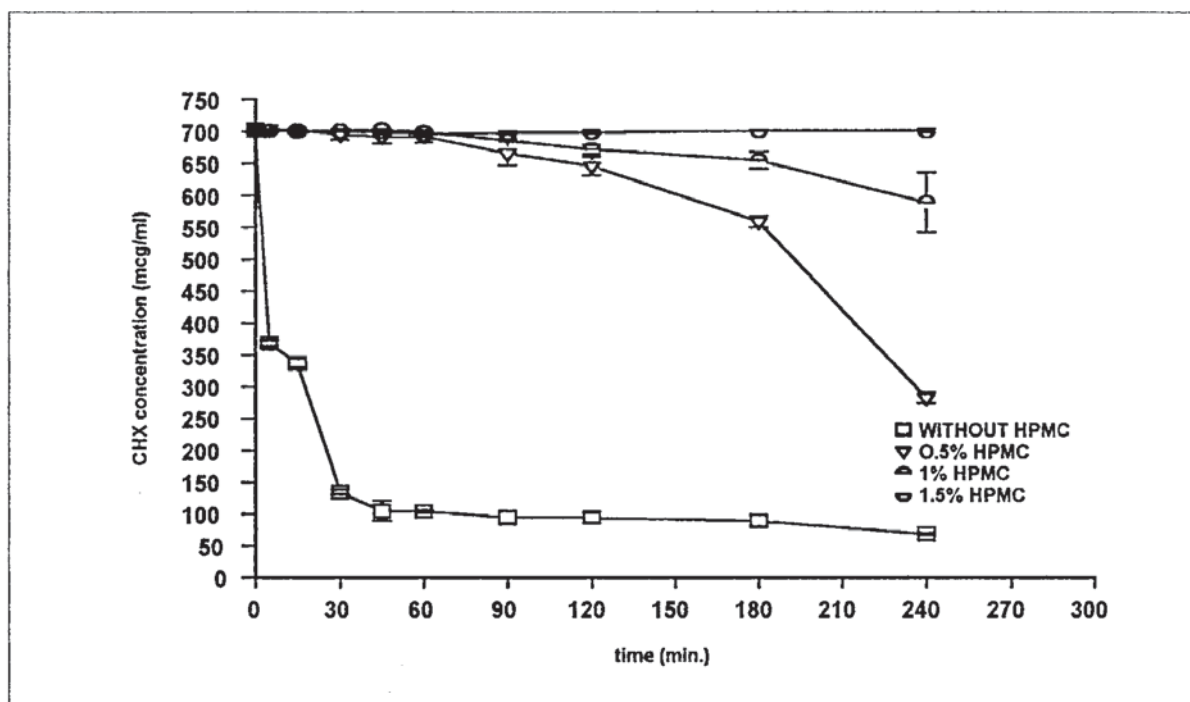
The next employed polymer was HPMC. HPMC has been described as a partly O-methylated and O-(2-hydroxypropylated) cellulose. It is available in several grades which vary in viscosity and extent of substitution. Grades may be distinguished by appending a number indicative of the apparent viscosity, in mPa, of a 2% w/w aqueous solution at 20°C. This polymer has been widely used as a suspending, stabilising and thickening agent in topical pharmaceutical formulations. HPMC structure has been shown in figure 4.10. A 2% w/w aqueous solution of HPMC used in this study had a viscosity of 200 centipose at 20°C.



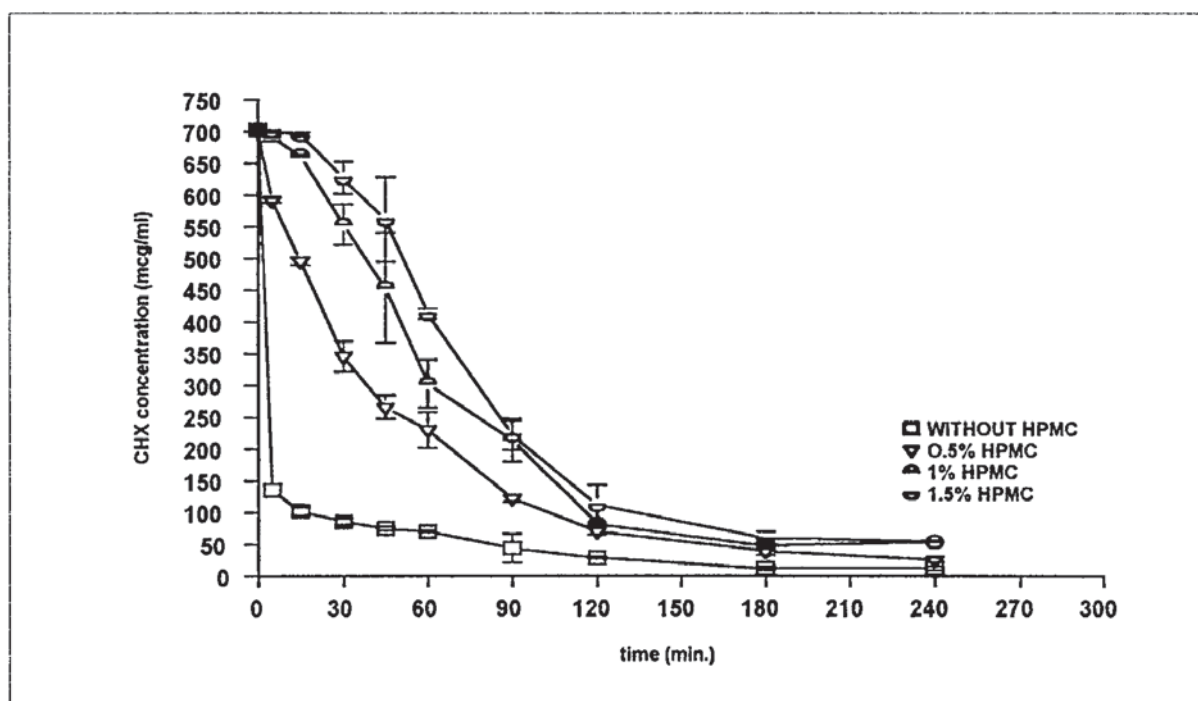
Where R is H, CH₃, or [CH₃CH(OH)CH₂].

Figure 4.10 Structure of HPMC

HPMC (0.5-1.5% w/v) was added to saturated solutions of CHX followed by NaCl (0.1-0.3 M). At different time points, 2 ml samples were withdrawn, filtered, diluted 20- to 50-fold with distilled water and then assayed by HPLC as described in section 2.2.1. HPMC showed substantially improved stability during the time of the experiment (4 h). Concentration of 0.5% w/v of HPMC inhibited the crystallisation of CHX in systems containing 0.1 M sodium chloride for at least 4 hours. The efficiency of HPMC increased as the polymer concentration was increased from 0.5% to 1.5%. 1.5% HPMC was able to stabilise the supersaturated systems containing 0.2 M NaCl for 4 hours. Results are depicted in figure 4.11. To compare the efficiency of HPMC with PVP, $t_{1/2}$ of graphs in figure 4.11 was calculated (table 4.4) and compared with $t_{1/2}$ values of systems containing PVP as the anti-nucleating agent.



(A)



(B)

Figure 4.11 Reduction of solubility of CHX on addition of (A) 0.2 M NaCl (B) 0.3 M NaCl in the presence of HPMC (Points represents the mean \pm s.d., $n=3$)

	Control (no polymer)	HPMC (% w/v)		
		0.5	1	1.5
System A	97	>240	>240	>240
$t_{1/2}$ (min) System B	10.5	225	>240	>240
System C	3	29	54	69

Table 4.4 $t_{1/2}$ of supersaturated systems with and without HPMC
($t_{1/2}$ = time when the initial concentration of CHX
(0.701mg/ml) is reduced by half; System A, B and C
contain 0.1, 0.2 and 0.3 M NaCl, respectively)

Comparing results presented in table 4.3 with table 4.4, it can be seen that 1.5% w/v HPMC is more effective than 10% PVP K30 in stabilising the supersaturated systems. However, the solutions containing HPMC showed higher viscosity than systems containing PVP which might have a negative effect on the permeation of CHX across the skin. The apparent viscosity of systems containing 0.5% HPMC, 10% PVP K30 and 10% PVP K25 were 15.7 mPa, 7.835 mPa and 5.395 mPa, respectively.

The mechanism by which anti-nucleating polymers inhibit or retard crystal growth in supersaturated solutions is not yet known; however, the adsorption of polymer on the hydrophobic surface of crystals has been well discussed (Law and Kayes, 1983). Therefore, it is likely that the retardation effect of polymers on the crystallisation of CHX arises from the adsorption of the polymer at the solid liquid interface at the stage at which the hydrophobic CHX crystal surface first forms. Hence, polymers prevent the seeds from growing beyond a critical size above which nucleation and crystallisation occurs. Another

mechanism could be increasing the viscosity of the solution. This can explain why PVP (K30), which has a higher molecular weight, was more efficient compared to PVP (K25). Increasing the solubility of the active chemical in the system could be another mode of action of anti-nucleating agents. To investigate if PVP and HPMC affect the solubility of CHX in the presence of chloride ions, solubility studies were carried out once again in the vehicles containing polymers and chloride ions.

4.7.4 Effect of Anti-Nucleating Polymers on the Solubility of CHX in the Presence of Increasing Concentrations of Chloride

The effect of anti-nucleating polymers (PVP and HPMC) on the solubility of CHX in the presence of increasing concentrations of chloride was studied. Concentrations of up to 10% PVP (K30) and only 0.5% HPMC were used since, above this concentrations, the system becomes so viscous that it might hinder the permeation of the CHX across the membrane. Solubility determinations were performed in the same manner as described in section 2.3.1. The anti-nucleating polymers (1-10% w/v PVP and 0.5% HPMC) were added separately to 10 ml distilled water followed by sodium chloride (0.1-0.3 M) and CHX (20 mg, excess powder). The solutions were left at the room temperature ($20\pm1^{\circ}\text{C}$) while stirring for 48 hours. 2 ml samples were withdrawn from each system, filtered through a presaturated filter, diluted appropriately (0- to 10-fold), and assayed for CHX with HPLC as described in section 2.2.1.

At a constant concentration of NaCl, solubility of CHX in the vehicle (distilled water and sodium chloride) increased with increasing the amount of polymer. 1% PVP K25 caused a 1.04-fold increase in the solubility of CHX in the vehicle containing 0.1 M NaCl. CHX solubility was further increased (1.31-fold and 1.54-fold) as the concentration of PVP K25 was increased to 5% and 10%, respectively. CHX solubility in the vehicles containing 0.2 M and 0.3 M NaCl was also enhanced by the addition of PVP K25. In systems containing 0.2 M NaCl, the CHX solubility was increased from $0.083\pm 7.2 \times 10^{-3}$ mg/ml (no polymer) to $0.111 \pm 5.28 \times 10^{-3}$ and $0.135 \pm 2.76 \times 10^{-4}$ mg/ml following the addition of 5% and 10% PVP K25, respectively. Results are depicted in figure 4.12.

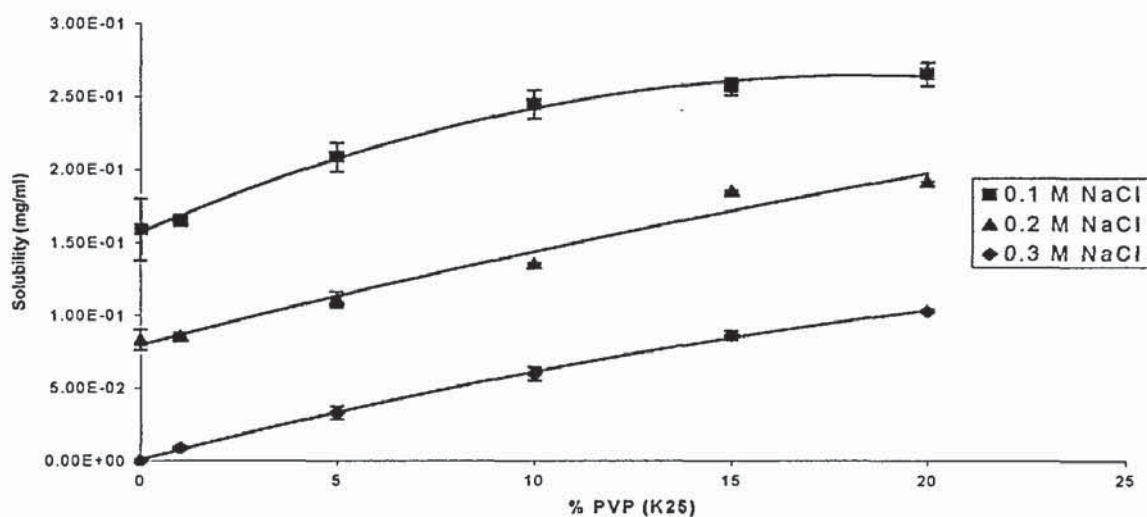


Figure 4.12 Saturated solubility of CHX in PVP (K25)/NaCl systems

CHX solubility in the vehicle (distilled water and chloride ions) were also enhanced in the presence of PVP K30. CHX solubility in 0.1 M aqueous solution of sodium chloride was enhanced from $0.159 \pm 2.12 \times 10^{-2}$ mg/ml (with no polymer) to 0.365 ± 0.0178 mg/ml when 10% PVP K30 was introduced to the systems. This polymer resulted in a higher enhancement factor compared to its lower molecular weight counterpart (PVP K25). At chloride ions concentration of 0.1 M, 10 % K30 induced a 2.3-fold increase in CHX solubility compared to 1.31-fold increase following the addition 10% K25 to the systems. Results are presented in figure 4.13.

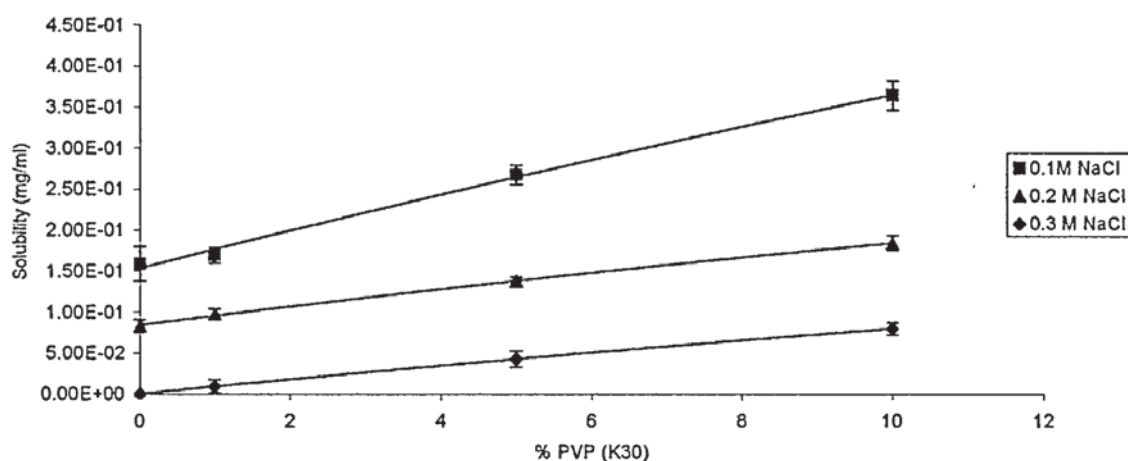


Figure 4.13 Saturated solubility of CHX in PVP (K30)/NaCl systems

Solubility studies were also performed with HPMC (0.5% w/v) in order to study the effect of this polymer on the CHX solubility in the presence of chloride ions. 0.5% HPMC was more efficient in increasing the CHX solubility in the presence of 0.2-0.3 M sodium chloride concentrations. At concentration of 0.1 M sodium chloride, the effect of HPMC on the solubility of CHX was similar to the effect of PVP K30 which was 2.24-fold enhancement (from $0.159 \pm 2.12 \times 10^{-2}$ mg/ml to 3.57 ± 0.011 mg/ml). At higher concentrations of sodium chloride (0.2 M), CHX solubility was enhanced 2.54 times when HPMC was introduced to the systems. However, increasing the concentration of HPMC from 0.5 % to 1.5 % did not increase the solubility of CHX any further despite the fact that the higher concentrations of HPMC were more efficient in the stabilising the

supersaturated systems. The inability of higher concentration of HPMC in enhancing the CHX solubility in the presence of sodium chloride could be due to an interaction between CHX and the polymer. Further studies need to be carried out to prove this assumption. Results are presented in figure 4.14.

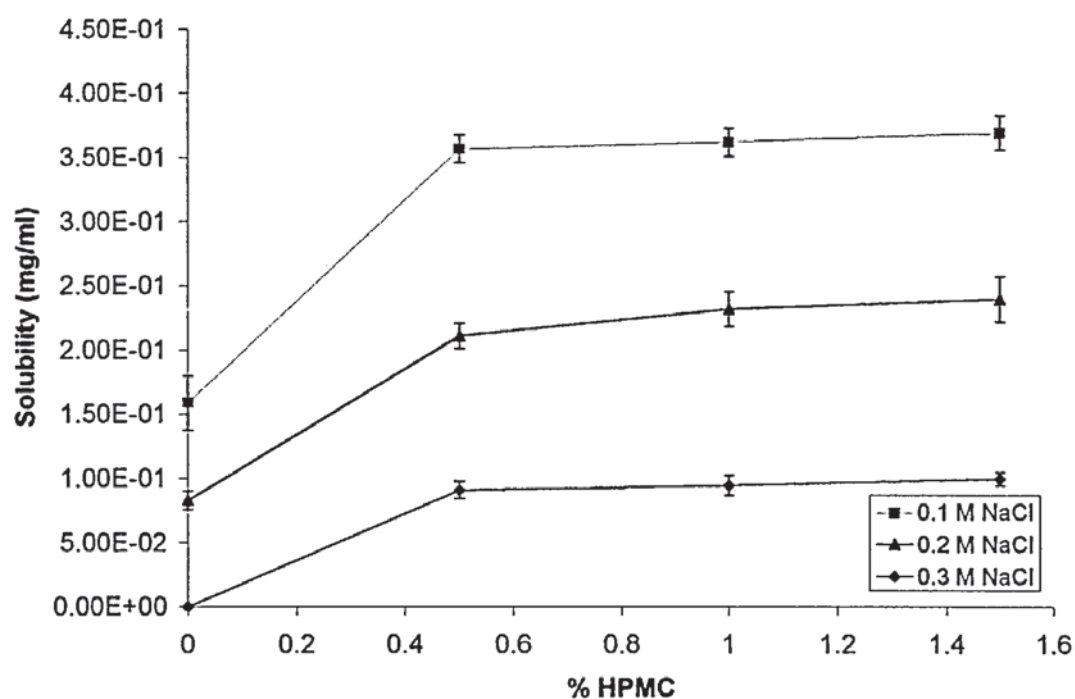


Figure 4.14 Saturated solubility of CHX in HPMC/NaCl systems

The solubility of CHX in the presence of increasing concentration of NaCl (0.1-0.3 M) is presented in table 4.5. The results show that all three polymers employed in this study are able to increase the solubility of CHX in the presence of chloride ions with HPMC showing a similar effect to 10 % PVP K30, which was better than PVP K25.

NaCl (M)	CHX Solubility (mg/ml)			
	Control	10% PVP K25	10% PVP K30	0.5% HPMC
0.1	0.159 (2.12×10^{-2})	0.245 (9.75×10^{-3})	0.365 (1.78×10^{-2})	0.357 (1.01×10^{-2})
0.2	0.083 (7.2×10^{-3})	0.135 (2.76×10^{-4})	0.185 (8.90×10^{-2})	0.211 (9.77×10^{-3})
0.3	*	0.0598 (4.56×10^{-3})	0.80 (7.80×10^{-3})	0.091 (6.70×10^{-3})

Table 4.5 Effect of polymers on the solubility of CHX in the presence of increasing chloride ion concentration, * below detection level (values in brackets represent the standard errors, n=3)

4.7.5 Permeation of Supersaturated Solutions

Depending on the degree of saturation, supersaturated solutions have been shown to enhance skin penetration of the drugs across the membrane (Schwarb *et al.*, 1999; Pellet *et al.*, 1997). In this study, the potential of the supersaturated systems in enhancing the flux of CHX across the human epidermis was studied. Solutions of CHX with the same concentration ($0.701 \pm 4.22 \times 10^{-3}$ mg/ml) but different degree of saturation were used as the donor solutions and their permeation across the skin was studied over 30 minutes using Franz-type diffusion cells (section 2.3.4). Supersaturated systems prepared with PVP K25 were dismissed as systems containing PVP K30 showed a better stability (section 4.7.3). CHX saturated solution was used as control in all permeation studies.

4.7.5.1 Permeation of CHX supersaturated solution containing 0.5% HPMC as the anti-nucleating agent

Permeation of supersaturated solutions of CHX prepared with 0.5% HPMC across the human arm epidermis was studied over 30 minutes. 1 ml of this solution was placed in the donor phase and at appropriate intervals, samples of the receptor were withdrawn and analysed by HPLC as described in section 2.2.1 (no dilution was performed). The cumulative amount of CHX penetrated across the skin was plotted against time and the flux was calculated from the initial part of the graph. Although the supersaturated solution of CHX had an initial degree of saturation of 4.4, no significant difference was observed in CHX flux from saturated and supersaturated systems ($0.011 \pm 4.14 \times 10^{-4}$ and $0.01 \pm 6.03 \times 10^{-4}$ mgcm⁻²min⁻¹, respectively) (figure 4.15 and table 4.6). This could be due to the increased viscosity (apparent viscosity = 0.0157 Pa.) of the supersaturated system by HPMC which can hinder the permeation process. Another possible reason could be the formation of a complex between CHX and HPMC as some polymers have been shown to helically incorporate a guest molecule (Kjarheim *et al.*, 1994). The latter reason is only an assumption as no information regarding the interaction of HPMC and CHX was found in the literature.

Based on the above result, no further studies were carried out on systems containing HPMC.

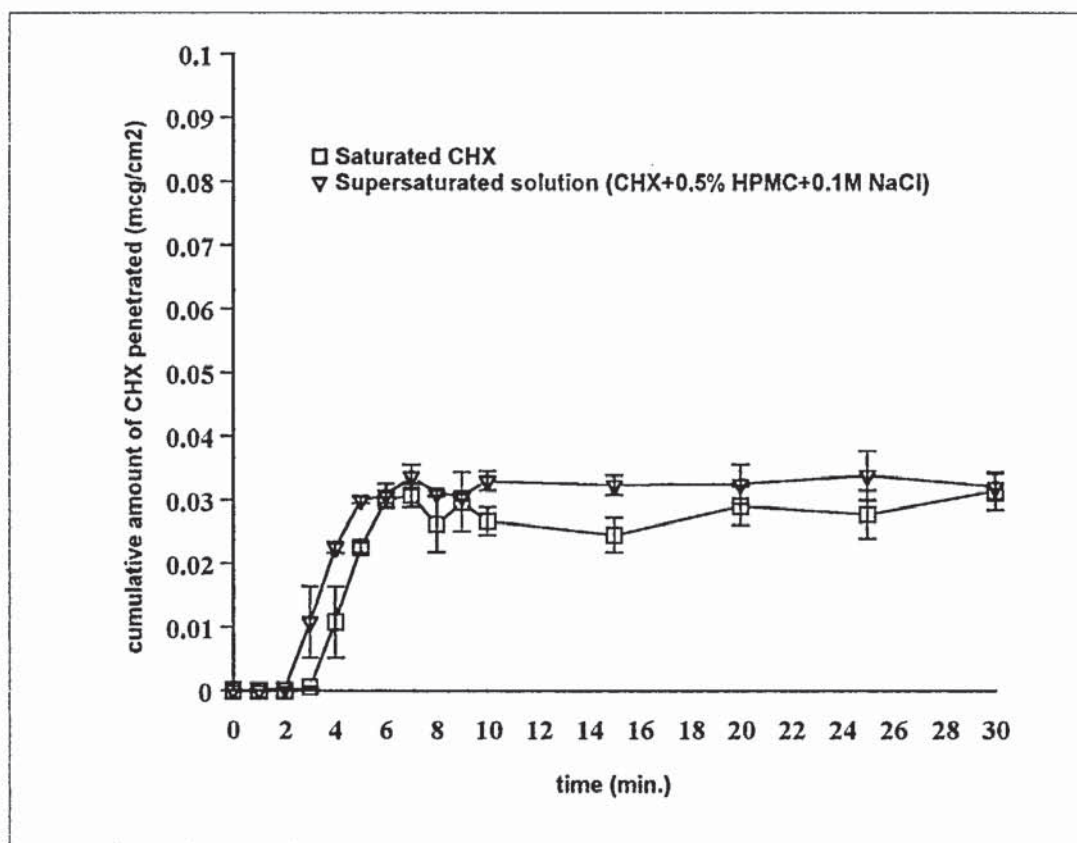


Figure 4.15 Permeation profile of CHX across human arm epidermis (Points represent the mean \pm s.d., n=6)

	Flux ($\text{mg cm}^{-2}\text{min}^{-1}$)	Permeability coefficient (cm min^{-1})
Saturated solution	0.0011 (4.14×10^{-4})	0.00157 (5.92×10^{-4})
Supersaturated solution (0.5% HPMC+0.1M NaCl)	0.0010 (6.03×10^{-4})	0.00143 (8.63×10^{-4})

Table 4.6 Permeation data for CHX saturated and supersaturated solutions (values in brackets represent the standard deviations)

4.7.5.2 Permeation of CHX supersaturated solution containing 10% PVP K30 as the anti-nucleating agent

Permeation of supersaturated systems with the same initial concentration (0.701 mg/ml) but different degree of saturation containing 10% PVP K30 as anti-nucleating agent across the skin was investigated. There was an initial increase in CHX flux across the skin with increasing degree of saturation. The supersaturated system with an initial degree of saturation of 4.4 enhanced the flux of CHX from $0.011 \pm 4.14 \times 10^{-4} \mu\text{g cm}^{-2}\text{min}^{-1}$ (control) to $0.016 \pm 6.43 \times 10^{-3} \mu\text{g cm}^{-2}\text{min}^{-1}$ which was further increased to $0.053 \pm 1.24 \times 10^{-4} \mu\text{g cm}^{-2}\text{min}^{-1}$ as the degree of saturation was enhanced to 8.4 (figure 4.14). Judging by the degree of saturation (table 4.2), one would expect the last supersaturated system (containing 0.3 M NaCl) to provide a higher flux than that was observed during permeation studies. This can be explained by the fact that all the stability experiments were performed in the ideal environment of a test tube where there were no rough surfaces or foreign particles and chemicals which could initiate nucleation. When such supersaturated solutions were placed on the relatively rough surface of the skin, crystallisation might have occurred at a faster rate. Hence, systems that were in a metastable state might show signs of decreasing thermodynamic activity by recrystallising from solution and would therefore not provide enhanced penetration as much as expected. The experimental fluxes, the corresponding permeability coefficients are given in table 4.7.

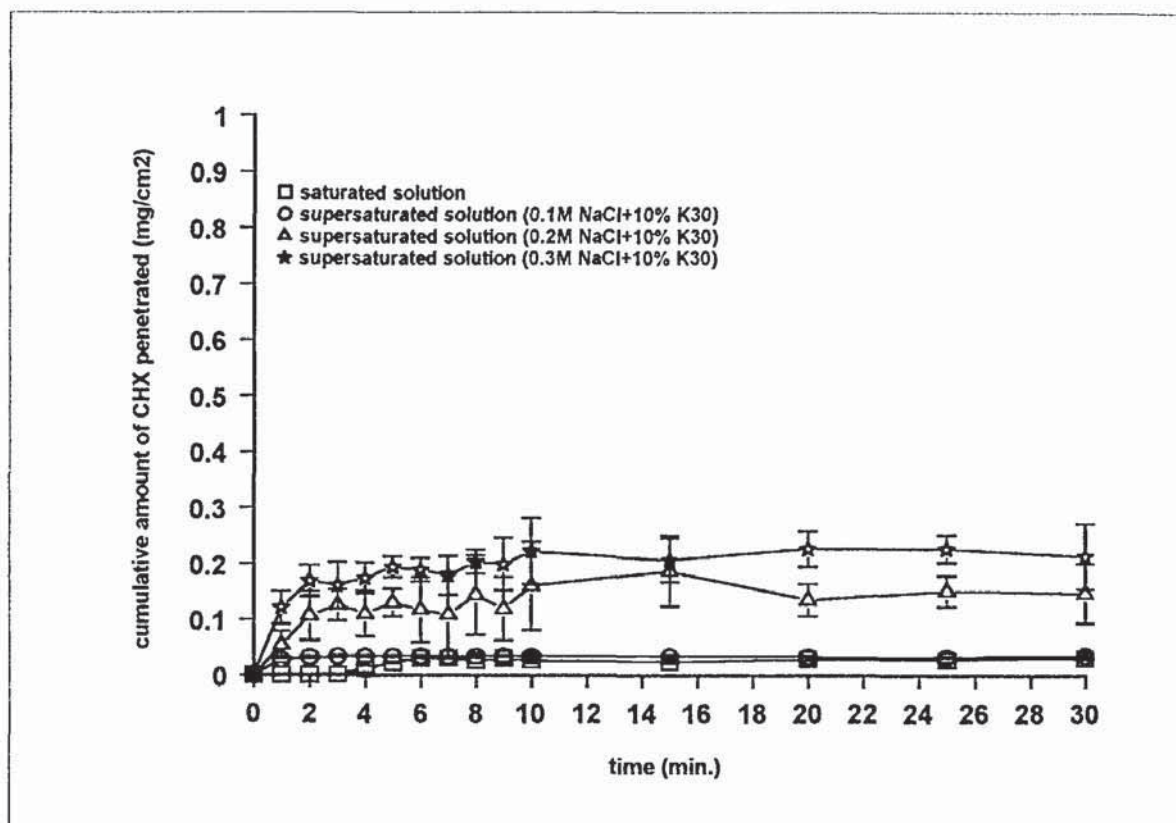


Figure 4.16 Permeation profiles of saturated and supersaturated solutions of CHX across human arm skin (Points represent the mean \pm s.d., n=6)

	Flux ($\mu\text{gcm}^{-2}\text{min}^{-1}$)	Permeability coefficient (cm min^{-1})	*ER
Saturated CHX	0.0011 (4.14×10^{-4})	0.0157 (5.92×10^{-4})	-
Supersaturated (10% K30+0.1M NaCl)	0.0016 (6.43×10^{-3})	0.0229 (9.203×10^{-3})	1.45
Supersaturated (10% K30+0.2M NaCl)	0.0053 (1.24×10^{-4})	0.0757 (2.633×10^{-4})	4.83
Supersaturated (10% K30+0.3M NaCl)	0.0084 (2.09×10^{-2})	0.12 (2.99×10^{-2})	7.65

Table 4.7 Permeability data for saturated and supersaturated solutions of CHX, *Enhancement factor (ER) = Flux of supersaturated system/flux of saturated system, values in brackets represent the standard error.

4.8 Summary

The results presented in this chapter demonstrate the effectiveness of the supersaturated systems on the acceleration of the CHX transport across the skin. In this chapter, a novel method, the common-ion effect, was employed to prepare the supersaturated systems. Different amounts of sodium chloride were added to the saturated solutions of chlorhexidine dihydrochloride which, due to the common-ion effect, resulted in the reduction of CHX solubility and thus, precipitation of CHX. Anti-nucleating polymers, such as PVP with different average molecular weight (24000 and 40000) and HPMC were employed for the stabilisation of the systems. HPMC proved to be a better choice for the purpose of stabilisation however, solutions containing HPMC showed no improved permeability. Increased viscosity and possibly the formation of a complex between HPMC and CHX were proposed as the reasons behind this observation.

PVP with the average molecular weight of 40000 (K30) showed to be a more effective antinucleating agent compared to its lower molecular weight counterpart. Permeations studies performed on the supersaturated systems containing PVP K30 showed an enhanced flux compared to the saturated system. The enhancement in the flux was also proved to be dependent on the degree of saturation.

CHAPTER FIVE

IONTOPHORESIS

5.1 Introduction

Iontophoresis is a technique that has been used to enhance the delivery of drugs particularly charged and polar molecules through the skin. It is a process which causes an increased penetration of molecules into or through the skin by the application of an electric field. A number of experiments have strongly suggested that ionic flow occurs through pores, such as sweat glands (Gibson and Cooke, 1959), sebaceous glands, and hair follicles (Cullander and Guy, 1991) and since skin microflora are also distributed in these sites of the skin (Chapter 1, section 1.8), it seemed appropriate to employ iontophoresis to target the bacteria residing in the pores.

Historically, the idea of applying electric current to increase the penetration of electrically charged drugs into surface tissues was originated by Veratti in 1747 (Turnell, 1921). Later in 1833, Faber-Palaprat claimed that he was able to drive medicine into the human body electrically. In the latter part of the 19th century, Morton conducted an experiment on himself in which finely powdered graphite was driven into his arm under a positive electrode, producing small black spots (Chien and Banga, 1989). This technique temporarily lost its popularity towards the end of the 19th century. However, it was revived at the beginning of the 20th century by Leduc who was the first, in 1908, to confirm that ions could be introduced into the skin by means of an electric current. He applied iontophoresis of strychnine and cyanide ions into rabbits and produced tetanic seizures and cyanide poisoning. Between then and 1921, numerous ophthalmological iontophoresis studies were carried out but led to problems, including corneal scarring, tissue burning, and some electrical shocking of patients. In 1936, iontophoresis was employed for the treatment of hyperhidrosis by Ichihashi, a condition characterised by excessive sweating. Conversely, in 1959, Gibson and Cooke used iontophoretic application of pilocarpine to induce sweating, and the technique has since been used for the diagnosis of cystic fibrosis (Gibson and Cooke, 1959) and currently, interest in iontophoresis has shifted toward its use as a drug delivery system.

5.2 Benefits of Iontophoresis

Iontophoresis enhances the advantages of transdermal drug delivery, which include bypass of hepatic first-pass effect and gastro-intestinal incompatibilities, ease of terminating drug delivery and increased patient compliance. It facilitates the transport of charged molecules and even those of high molecular weight which do not normally penetrate across the skin. This technique offers a high degree of control over the drug delivery rate and the regimen can be tailored on an individual basis to deliver drug at preprogrammed rates (Banga and Chien, 1988). Its advantage in treating local conditions lies in the reduced incidence of systemic side effects due to minimal systemic uptake of drugs and high local drug concentrations (Sloan and Soltani, 1986). This drug delivery system also minimises the risk of infection and trauma and is an alternative to parenteral injection.

5.3 Basis for Iontophoresis

Iontophoresis is based on the general principle of electrical attraction, opposite charges attract each other and like charges repel each other. An iontophoretic transdermal drug delivery device consists of a power supply connected to electrodes and two reservoirs, one containing drug ions and the other containing a biocompatible salt or materials that can conduct electricity to complete the circuit (figure 5.1). Delivering a positively charged drug requires that the drug be placed under the anode where it is repelled and is attracted to cathode placed elsewhere on the body (anodal iontophoresis). In cathodal iontophoresis, the electrode orientation is reversed.

The transport of neutral molecules across the skin can also be enhanced by this technique by the process of electro-osmosis (Burnette and Marrero, 1986; Burnette and Ongpipattanakul, 1987; Srinivasan *et al.*, 1989; Wearley and Chien, 1990).

When a concentration gradient and electric field both exist, the ionic flux is a linear sum that would arise from each effect alone. The total flux of a component under the influence of a uniform electric field can be expressed as the sum of the flux due to passive diffusion (J_p), the flux due to electrodiffusion (J_e), and the flux due to electro-osmosis convective

effect (J_e) which originated due to the net negative charge on the skin, its permselectivity to cations, and the resulting induced solvent flow:

$$J_{\text{total}} = J_p + J_e + J_c \quad (5.1)$$

This equation does not predict the effects of any changes in transport numbers. The most commonly used equation for the steady-state flux of an ion is described by the Nernst-Planck which is given as:

$$J_i = -D \frac{\partial C}{\partial x} - D \frac{CzF}{RT} \frac{\partial E}{\partial x} \pm CJ_v \quad (5.2)$$

J_i = Flux of the ion across the membrane

D = Diffusion coefficient of solute

$\frac{\partial C}{\partial x}$ = Solute concentration gradient

C = Concentration of the ion

z = Charge of the ion

F = Faraday Constant

R = Gas Constant

T = Absolute temperature

$\frac{\partial E}{\partial x}$ = Electrical potential difference

J_v = Velocity of the solvent flow (electro-osmotic flow)

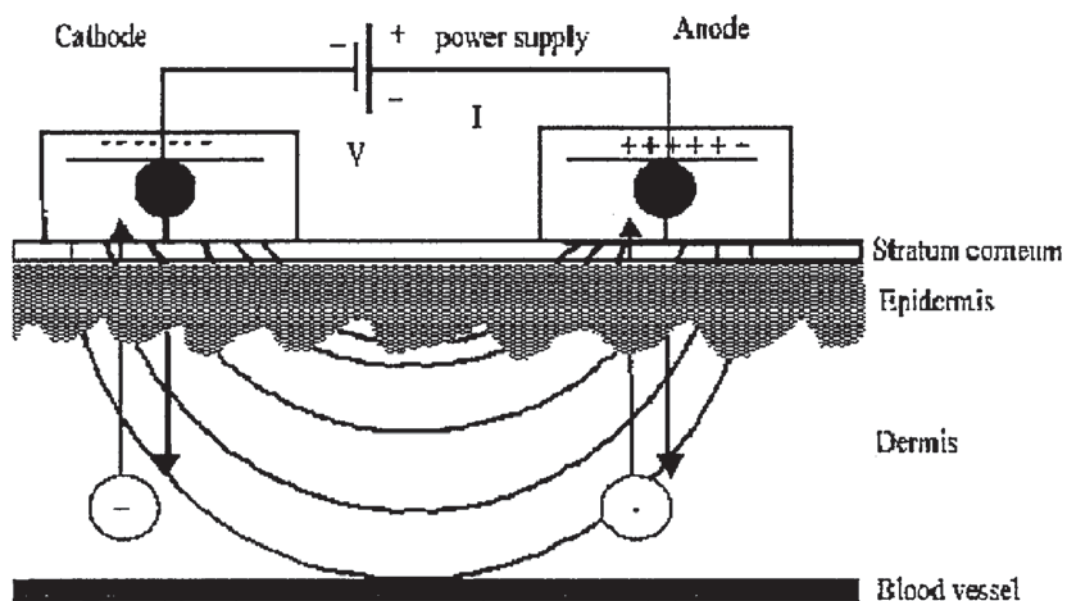


Figure 5.1 Schematic diagram of iontophoretic process

5.4 Pathways of Iontophoresis

The process of percutaneous absorption can be defined as the mass movement of substances from the skin surface to the general circulation. When an electrical potential is imposed across the skin, ions will move along the pathways of the lowest electrical resistance. A number of experiments have strongly suggested that ionic flow occurs through pores, such as sweat glands, sebaceous glands, and hair follicles. Iontophoresis of pilocarpine is routinely used to induce sweating in the diagnosis of cystic fibrosis (Gibson and Cooke, 1959) and it is likely that at least some of the drug travels down the eccrine duct. Burnette and Ongpipattanakul (1988) demonstrated that the iontophoretically driven movement of a negatively charged, fluorescent dye occurred through skin pores. The iontophoretic transport of a series of radioactively labeled alkanoic acids sodium salts through closely clipped furry rat skin was also found to be 12-66% greater than electrically facilitated flow through nude rat skin, which has very few hairs (Terzo *et al.*, 1989). Abramson and his co-workers showed that the iontophoretic transport of various charged dyes into human skin *in vivo* resulted in the formation of a dot pattern which

were then identified as the surface openings of sweat glands (Abramson and Gorin, 1940). Cullander and Guy (1991) used a vibrating probe electrode, which can rapidly detect currents on the skin surface, to measure current flow through hairless mouse skin and found that the largest current occurs in the vicinity of residual hairs. Similar results have been obtained in the work by Scott and co-workers using a scanning electrochemical microscope to detect the flux of Fe^{2+} and Fe^{3+} through hairless mouse skin (Scott *et al.*, 1991).

There is also evidence for paracellular but not for transcellular iontophoretic pathways. By using confocal microscopy, after iontophoresis of calcein (a polyanionic fluorescein derivative) and NBD-diethanolamine (a fluorescent alcohol), it was shown that a paracellular pathway for charged and polar species exists (Cullander and Guy, 1992). High-resolution transmission microscopy of skin tissues following iontophoretic transport of heavy metal cations also supports paracellular transport, in which molecules are transported through the lipid bilayer region surrounding corneocytes (Monteiro-Riviere *et al.*, 1994). It should be noted, however, that the above techniques, which rely on detecting the presence of the penetrant in the sample after exposure, may in fact reflect the relative affinity of the penetrant for particular sites within the skin rather than showing the actual pathways of penetration.

A nonappendageal pore pathway has also been suggested, which probably implies the current flow through artificial shunts as a result of temporary disruption of the organised structure of stratum corneum (Cullander, 1992). A potential-dependent pore formation in the stratum corneum has also been reported. According to this theory, an electric potential applied across the stratum corneum causes α -helical keratin polypeptide molecules to reorient or flip-flop into a parallel arrangement. Pores are formed between neighbouring keratin helices as a result of molecular realignment and repulsion of neighbouring dipoles (Singh and Maibach, 1994)

5.5 Factors Affecting Iontophoresis

The major factors affecting iontophoretic transport include physicochemical properties of the permeant, formulation factors, type of the equipment, biological variations, and other factors, such as duration of the treatment.

5.5.1 Physicochemical Factors

Molecular Size

As it appears that iontophoresis follows a porous pathway, one expects that the degree of enhancement will be penetrant size-dependent, and that, as size increases, the flux will decrease. Upon the existence of the paracellular pathways (Cullander and Guy, 1992), it has been shown that the permeability coefficient of a series of positively charged, negatively charged, and uncharged molecules across excised human skin decrease as the molecular sizes increase (Yoshida and Roberts, 1993). However, the upper limit has not been established as recent reports indicate that insulin, which is a relatively large molecule ($M_R = 5807.7$), can be delivered across the skin (Siddiqui *et al.*, 1987; Meyer *et al.*, 1989).

Drug Concentration

The effect of concentration can be predicted with the aid of the Nernst-Planck equation (equation 5.2). The flux of a number of solutes has been found to increase with increasing concentration in the donor compartment (Van der Geest *et al.*, 1997; Lelawongs *et al.*, 1989; Miller and Smith, 1989). Bellanton *et al.* (1986) showed a linear increase in benzoate ion flux with donor concentration. An increase in concentration of sodium butyrate in the donor compartment was found to produce a proportional increase in their fluxes across skin (Del Terzo *et al.*, 1989).

Charge of Drug Ion

The drug molecules must be in an ionised state with either a positive or negative charge. As evidenced by equation 5.2, the number of charges on the molecular ion has a direct proportional effect on the rate of its transfer in an electric field. However, Phipps *et al.*

(1989) showed that the delivery of the Mg^{2+} (divalent) ion was approximately half that of the Na^+ (monovalent). This can be attributed to the stronger binding of the divalent cations to negative sites in the skin as skin is believed to be negatively charged at pH values above 4. Furthermore, it was shown that the permeation of nonionised solutes could also be enhanced. Burnette and Marrero (1986) reported higher transport rates of thyrotropin releasing hormone at pH 8 (un-ionised) than at pH 4 (ionised). Iontophoresis has also been observed to enhance penetration of a number of zwitterionic amino acids such as glycine, alanine, leucine, valine, and phenylalanine (Green *et al.*, 1991). This enhanced flux is due to convective or electroosmotic water-flow.

Conductivity of Drugs

Many drugs are ionic in nature and in aqueous solution will dissociate into constituents which are free to carry electrical currents when placed in an electric field. The ease with which a current is passed will depend on the conductivity of the electrolyte, which depends on the number of ions in a given volume of solution and the interaction between them. If electrolytes in solution were fully dissociated and no interaction existed between the ions then conductivity would be proportional to concentration, however, the relationship is not so straightforward. Hence, it is often convenient to express the conductivity of an electrolyte solution in terms of molar conductivity:

$$\Lambda = \frac{k}{C} \quad (5.3)$$

Λ = Molar conductivity ($\Omega^{-1} m^2 mol^{-1}$)

k = Conductivity ($\Omega^{-1} m^{-1}$)

C = Concentration of electrolyte ($mol m^{-3}$)

The variation in molar conductivity with concentration will depend on whether the electrolyte dissociates completely in solution (a strong electrolyte) or only partly (a weak

electrolyte). Gangarosa *et al.* (1978) reported the specific conductivity for a number of drugs, on the basis of which many agents could be suggested for iontophoresis.

Permeant Lipophilicity

It was shown by the work of Del Terzo *et al.* (1989) that iontophoresis is an approach targeted at the augmented delivery of polar compounds. They used a series of n-alkanols and n-alkanoic acids to investigate the effect of lipophilicity on iontophoretic enhancement and found that the enhancement of both chemical classes decreased as permeant lipophilicity increased.

Transport Number

The transport number of the drug has a direct effect on the mobility of the drug ion. The higher the number, the larger the fraction of total applied current it carries and the higher the rate of migration through the permeation medium.

5.5.2 Formulation Factors

Ionic Strength

The ionic strength is related to the concentration of various ions present in the solution. In iontophoresis, the applied drug solution must contain a reasonable concentration of electrolyte so that the current can be efficiently conducted. If the drug is neutral, the presence of electrolyte is also essential for the electroosmotic effect. However, the addition of electrolyte will cause the transport number of the drug ion to decrease. Del Terzo *et al.* (1989) studied the iontophoretic transport of butyrate in the presence of inorganic salts in both the donor and the receiver solutions. Potassium ions caused a greater reduction in butyrate transport than sodium ions. Thus, the greater mobility of the potassium ion will enable it to compete more effectively to reduce the transport of butyrate more than sodium ion. Bellantone *et al.* (1986) observed reduction in benzoate

flux to more than half when an equimolar amount of sodium chloride was added to the donor solution.

Buffer Components

The purpose of having a buffer in the electrolytes used in iontophoresis is to maintain the pH, as water can be electrolysed during current passage. Buffers are usually salt solutions containing small inorganic ions. These ions compete for charge transfer with the drug ions and therefore, the fraction of current carrying by the drug ions as well as drug flux would be reduced. This problem can be overcome by employing a buffer system consisting of ions with low mobility or conductivity, such as HEPES buffer in which the principal buffering ion is a large zwitterionic molecule, with $pK_a=7.4$. Hence, at physiological pH, it has relatively small effect on the transport number of the drug.

pH

Donor solution pH influences delivery in several ways which have a complex interrelationship. These include hydronium and hydroxide transport as competitive co-ions and influences on the charge state of drugs that are weak bases and acids. Most drug molecules are weak electrolytes and therefore, pH control is required to maintain the species in an ionic form.

Siddiqui *et al.* (1985) investigated the effect of pH of the aqueous vehicle on the rate of lidocaine iontophoresis through human stratum corneum and showed that decreasing the vehicle pH, which increased the percentage ionised form of the drug, caused the flux to rise. Several other workers have also demonstrated the pH-dependent iontophoretic flux of solutes across the skin (Pikal and Shah, 1990; Srinivasan *et al.*, 1990).

However, pH affects the permselectivity of the skin and extremes of either high or low pH can cause tissue damage. Several authors have reported evidence that skin is permselective, favoring the transport of cations over anions at neutral pH (Pikal, 1990; Wearley *et al.*, 1989) and this permselectivity is pH-dependent. The isoelectric point of human skin lies between 4 and 5 (corresponding probably to the ubiquitous and weakly acid lipid or protein-based carboxylate groups). Thus, under physiologic conditions, the skin is a negatively charged membrane and is permselective to cations; that is, positively

charged ions are preferentially attracted to the skin, whereas anions experience a repulsive force. This ionic selectivity causes a net solvent flow in the direction of cation movement because the rapidly ions collide with water molecules in their path and transfer to them some of their momentum. This phenomenon, electroosmosis, provides a means to deliver uncharged molecules. For cationic drugs, the relative contribution of electroosmosis compared to electrorepulsion effect becomes increasingly significant with increasing molecular weight (Turner *et al.*, 1997; Delgado-Charro and Guy, 1994; Pikal and Shah, 1990). Therefore, decreasing pH can neutralise the charge on the skin which consequently decreases the electroosmotic flux. Burnette and Marrero (1986) showed that the iontophoresis of tripeptide thyrotropin at pH 8, in which the drug exists in neutral form, was higher than the ionised form at pH 4.

5.5.3 Equipment Factors

Current Density

The steady-state flux of the drug under the influence of an applied electrical potential is proportional to the current density. A linear relationship between the flux of benzoate and current density has been shown by Bellantone *et al.* (1986). Wearnly and Chien (1990) showed a correlation between the flux of verapamil and the applied current. The pyridostigmine plasma concentration after iontophoresis in pigs *in vivo* has also been shown to be a function of applied current (Phipps *et al.*, 1989).

However, patient safety considerations may limit the maximum strength of current that can be used and the upper limiting value of current has been suggested to be 0.5 mA/cm^2 .

Types of Electrodes

The electrochemistry at the electrode-solution interface where electronic current is converted to ionic current is controlled by the electrode material used. The desirable properties of electrodes include good conductive material, and producing minimal changes in pH. Two types of electrodes (inert and reversible) are used in iontophoretic devices. The inert electrodes cause the electrolysis of water leading to the production of hydrogen ions at the anode and hydroxyl ions at the cathode.

At anode $\text{H}_2\text{O} \rightarrow 2\text{H}^+ + \frac{1}{2} \text{O}_2 + 2\text{e}^-$

At cathode $2\text{H}_2\text{O} + 2\text{e}^- \rightarrow \text{H}_2 + 2\text{OH}^-$

This may result in the pH changes in the system. Such changes may cause damage to the skin and degrade the solute molecules. Examples of such electrodes are nickel, stainless steel, carbon graphite and platinum. A significant degradation of propranolol hydrochloride was observed when a high current was passed through platinised electrodes and the solutions in both the receptor and donor compartments were discoloured at the end of the *in vitro* experiment (D'Emanuele and Staniforth, 1992).

The other type, reversible, electrode is Ag/AgCl. The redox potential for this system is lower than for water enabling electroneutrality to be maintained at anode and cathode. At the anode, the silver oxidizes under the influence of an applied current and when chloride ion is present it reacts to form insoluble silver chloride which precipitates on the anode surface, and an electron is released to the electrical circuit. Simultaneously, the silver chloride cathode is reduced, using an electron from the circuit, to silver metal that precipitate at the electrode surface and the resulting chloride ion is free to migrate into the body.

At anode $\text{Ag} + \text{Cl}^- \rightarrow \text{AgCl} + \text{e}^-$

At cathode $\text{AgCl} + \text{e}^- \rightarrow \text{Ag} + \text{Cl}^-$

Pulsed or Continuous Current

Use of continuous current may result in skin polarisation which can reduce the efficiency of the iontophoresis. This can be overcome by using pulsed d.c. During the off-time the skin gets depolarised and returns to its near initial electric condition (Banga and Chien, 1988). Enhanced iontophoretic transport of peptide and protein drugs using pulsed d.c. compared to continuous d.c. has been reported by Chien *et al.* (1989). The *in vivo* transdermal iontophoresis of vasopressin in rabbits was facilitated two-fold using pulse current as compared to simple d.c. using the same current density for the duration of application.

However, enhanced skin depolarisation can decrease the efficiency of pulsed transport if the frequency is high. Bagniefski and Burnette (1990) compared the pulsed and continuous current iontophoresis and they found that enhanced depolarisation of the skin decreases the pulsed flux.

5.5.4 Physiological Factors

The physiological factors, such as region, age, sex, race, skin hydration, fluidisation of lipids by enhancers that affect passive diffusion may also influence the iontophoretic delivery of drugs. Srinivasan *et al.* (1989) examined a synergism of iontophoresis and ethanol pretreatment of the skin in order to deliver insulin and they found a several-fold increase in the permeation of insulin with this combination. The influence of dermal blood-flow on transdermal iontophoresis has been evaluated by monitoring lidocaine flux in pigs in the presence of vasoactive chemicals by Riviere *et al.* (1992). In their findings, the vasoconstrictor epinephrine decreases the lidocaine flux due to a vasoconstricting effect, whereas the vasodilator tolazoline increases the lidocaine flux due to vasodilatory effect on the dermal vasculature.

5.6 Disadvantages of Iontophoresis

Although iontophoresis has much to offer, there are some disadvantages associated with this technique. A slight feeling of warmth and tingling generally accompanies all iontophoresis procedures (Ledger, 1992; Maloney *et al.*, 1992). Other side-effects, such as itching, erythema, and general irritation have also been reported (Ledger, 1992). Toxic effects are not clear when metal ions resulting from dissolution of the metallic electrodes get into the skin. Burn and shock produced by high-current densities are another problem. In addition, electrochemical stability of the drug could be a serious problem and the delivery system is much more complicated than passive diffusion. The cost of developing and manufacturing iontophoretic drug delivery is also higher than passive transdermal systems.

5.7 Electrical Properties of Skin

In addition to its many functions, the skin also serves as an electrical barrier. Indeed, dry skin has been observed to show a very high resistance relative to other biological tissues (Yamamoto and Yamamoto, 1976). They also demonstrated, by stripping away the outer tissue layers, that the *in vivo* resistance of human skin is almost entirely located within the SC. The stratum germinativum and the deeper tissues do not act as a barrier to electrical conduction. This resistance has been attributed to the lower water content of the SC, which being composed of dead keratinised cells, is a good insulator and consequently a barrier to electrical conductivity. Any electrical conduction by the skin would thus depend on the movement of ions through the SC. The bulk resistance of excised SC has been observed by many investigators to vary considerably between individuals (Inada *et al.*, 1994; De Nuzzio and Berner, 1990). Significant variation is also evident in resistance measurements taken at different epidermal sites on the same individual. This property of skin is especially important in iontophoretic drug delivery.

Apart from being electrically resistant, the skin tissue also exhibits an ability to store electrons. It therefore displays a capacitance as well as a resistance when subjected to an electric field (Barel *et al.* 1991).

As a result of combined capacitive and resistive elements, the skin is described as being reactive to electrical flow, giving rise to an impedance (Banga and Chein, 1988). Skin impedance represents the total electrical opposition to the passage of current through skin. Studies carried out by Burnett and Ongpipattanakul (1987) to elucidate the mechanism of ion transport and Bommannan *et al.* (1993) suggested that skin capacitance could be attributed to the lipid matrix of the SC, while skin resistance was due to the resistance encountered in the ion shunt pathways (sweat ducts, hair follicles and imperfections in the skin).

5.8 Skin Alteration and Recovery

One of the factors which are critical for iontophoresis, is the reversibility of skin permeability. Some studies have reported that the skin permeation flux does return to a

much lower rate when the current is turned off (Kasting *et al.*, 1988; Bellantone-Harper *et al.*, 1986); however, other experiments indicate that complete reversibility does not occur (Li *et al.*, 1998; Burnette and Ongpipattanakul, 1988). Wearley *et al.* (1989) chose verapamil hydrochloride to investigate factors affecting the reversibility of skin permeability following iontophoresis-facilitated transdermal delivery. They concluded that the skin permeability of verapamil after iontophoresis is not immediately reversible and the time at which reversibility is observed is dependent on the concentration of the drug in the skin. Moreover, the intensity and duration of the current also affect the skin permeation reversibility. Reasons for this result could be as follows:

- Immediately after the electric field is removed, the concentration gradient at the skin/receptor interface could be substantially greater than that resulting from passive diffusion. The concentration gradient will gradually change with time until it becomes equal to that characteristic of passive diffusion. However, the length of time required depends on the concentration, diffusivity and binding affinity of the permeant ion in the skin.

- The application of an electric field may change the chemical potential of the molecules in the skin and provide sufficient energy for them to make conformation changes, which may facilitate the entry of ions. Such conformational changes could occur in structural proteins or lipids in the skin and they may or may not be immediately reversible.

- The application of an electric field increases the movement of water into the skin. This increase in the rate of hydration of the skin may increase the permeation rate (Knutson *et al.*, 1985) or decrease it (Burnette and Ongpipattanakul, 1988) depending upon the major pathways taken by the ions in traversing the skin.

5.9 Experimental Considerations

5.9.1 Membrane

It is most desirable to carry out permeation studies in the ultimate model human *in vivo*; however, this is not a practical approach. The next choice is the use of excised human

skin which itself poses difficulties, especially in terms of the availability and controlling the gender, age, race, site and quality of the donor. Hence, a large number of animal models have been explored, yet the search for the most suitable model continues. Some of the animal models studied are hairless mouse (Langkjaer *et al.*, 1998; Bellantone *et al.*, 1986), nude rat (DelTerzo *et al.*, 1989), furry rat (Siddiqui *et al.*, 1987) and guinea pig. However, based on histological similarity and dissimilarity to human skin Behl *et al.* (1989) believe that the hairless animal skin provides a better model for human skin in iontophoretic experiments.

5.9.2 Apparatus

Several *in vitro* diffusion cells have been employed in iontophoretic studies. Most of the *in vitro* apparatuses utilize a system of two electrodes, one in each compartment of the apparatus. However, most of those systems are not wholly representative of the *in vivo* situation since an electrode should be placed under the skin. A more sophisticated version of this design, which utilizes four electrodes, was developed by Masada *et al.* (1989). This four-electrode system is advantageous in that it measures the voltage drop across the skin surface and thus provides a more precise determination of the voltage. In another type of diffusion cell, which has been developed by Gikfeld *et al.* (1988), the electrodes are placed on the same side of the skin and the two electrode compartments are separated by a glass wall (Figure 5.2).

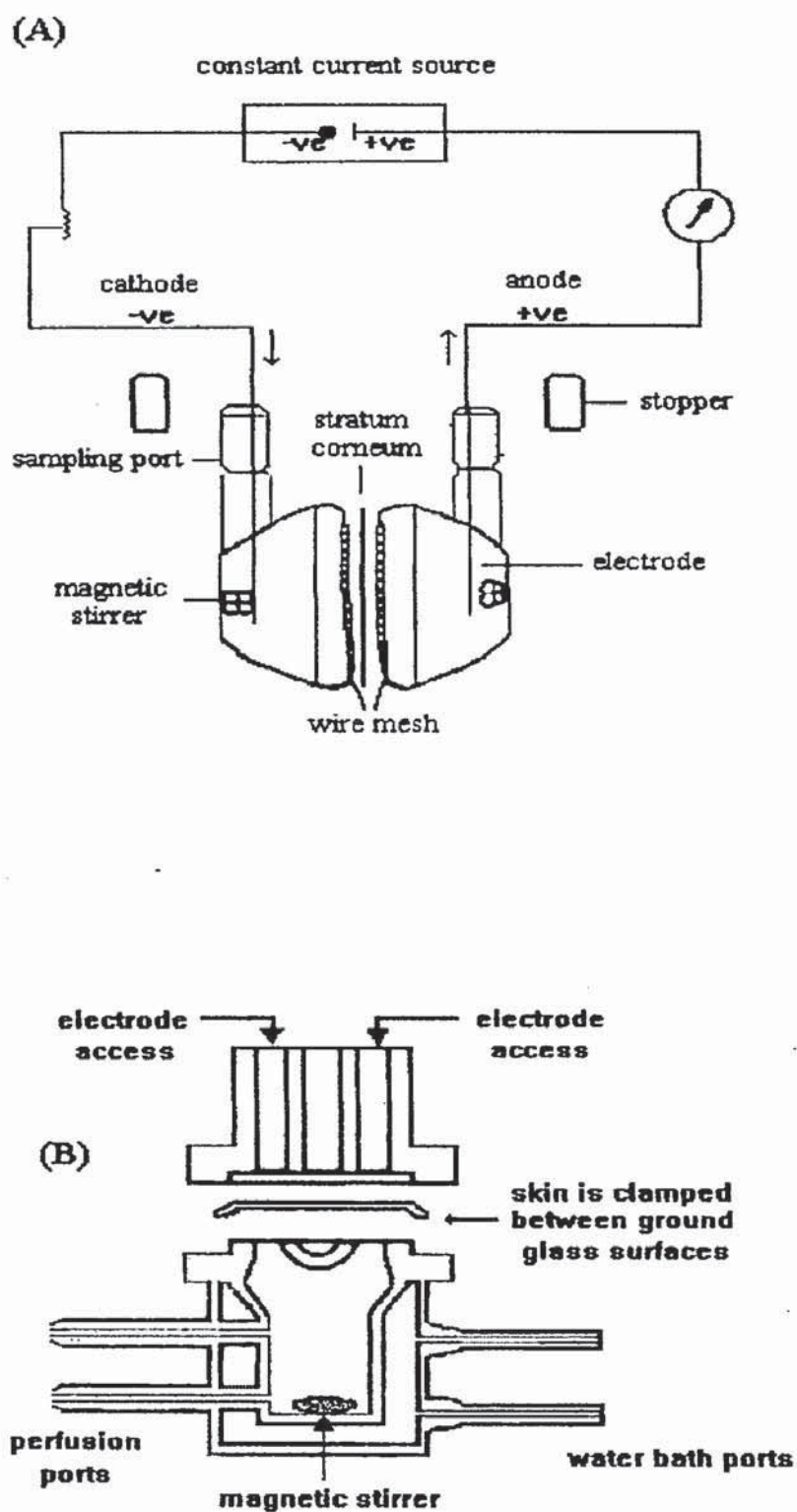


Figure 5.2 Diffusion cells (A) side-by-side diffusion cells (B) iontophoretic cells

5.10 Commercially Available Iontophoresis Devices

While wearable electrical patches are mostly still in development for systemic delivery, external palm or Walkman-sized iontophoresis devices have already been used for topical delivery. Devices available on the market for delivery of local anaesthetics and corticosteroids include Phoresor II (Iomed) (figure 5.3), Empi Dupel, LifeTech Iontophor and Henley Intl Dynaphor. In addition, devices for iontophoresis of pilocarpine for diagnosis of cystic fibrosis are on the market, and these include CF Indicator (Scandipharm) and the system based on the Webster sweat inducer with Pilogel discs for iontophoresis of pilocarpine (Wescor) (Banga, 1998).



Figure 5.3 The Phoresor II system, Iomed (Banga, 1998)

Several companies are trying to commercialise the miniature patch systems. A partial list includes the Alza Corporation (USA), Becton Dickinson (USA), Fournier (France), Hisamitsu (Japan) and Cygnus (USA). Alza currently has an E-TRANS fentanyl product under development which is an on-demand delivery system intended to allow a patient to manage acute pain by self-titrating the level of fentanyl administered according to his or her need. An E-TRANS electrotransport delivery of insulin is also under development by this company. A schematic representation of the E-TRANS system is shown in figure 5.4. The size of these systems will vary from 5-50 cm², depending on marketing considerations. These systems can deliver drug for periods from hours to days at therapeutic levels.



Figure 5.4 A schematic representation of the E-TRANS system (Banga, 1998)

5.11 Combined Use of Iontophoresis and Chemical Enhancers

Penetration enhancers may moderate the iontophoretic regimen required to achieve the target flux, thus improving the tolerability of skin to iontophoretic regimen. Few workers have examined the effect of iontophoresis in combination with permeation enhancers as a potential means to enhance the transdermal delivery of drugs (Bhatia and Singh, 1999; Srinivasan *et al.*, 1990). Enhancers in combination with iontophoresis increased the permeability coefficient of methotrexate and metoprolol through human epidermis (Singh and Singh, 1995; Ganga *et al.*, 1996). A synergism of iontophoresis and ethanol as enhancer was also reported on the transport of peptides through human epidermis (Srinivasan *et al.*, 1990). Kanikkannan *et al.* (2000) also investigated the effect of Azone on the iontophoretic transport of timolol maleate in rabbits. They showed that Azone eliminated the lag time and the duration of effect was prolonged compared to the effect of iontophoresis alone. However, some of the penetration enhancers had no effect or decreased the iontophoretic transport (Hirvonen *et al.*, 1993; Wearley and Chien, 1990).

To explore the possibility of a synergistic effect of a chemical penetration enhancer and iontophoresis on the permeation of CHX, several enhancers were employed in this study to pre-treat skin before iontophoretic studies.

5.12 Aims and Objectives

The research presented in this chapter involves an assessment of the application of iontophoresis to increase CHX transport across the skin *in vitro*. Furthermore, the possibility of combining the iontophoretic and chemical enhancement techniques to facilitate a more dramatic increase in drug delivery is also assessed.

5.13 Materials and Methods

5.13.1 Materials

Silver wire (99.99%), silver chloride (99.99%), HEPES were purchased from Sigma. Sodium chloride was obtained from Aldrich (UK). PVC insulating tubes were purchased from Fisher (UK).

5.13.2 Equipment

Side-by-side diffusion cells with donor and receptor volumes of 10 ml were employed in permeation studies. The diffusion cells provided an average diffusional surface area of $2.56 \pm 0.23 \text{ cm}^2$. Since a four-channel power supply was unavailable, two 2-channel power supply were used in this study. The first power supply was a Kepco power supply with voltage range of 0-1000 V and current range of 0-20 mA. The second power supply was a Vokam (SAE 2761) power supply with voltage range of 0-400 V and current range of 0-80 mA.

5.13.3 Assay Procedure

CHX assays were carried out using HPLC as discussed in section 2.2.1. Where needed, samples were diluted (20 to 50-fold) with distilled water prior to the injection to HPLC.

5.13.4 Donor Solution

In order to maintain a constant thermodynamic activity in the applied donor phase, saturated aqueous solution of CHX in 25mM HEPES was prepared as described in section 2.3.1. 10 mM NaCl was added to the above solutions prior to the experiment and were used as the donor solutions. The small amount of sodium chloride added to the saturated solution of CHX did not cause CHX to precipitate out of the system over a month and thus, the donor solution was considered not to be supersaturated (chapter 4, section 4.6.3). The final solution had a pH of 5 which then was adjusted to the desired value using either sodium hydroxide or phosphoric acid.

Saturated solutions of CHX in 10-20% ethanol were also prepared using the same procedure as described in section 2.3.1 and used as donor solution where needed.

5.13.5 Membrane Preparation

Intact human epidermis was prepared as described in section 2.3.2.2. Whenever the pre-treated skin was needed, the pre-treatment procedure was performed as described in section 3.4.5.

5.13.6 Electrode Preparation

Silver/silver chloride electrodes were prepared using a pure silver wire (1 mm in diameter, 4 cm long). The wire was first cleaned with a cloth and one end was manipulated into a small loop (2-3 mm in diameter). The latter was then coated with silver chloride by dipping the loop into molten AgCl, which was prepared by placing 5 g of AgCl powder in a crucible and melting it on the fire. After cooling, the non-coated part of the Ag wire was protected from contact with the electrolyte by PVC insulating tubing. The resulting electrodes (cathodes) were reasonably consistent in surface area.

As anode, silver wire could have been used however, in this study anodal electrode was prepared by reconditioning of some of the AgCl-coated silver wires in 1 M NaCl solution. This process was performed to increase the surface area of the anode and was as follows:

AgCl-coated silver wire was placed in 1 M sodium chloride solution together with a platinum bar. The former was then connected to the negative pole and the latter to the positive pole of a power supply and a constant current of $0.5\text{mA}/\text{cm}^2$ was applied for 12 hours using the same power supply as described in section 5.13.2. The re-conditioned electrode was then withdrawn from the solution and dried and was ready for use.

5.13.7 Permeation Studies

The effect of current on the flux of CHX across the skin from a saturated solution was investigated. Heat-separated human epidermis was mounted in side-by-side diffusion

cells, which provided surface areas of $2.56 \pm 0.23 \text{ cm}^2$ and donor and receptor volumes of 10 ml. 10 mM NaCl was added to the saturated solution of CHX in 25mM HEPES ($0.708 \pm 0.046 \text{ mg/ml}$) and was placed in the donor compartment, facing the stratum corneum. The receptor was filled with deionised water to eliminate the participation of extraneous ions in the receptor. Ag (anode) and AgCl (cathode) electrodes were also placed in the donor and receptor chambers respectively. Electrodes were connected to an in-house built power supply. The permeation study was then carried out in the same manner as described in section 2.3.4 over 150 minutes. During the first 60 minutes, no current was applied to the systems and the passive diffusion of the donor solutions was studied (stage I). After this time, the power was switched on and the iontophoretic delivery was studied for another hour (stage II). The power was switched off after 60 minutes (time 120 min from the start of the permeation study) and the passive diffusion of the CHX was studied once again (stage III). The three-staged method allowed each cell to act as its own control. At appropriate intervals, 1 ml samples were removed from the receptor, diluted (20-fold) where needed and injected to HPLC for CHX assay in the manner as described in section 2.2.1.

Permeation experiments were also performed in the same manner as described above to determine flux as a function of pH of the donor solution. The pH of donor solution (saturated solution of CHX in 25 mM HEPES) was adjusted to 3, 4, 5, 6 and 7 using either sodium hydroxide or phosphoric acid.

Another set of experiments were also performed in order to study the effect of chemical enhancers and/or donor vehicle on the iontophoretic transport of CHX. The procedure was identical to the above process except for the membrane (epidermis), which was pretreated with the relevant enhancer prior to the experiment. In the case of effect of donor vehicle, a saturated solution of CHX in 20% ethanol was used as the donor solution.

All iontophoresis experiments were performed in four replicates and at a constant electric current of 0.5 mA/cm^2 . The voltage was initially adjusted to 30 V, however, during the experiment as the resistance changed the voltage also changed in order to provide a constant current of 0.5 mA/cm^2 .

5.14 Results and Discussion

The delivery of many ionised compounds across membranes is precluded by their inability to enter the membrane in sufficient concentration after topical application. Iontophoresis offers a means of facilitating the transport of drugs unable to cross by diffusion alone. Hence, in this study, iontophoresis was employed to enhance the permeation of CHX across the skin in order to eliminate the bacteria in the skin, which can act as a source of infection during the insertion of catheters. For this purpose, excised human skin was mounted between two halves of the side-by-side diffusion cell, which provided an average surface area of $2.56 \pm 0.23 \text{ cm}^2$. The receptor compartment was filled with 10 ml de-ionised water and the donor chamber with 10 ml of the CHX solutions under the study. Silver (Ag) and silver chloride (AgCl) electrodes were placed in the donor and receptor, respectively. The electrodes were then connected to a power supply, which provided a constant current of 0.5 mA/cm^2 . The voltage was first set to 30 V, however, during the experiment, the voltage could probably change due to the changes in the resistance of the system (we were unable to record the voltage changes due to the kind of the power supply used). At appropriate intervals, 1 ml samples were withdrawn from the receptor chamber which was replenished immediately with 1 ml de-ionised water. Removed samples were then diluted if needed (20 to 50-fold) and the CHX assay was performed using HPLC as discussed in section 2.2.1.

5.14.1 Effect of constant current on CHX transport

The effect of a constant current of 0.5 mA/cm^2 on CHX transport across excised human skin from a saturated solution of drug in HEPES ($0.708 \pm 0.046 \text{ mg/ml}$, pH=5) was studied using side-by-side diffusion cells. Ag/AgCl electrodes, which were connected to the power supplies, were placed in donor and receptor cells. 1 ml samples were withdrawn from the receptor chambers at appropriate intervals. The samples were analysed by HPLC for CHX quantification as discussed in section 2.2.1. When needed, samples were diluted 20 times using distilled water. Passive diffusion was studied for an hour followed by the application of constant current for another hour and a further 30 minutes of passive diffusion. The resulting permeation profile is shown in figure 5.5. It is evident that the application of a constant current substantially enhances the transport of

CHX over the corresponding passive diffusion. The cumulative amount of CHX penetrated across the skin was enhanced from $4.5 \times 10^{-4} \pm 7.07 \times 10^{-5} \text{ mg/cm}^2$ (passive diffusion) to $0.069 \pm 3.18 \times 10^{-2} \text{ mg/cm}^2$ upon the application of the current showing a 153.3-fold increase. This increase indicates the effectiveness of iontophoresis in enhancing the delivery of CHX across the skin. Hence, the bacteria residing in deeper layers of the skin, such as pilosebaceous units, sweat ducts and skin cervices, where lipids and superficial cornified epithelium protect them, could be removed and thus the complete sterilisation of skin could probably be achieved.

Upon termination of the current after one hour, no increase in receptor CHX concentration was observed. This indicates no current- or water-induced perturbation of the SC by the iontophoresis procedure.

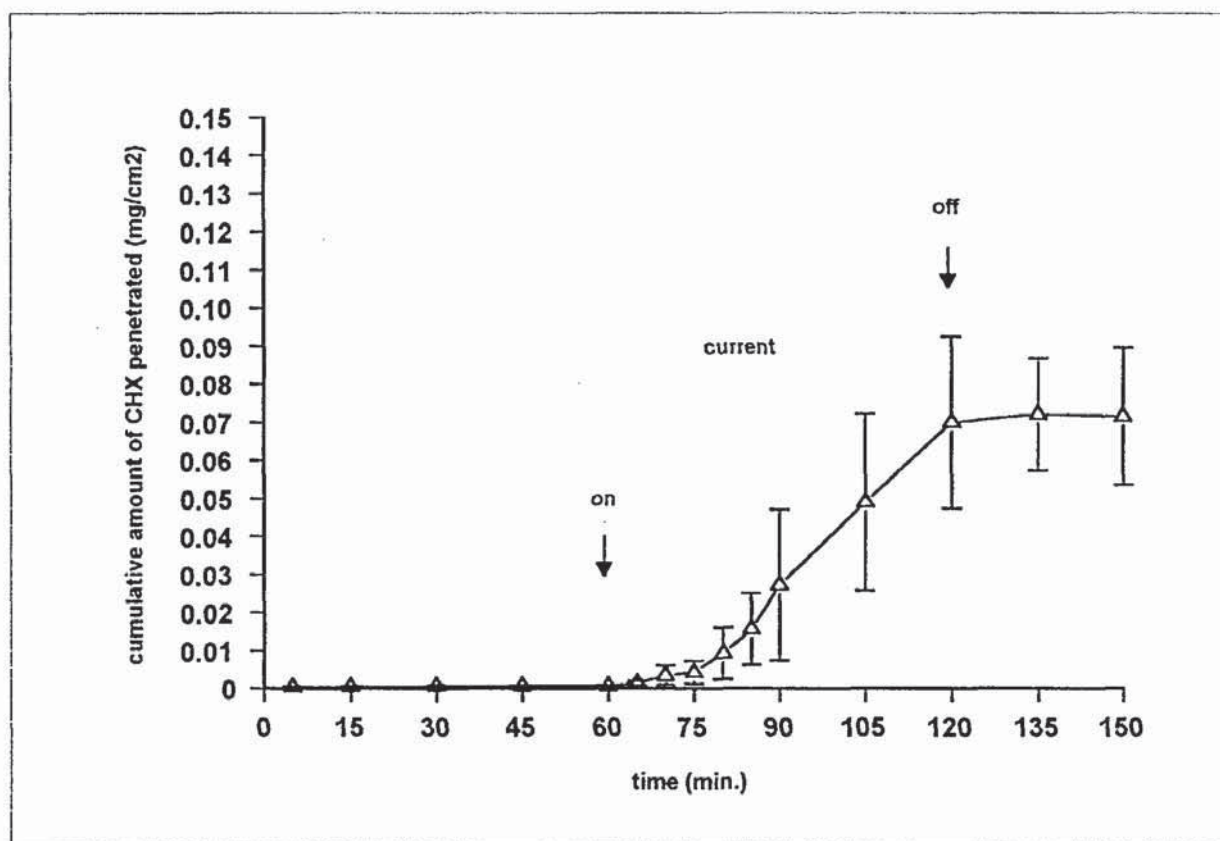


Figure 5.5 Passive and iontophoretic permeation profile of CHX across human epidermis (each point represents mean \pm s.d, $n=4$)

5.14.2 Effect of pH on iontophoretic transport of CHX

As discussed in section 5.3, the total flux from an iontophoretic system can be divided to three components: flux due to passive diffusion, flux due to electrodiffusion and flux due to the electro-osmosis connective effect which originates due to the net negative charge on the skin, its permselectivity to cations, and the resulting induced solvent flow. Hence, to delineate the contributions of electrorepulsion and electroosmosis to the iontophoretic flux of CHX, the pH of donor solution (saturated solution of CHX in 25 mM HEPES with the concentration of 0.708 ± 0.046 mg/ml) was adjusted to 3, 4, 5, 6 and 7 using sodium hydroxide or phosphoric acid and the *in vitro* permeation experiments were performed once again as described in section 5.13.7. Results are depicted in figure 5.6 and show that the penetration of CHX decreases as the pH of the donor solution decreases. Reducing the pH of the donor solution from 5 (the normal pH of saturated solution of CHX in 25 mM HEPES) to 3 resulted in 1.8-fold decrease in the cumulative amount of CHX penetrated across the skin over 60 minutes of iontophoresis ($0.039 \pm 2.4 \times 10^{-3}$ and $0.069 \pm 1.71 \times 10^{-3}$ mg/cm², respectively). One possible explanation for the observed decrease in the permeation rate of CHX could be the fact that decreasing pH from 5 to 3 can neutralise the negative charge on the skin, therefore lowering electroosmotic flux. Since the CHX permeation was reduced by decreasing the pH of the donor solution, we can conclude that electrorepulsion is not the sole delivery mechanism in CHX iontophoresis but electroosmosis also contributes in CHX iontophoresis.

Furthermore, increasing the pH from 5 to 7 resulted in 1.6-fold increase in the amount of CHX penetrated across the human epidermis. The values of cumulative CHX at each pH are $0.069 \pm 1.71 \times 10^{-2}$ mg/cm² and $0.113 \pm 9.45 \times 10^{-3}$ mg/cm², respectively (table 5.1). Flux of CHX at each pH was calculated from the linear part of the graphs and is presented in table 5.2. As the results show, the highest flux ($2.55 \times 10^{-3} \pm 6.57 \times 10^{-5}$ mg cm⁻² min⁻¹) was achieved from a donor solution with pH value of 7. Permeability coefficients were also calculated by dividing the flux by the initial donor concentration (0.708 ± 0.046 mg/ml). As the pH of the donor solution increased, the permeability coefficient also increased with solution with pH 7 having the highest flux. The permeation enhancement observed with increasing pH can not be due to the effect of pH on the ionisation of the drug as CHX ionisation is little affected in this pH range (pK_a of CHX = 2.2 and 10.3). The effect of pH on the ionization of CHX has been discussed in

details in section 2.4.1.3. A possible explanation could be the effect of pH on the membrane ionisation (Guy, 1992). According to Guy, increasing the pH would result in enhanced membrane ionisation, which would favour the electroosmotic flux. As electroosmosis is one of the delivery mechanisms in CHX iontophoresis, increasing pH would consequently enhance the iontophoretic delivery of CHX.

Another variable which may impact on the observed trend in the rates of penetration is the varying thermodynamic activity of each vehicle. Looking at the passive diffusion of CHX (stage I), where no current was applied, we can see that the CHX transport was enhanced as the pH of the donor solution was increased. This could be due to the higher thermodynamic activity of CHX solutions with higher pH value. As this study was performed using solutions with the same concentration ($0.708 \pm 0.046 \text{ mg/ml}$), and increasing pH decreases the solubility of CHX (chapter 2, table 2.4), therefore, the thermodynamic activity of the permeant will be enhanced with increasing pH. The higher the pH, the higher thermodynamic activities of donor solutions.

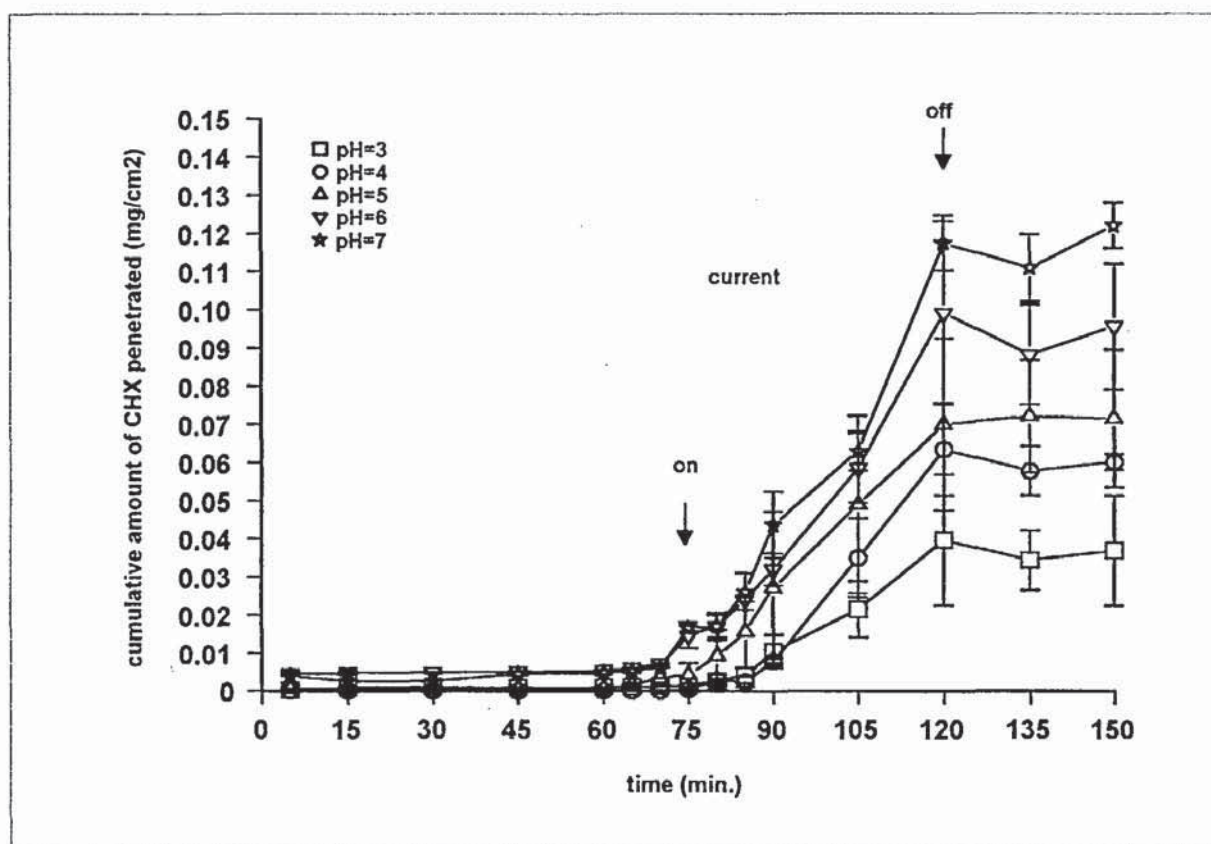


Figure 5.6 Effect of pH on iontophoretic transport of CHX across human breast epidermis (each point represents mean \pm s.d, n=4)

The effectiveness of iontophoresis was determined by comparing cumulative amount of CHX penetrated after 60 minutes (Q_{60}) in the presence and absence of current (passive diffusion), this was defined as the enhancement ratio (ER) (Saija *et al*, 1998):

$$\text{Enhancement Ratio} = \frac{\text{CHX } Q_{60} \text{ in the presence of current}}{\text{CHX } Q_{60} \text{ with no current (passive diffusion)}} \quad (5.4)$$

The cumulative amount of CHX penetrated after 60 minutes in the presence of the current has been corrected to exclude any passive diffusion contributions. The corrections were made by deducting the amount of CHX penetrated during passive diffusion from the amount penetrated in the presence of current. Table 5.1 gives the enhancement ratios for CHX with iontophoresis. As evident, the highest enhancement was achieved at pH 7. Since the percentage of CHX ionised at this pH (99.45%) is not greatly different from the percentage of CHX ionised at pH 4, 5 or 6 (98.44%, 99.84% and 99.98%, respectively) the mechanism of enhancement under this condition could be attributed to (1) enhanced electroosmotic flow due to the increased membrane ionisation, (2) an increase in the rate of hydration due to the movement of water into the skin, and (3) permeability changes in the skin in the presence of an electric field which can also happen by changing the pH of the solution.

Statistical analysis of the cumulative amount of CHX penetrated across the skin after 60 minutes of iontophoresis showed a significant difference between the values of 3, 4, 6 and 7 ($P < 0.05$). However, no significant difference was observed between the cumulative amount of CHX penetrated across the skin from the vehicle with pH 4 and vehicle with pH 5 ($P > 0.05$).

pH	$Q_{60} \text{ (mg cm}^{-2}\text{)}$		ER
	Passive	Iontophoresis*	

3	3.95×10^{-4} (1.21×10^{-4})	0.039 (2.4×10^{-2})	98.73
4	4.2×10^{-4} (1.41×10^{-4})	0.063 (1.71×10^{-2})	150
5	4.5×10^{-4} (7.07×10^{-5})	0.069 (3.18×10^{-2})	153.3
6	5.1×10^{-4} (7.07×10^{-5})	0.094 (3.39×10^{-2})	184.31
7	5.5×10^{-4} (7.07×10^{-4})	0.113 (9.45×10^{-3})	205.45

Table 5.1 Effect of pH on the amount of CHX penetrated across the skin, ER is the enhancement ratio, * corrected Q_{60} value (amount of CHX penetrated during passive diffusion has been deducted from the final value). Values in brackets present the standard error.

pH	Iontophoretic Flux (mg cm min^{-1})	k_p (cm min^{-1})
3	9.79×10^{-4} (7.55×10^{-4})	1.383×10^{-3} (1.395×10^{-4})
4	1.53×10^{-3} (5.53×10^{-5})	2.16×10^{-3} (1.61×10^{-4})
5	1.77×10^{-3} (7.21×10^{-5})	2.50×10^{-3} (1.92×10^{-4})
6	2.13×10^{-3} (1.78×10^{-4})	3.0×10^{-3} (3.18×10^{-4})
7	2.55×10^{-3} (6.57×10^{-5})	3.6×10^{-3} (2.52×10^{-4})

Table 5.2 Effect of pH on the iontophoretic flux and permeability coefficient of CHX, (values in brackets present the standard error).

5.14.3 Effect of vehicle on the iontophoretic transport of CHX across human skin

As discussed in chapter 2 (section 2.4.3.2), a general approach to affect permeation rate is to incorporate solvents that either lead to an increase in the thermodynamic activity of the drug in the vehicle and thereby promoting the interfacial drug transfer into the SC or to include solvents that penetrate the SC themselves and in this way alter the barrier properties of the SC (Chandrasekaran and Shaw, 1978). Results obtained in chapter 2 (section 2.4.3.2), indicated that incorporating ethanol in the system would result in enhancing the permeation of the CHX across the skin. Hence, in this study, saturated solutions of CHX in 10% and 20% v/v ethanol were prepared in the same manner as discussed in section 2.3.1 and their effect on the iontophoretic transport of CHX across the human epidermis were assessed. CHX saturated solutions in distilled water were also used as the donor solutions in control diffusion cells. The permeation studies were performed in three stages as explained in section 5.13.7. 1 ml samples were withdrawn from the receptor at appropriate intervals. The CHX assay was performed using HPLC as described in section 2.2.1. (Receptor samples withdrawn at stage II of the permeation experiment, where the current of 0.5 mA/cm^2 was applied, were diluted 50 times using distilled water).

Permeation profiles of saturated solutions of CHX in distilled water, 10% and 20% ethanol are illustrated in figure 5.7. The CHX iontophoretic flux was promoted from $1.77 \times 10^{-3} \pm 7.21 \times 10^{-5}$ to $2.87 \times 10^{-2} \pm 3.59 \times 10^{-4} \text{ mg cm}^{-2} \text{ min}^{-1}$ by introducing 10% v/v ethanol to the system which was further enhanced to $6.44 \times 10^{-2} \pm 1.37 \times 10^{-4} \text{ mg cm}^{-2} \text{ min}^{-1}$ as the concentration of ethanol in the system was increased to 20% v/v. Although the transport of CHX from ethanolic solutions was accelerated during the passive diffusion stage, the enhancement became greater as the current was applied. This indicates a synergistic effect between ethanol and iontophoresis. In the presence of the current, 10% ethanol induced a 16.2-fold enhancement in the amount of CHX penetrated across the skin compared to distilled water (vehicle). 20% ethanol showed a higher effect on the amount of CHX penetrated across the skin compared to 10% ethanol and distilled water (as vehicle) during the iontophoresis. The enhancement ratio was 36.4 compared to the distilled water as the vehicle and 5.5 compared to 10% v/v ethanol as the vehicle.

The enhanced flux could be due to the higher concentration of CHX in ethanol which causes an increase in the concentration gradient across the skin (CHX solubility = 0.88 ± 0.049 mg/ml in 10% ethanol and 1.23 ± 0.085 mg/ml in 20% ethanol). According to Fick's first law (equation 1.1), the flux is proportional to the concentration gradient therefore, by increasing the concentration gradient an enhanced diffusion would be expected. Another explanation for the increased flux of CHX could be the effect of ethanol on the barrier properties of the SC. It has been shown that ethanol can act as a penetration enhancer and thus, increase the penetration of chemicals across the skin (Yum *et al.*, 1994; Pershing *et al.*, 1990). The proposed mechanism of action was the ability of ethanol to alter the structure of the skin such as, extracting SC lipids and osmotic expansions, in ways that appreciably increase solute diffusivity. This effect of ethanol has been discussed in more detail in chapter 3 where ethanol is used as a penetration enhancer.

Using equation 5.4, the effectiveness of iontophoresis on transport of CHX from ethanolic solutions was determined. Results are presented in table 5.4 and show that over 60 minutes, iontophoresis can enhance the cumulative amount of CHX penetrated across the epidermis from $2.45 \times 10^{-3} \pm 2.19 \times 10^{-3}$ to $0.126 \pm 4.73 \times 10^{-2}$ mg/ cm² in systems containing 10% ethanol.

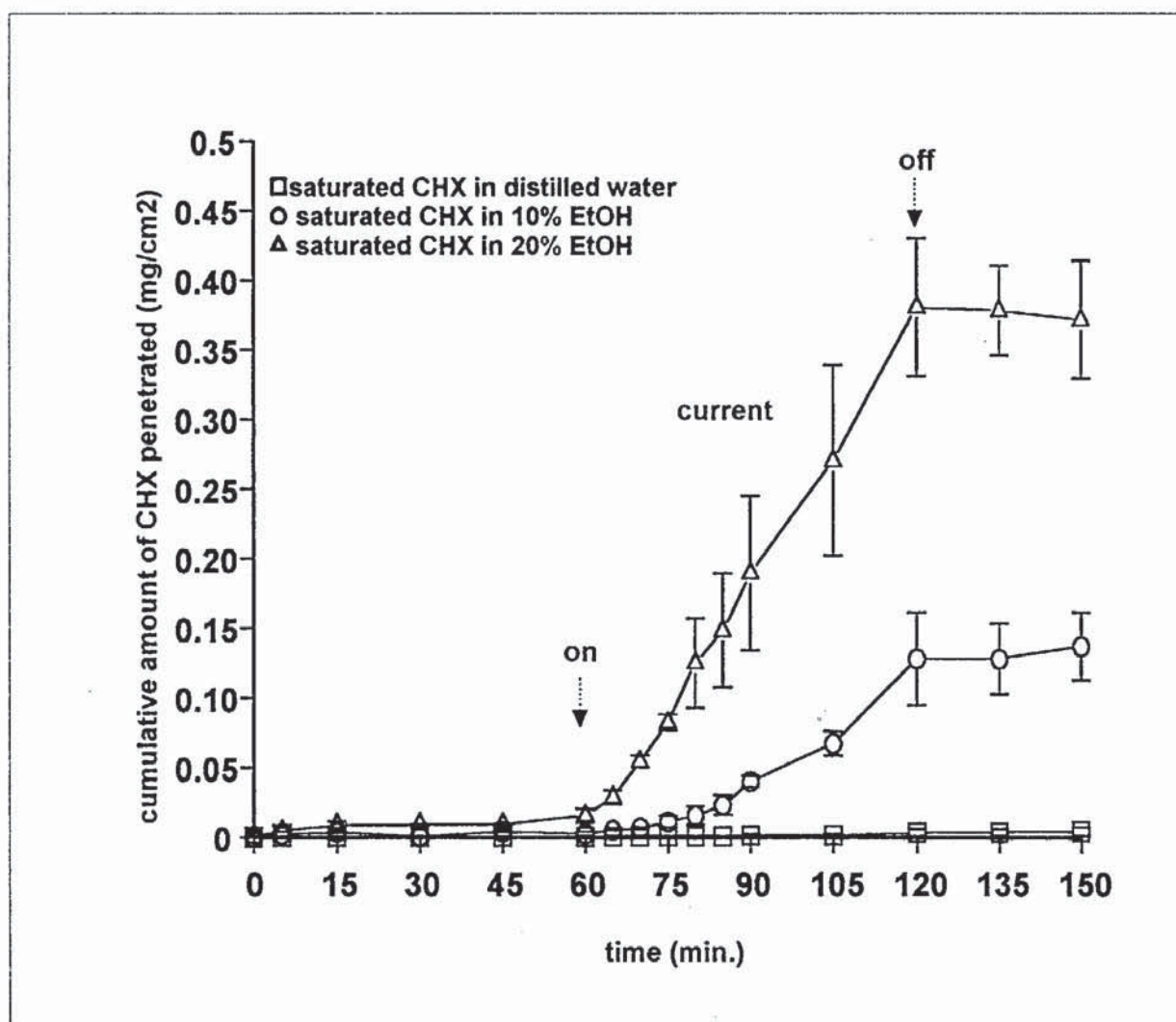


Figure 5.7 Effect of donor vehicle on the iontophoretic transport of CHX across human breast epidermis (each point represents mean \pm s.d, n=4)

Vehicle	Q ₆₀ (mg.cm ⁻²)		ER
	Passive	*Iontophoresis	
Distilled water	6.5×10 ⁻⁴ (7.1×10 ⁻⁵)	6.9×10 ⁻² (7.07×10 ⁻⁵)	106.15
10% Ethanol	2.45×10 ⁻³ (2.19×10 ⁻³)	0.126 (4.73×10 ⁻²)	51.43
20% Ethanol	4.8×10 ⁻³ (9.9×10 ⁻⁴)	0.693 (2.11×10 ⁻²)	144.38

Table 5.3 CHX Permeation data across human breast epidermis, ER is the enhancement ratio (iontophoretic Q₆₀/passive Q₆₀), * corrected Q₆₀ value (amount of CHX penetrated during passive diffusion has been deducted from the final value), values in brackets present the standard error.

Vehicle	Iontophoretic Flux (mg cm min ⁻¹)	k _p (cm min ⁻¹)	ER
0% Ethanol (distilled water)	1.67×10 ⁻³ (7.61×10 ⁻⁵)	2.50×10 ⁻³ (1.51×10 ⁻⁴)	-
10% Ethanol	2.87×10 ⁻³ (3.59×10 ⁻⁴)	3.26×10 ⁻³ (4.46×10 ⁻⁴)	1.62
20% Ethanol	6.44×10 ⁻³ (1.37×10 ⁻⁴)	5.24×10 ⁻³ (3.79×10 ⁻⁴)	3.64

Table 5.4 Effect of vehicle on the iontophoretic flux of CHX across the human epidermis, ER is the enhancement ratio (iontophoretic flux from ethanolic vehicle/iontophoretic flux from distilled water as the vehicle), values in brackets present the standard error.

5.14.4 Combined use of iontophoresis and chemical enhancers

In an attempt to increase the iontophoretic transport of CHX, chemical enhancers were used for pre-treatment of skin for 1 hour followed by one hour of iontophoresis (0.5 mA/cm² and 30 minutes of passive diffusion. The first enhancer employed for the pretreatment of human epidermis was 10% oleic acid (OA) in ethanol (EtOH). Human epidermis was pretreated with 10% OA/EtOH for 1 hour as discussed in section 3.5.5. After being washed with distilled water, the epidermis sheets were mounted on the diffusion cells. Donor chambers were filled with 10 ml of saturated solution of CHX in 25 mM HEPES having the concentration of 0.708 ± 0.046 mg/ml. De-ionised water (10 ml) was placed in the receptor chambers and the passive permeation of CHX was studied over 60 minutes. OA /EtOH pre-treatment promoted the amount of CHX penetrated across the skin from $6.5 \times 10^{-4} \pm 7.1 \times 10^{-5}$ to $5.55 \times 10^{-3} \pm 1.77 \times 10^{-3}$ mg/cm², indicating 8.5-fold increase. After 60 min of passive diffusion, Ag/AgCl electrodes were placed in the diffusion cells and a current of 0.5 mA/cm² was applied to the system. The cumulative amount of CHX penetrated across the skin was further enhanced to $8.13 \times 10^{-2} \pm 2.62 \times 10^{-3}$ mg/cm² in the presence of the current, indicating 125-fold increase in the CHX amount penetrated compared to the passive flux. Results are depicted in figure 5.8 and table 5.5.

To compare the effect of oleic acid/ethanol pre-treatment on the iontophoretic transport, the data obtained with iontophoresis of saturated solution of CHX across the untreated skin was replotted. Iontophoretic flux values were calculated from the linear part of the graphs and are summarised in table 5.5. No significant difference was seen between the flux of CHX penetrated iontophoreically across the untreated skin and pretreated skin ($P > 0.05$). Hence, we can conclude that 10% OA/EtOH pretreatment did not have any synergistic effect with iontophoresis of CHX.

Oleic acid has been reported to enhance the passive diffusion of a variety of drugs (Menon and Elias, 1997). The changes in the intercellular structure and reduction in the skin impedance caused by oleic acid could be the reasons for the enhancement of passive transport of CHX across the skin. However, when combined with iontophoresis, no synergistic effect was observed between iontophoresis and ethanolic solution of oleic acid. This could be due to the alteration of the electrical properties of the SC by oleic

acid/ethanol. It has been shown that upon the application of ethanolic solutions of oleic acid to the SC, the resistance of the tissue and the extent of the increase is dependent on the concentration of fatty acid present in the solution (Conaghey, 1995). Increasing the concentration of oleic acid from 1% to 3% resulted in an increase of 30% in the resistance of the tissue. The reason behind this observation could possibly be the replacement of removed SC lipids (due to ethanol pretreatment) by oleic acid. Hence the lipophilic acid, which itself is not conductive, can substantially contribute to the resistance of the tissue.

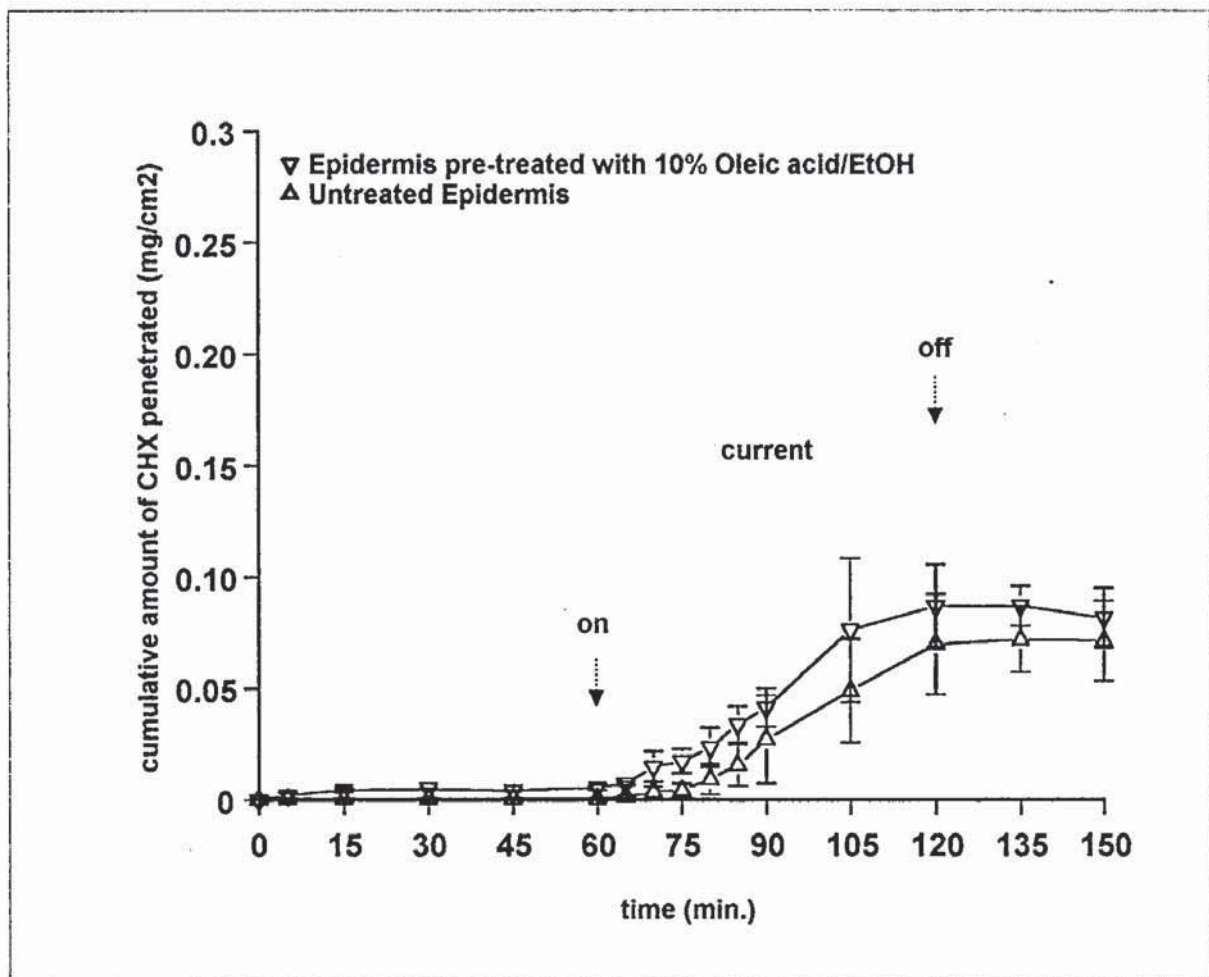


Figure 5.8 Cumulative amount of CHX penetrated across the untreated and pretreated human breast epidermis (each point represents mean \pm s.d, n=4)

The epidermis was next pretreated with 10% Azone in ethanol. Azone is a chemical that was developed specifically as a skin penetration enhancer in order to improve the permeation of drugs through the skin. In this study, human epidermis was pretreated with 10% Azone/ethanol for 1 h which was then washed thoroughly with distilled water to remove any remaining enhancer solution from the skin. Passive diffusion of CHX was studied across the pretreated skin for 1 h. The amount of CHX penetrated across the skin was enhanced 10.85 times (from $6.5 \times 10^{-4} \pm 7.1 \times 10^{-5}$ to $7.05 \times 10^{-3} \pm 7.07 \times 10^{-5}$ mg/cm²) following the pre-treatment. After 1 h of passive diffusion, 0.5 mA/cm² current was applied to the system and the transport of CHX was investigated for another hour. Figure 5.9 and table 5.5 show the results of pretreatment of Azone/ethanol on the iontophoretic delivery of CHX. To investigate the effect of the pretreatment on the iontophoretic flux of CHX across the skin, flux values were calculated from the linear part of the graphs and are summarised in table 5.6. Results show that the iontophoretic flux of CHX was enhanced 1.5 times following Azone/ethanol pre-treatment of the skin. Statistical analysis (t-test) of the data obtained with iontophoresis of CHX across the pretreated skin and the data obtained with iontophoresis across the untreated skin showed that Azone/ethanol pre-treatment enhances the iontophoretic flux of CHX significantly ($P < 0.05$). This result indicates the existence of a synergistic effect between iontophoresis and Azone/ethanol pre-treatment. However, this enhancement can not be related to Azone solely as ethanol itself can act as an enhancer.

To delineate the contributions of Azone and ethanol to the enhanced iontophoretic delivery of CHX, neat ethanol was used to pretreat the skin. Skin was pretreated with neat ethanol for an hour after which it was washed off the skin by distilled water. Permeation studies were performed as discussed in section 5.12.7 for the total of 150 min (60 min of passive diffusion followed by 60 min of iontophoresis and another 30 min of passive diffusion). Ethanol pre-treatment had a significant effect on the CHX iontophoretic flux ($P < 0.05$). The iontophoretic flux was increased from $1.77 \times 10^{-3} \pm 7.21 \times 10^{-5}$ to $2.42 \times 10^{-3} \pm 1.01 \times 10^{-4}$ mg cm⁻² min⁻¹) upon the pretreatment of the skin with neat ethanol (figure 5.9 and table 5.5). The enhancement could be due to the effect of ethanol on the SC structure (Levang *et al.*, 1999). Moreover, it has been shown that the application of neat ethanol almost completely removes the resistance of the tissue (Conaghey, 1995). A 2-hour pre-treatment with absolute ethanol reduced the resistance of the SC from 50k Ω to just a few

hundred ohms. This can be another explanation for the observed enhancement ratio with ethanol pre-treatment.

The comparison of the enhancement ratio (cumulative amount of CHX iontophoretically penetrated across enhancer pretreated skin/cumulative amount of CHX iontophoretically penetrated across untreated skin) obtained with neat ethanol with 10% Azone in ethanol showed no significant difference ($P>0.05$). Hence, we can conclude that the results observed with 10% Azone/ethanol were probably due to ethanol not Azone. The exact reason is not yet known. However, Kontturi *et al.* (1993) reported that treatment of human SC with lipophilic penetration enhancers such as Azone and dimethylaminoacetate (DDAA) could increase the resistance of the tissue to the current flow compared to the neat ethanol. In the analysis of their data, the authors proposed a modification to the equivalent circuit for the skin which incorporated the enhancer as an additional resistor.

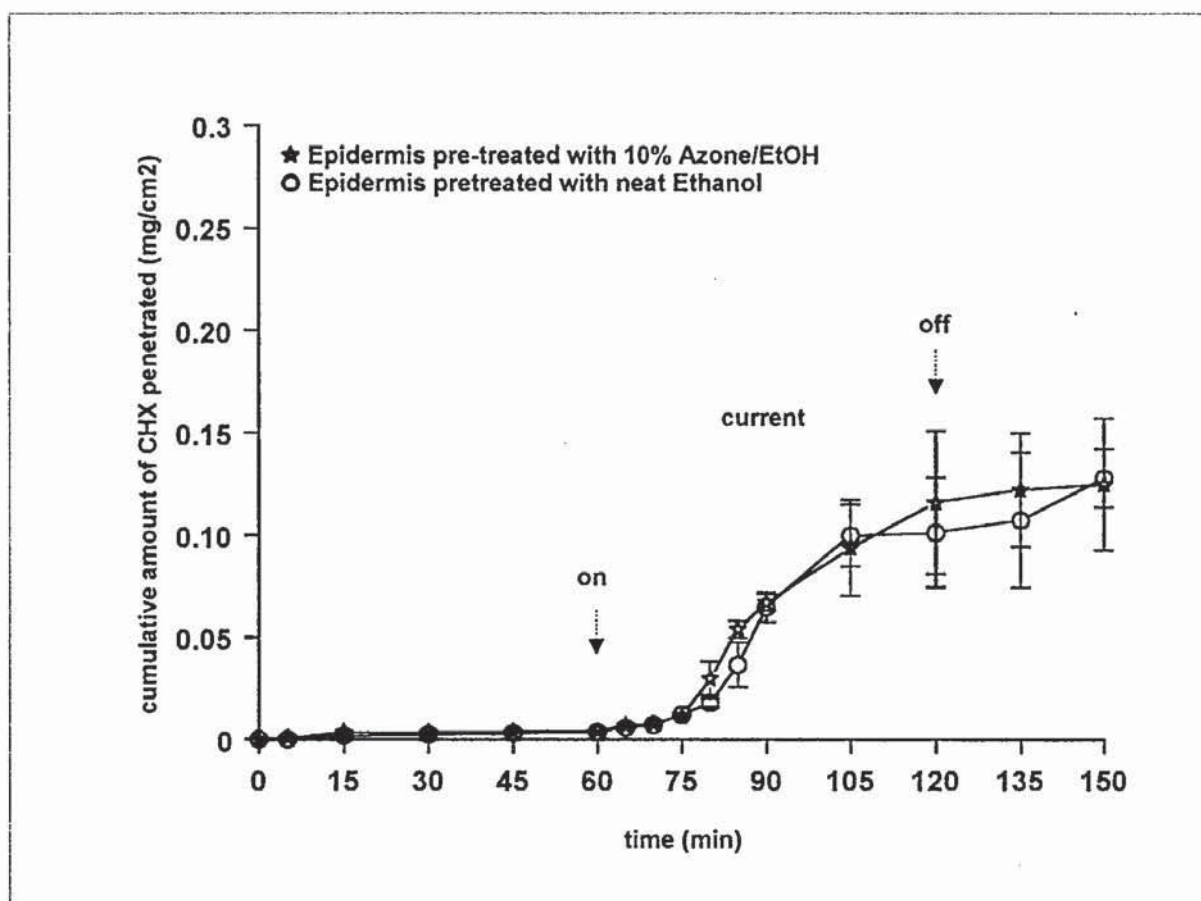


Figure 5.9 Effect of enhancer and iontophoresis on the transport of CHX across human breast epidermis (each point represents mean \pm s.d, $n=4$)

Another set of experiments were performed on human epidermis pretreated with either 10% oleic acid (OA) in PG or 10% Azone in PG as it has been reported that Azone and OA are more effective when used with PG (Tanojo *et al.*, 1998). The pre-treatment procedure was the same as the one described in section 3.5.5. Permeation studies were performed in the same manner as discussed in section 5.13.7 at three different stages (60 min of passive diffusion followed by 60 min of iontophoresis and another 30 min of passive diffusion). OA/PG pretreatment accelerated the transport of CHX across the skin. The amount of CHX penetrated across the skin was enhanced 10.5-fold following OA/PG skin pretreatment compared to untreated skin. As the current applied (0.5 mA/cm^2), the amount of CHX penetrated was promoted to $0.25 \pm 8.53 \times 10^{-2} \text{ mg/cm}^2$, indicating a 3.62-fold enhancement compared to the amount penetrated iontophoretically across the untreated skin. Results are presented in figure 5.10 and table 5.5.

Pretreatment of the skin with 10% Azone in PG showed a higher enhancement effect. The passive cumulative amount of CHX was enhanced from $6.5 \times 10^{-4} \pm 7.1 \times 10^{-5}$ (untreated skin) to $1.64 \times 10^{-2} \pm 4.88 \times 10^{-3} \text{ mg cm}^{-2}$. The iontophoretic amount of CHX penetrated was also enhanced from $1.77 \times 10^{-3} \pm 7.21 \times 10^{-5}$ to $0.368 \pm 8.25 \times 10^{-2} \text{ mg cm}^{-2}$, indicating a 208-fold increase (figure 5.10 and table 5.5). The iontophoretic flux of CHX calculated from the linear part of the graph and the permeability coefficient are summarised in table 5.5. The values show that the combination of skin pretreatment and iontophoresis would enhance the iontophoretic flux of CHX across the skin by 25.2-fold.

This work was in agreement with the work of Kanikkannan *et al.* (2000), who studied the effect of the skin pre-treatment with Azone and iontophoresis on the pharmacodynamic effect of timolol maleate *in vivo* in albino rabbits. They showed that the pre-treatment of skin with Azone eliminated the lag time and prolonged the duration of action of iontophoresis from 4 to 6 h. Kalia and Guy (1997) investigated the interaction between penetration enhancers and iontophoresis and their effect on human skin impedance. Using impedance spectroscopy, they concluded that Azone/PG had a profound effect on post-iontophoretic impedance, considerably amplifying the effect of current passage however, oleic acid showed a smaller decrease in skin impedance. This result can explain the lower enhancement ratio obtained with oleic acid/PG compared to Azone/PG.

Since oleic acid and Azone were applied to the skin in PG as the solvent, the effect of PG on the iontophoretic delivery of CHX was studied to exclude the contributions of PG to the enhancement ratio obtained with oleic acid and Azone. The epidermis was pretreated with neat PG and the permeation studies were once again performed over 150 minutes. PG pretreatment enhanced the amount of CHX penetrated across the skin by 7-fold during stage I (passive diffusion) and by 2-fold during the stage II (iontophoresis) of the permeation experiment (figure 5.10 and table 5.5). The mechanism by which PG increases the permeation of drugs through the skin can be explained by the effect of PG on the SC structure (denaturing the SC proteins) (Barry, 1987). This effect has been explained in more details in chapter 3 section 3.3.1.

Comparing the enhancement ratio obtained with the oleic acid/PG pre-treatment (3.62 times) and Azone/PG pre-treatment (5.33 times) with the neat PG treatment (2 times), we can conclude that the obtained enhancement was the result of both the enhancer and the solvent (PG).

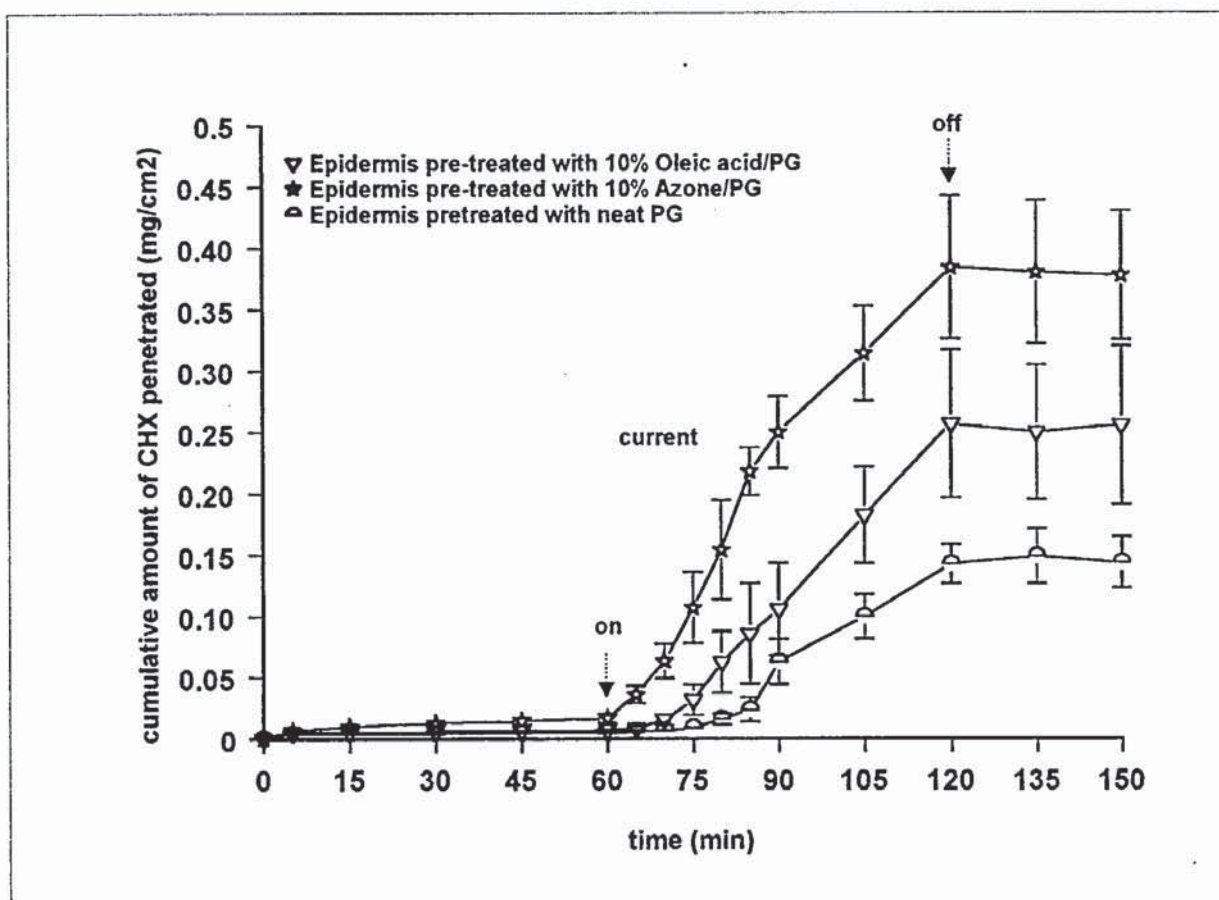


Figure 5.10 Effect of enhancer and iontophoresis on the transport of CHX across human breast epidermis (each point represents mean \pm s.d, n=4)

In the next set of experiments, the human skin was pretreated with surfactants and the effect of these surfactants on the iontophoretic delivery of CHX was studied. The studied surfactants were sodium dodecyl sulphate (SDS, an anionic surfactant) and dodecyl triammonium bromide (DTAB, a cationic surfactant). The epidermis sheets were pretreated with 10% SDS in PG and 10% DTAB in PG separately and the permeation studies were performed as discussed in section 5.13.6 over 150 min. Results are depicted in figure 5.11 and table 5.5 and show that SDS, an anionic surfactant, was more effective in enhancing the amount of CHX penetrated across the skin than its cationic counterpart (DTAB) in the presence of current. The enhancement ratios were 2.78 and 2.23, respectively. This result was not consistent with the literature reporting greater enhancing effects attributable to cationic rather than anionic surfactants (Ashton *et al.*, 1992). One explanation could be the effect of DTAB on the negative charge the skin carried at physiological pH (Burnette and Ongpipattanakul, 1987). DTAB may lower SC negativity and consequently reduce its permselectivity to cations. Hence, the flux due to electro-osmosis effect, which is one of the delivery mechanisms in CHX iontophoresis (section 5.14.2) would be reduced.

Comparing the amount of CHX penetrated across the neat PG pretreated skin ($0.138 \pm 2.26 \times 10^{-2}$ mg cm⁻²) to the amount of CHX penetrated across the surfactant pretreated skin ($0.192 \pm 1.26 \times 10^{-2}$ for SDS/PG pre-treatment and $0.154 \pm 4.60 \times 10^{-2}$ mg cm⁻²), we can see that there was a significant difference between results obtained with PG and the results obtained with 10% SDS/PG ($P < 0.05$). This indicated that SDS itself contributed towards the enhancement in the delivery of CHX. However, there was no significant difference between the results obtained with PG pretreated skin and DTAB/PG pretreated skin ($P > 0.05$). This indicated that DTAB had no effect on the enhancing the amount of CHX penetrated across the skin and the observed ER was due to PG alone.

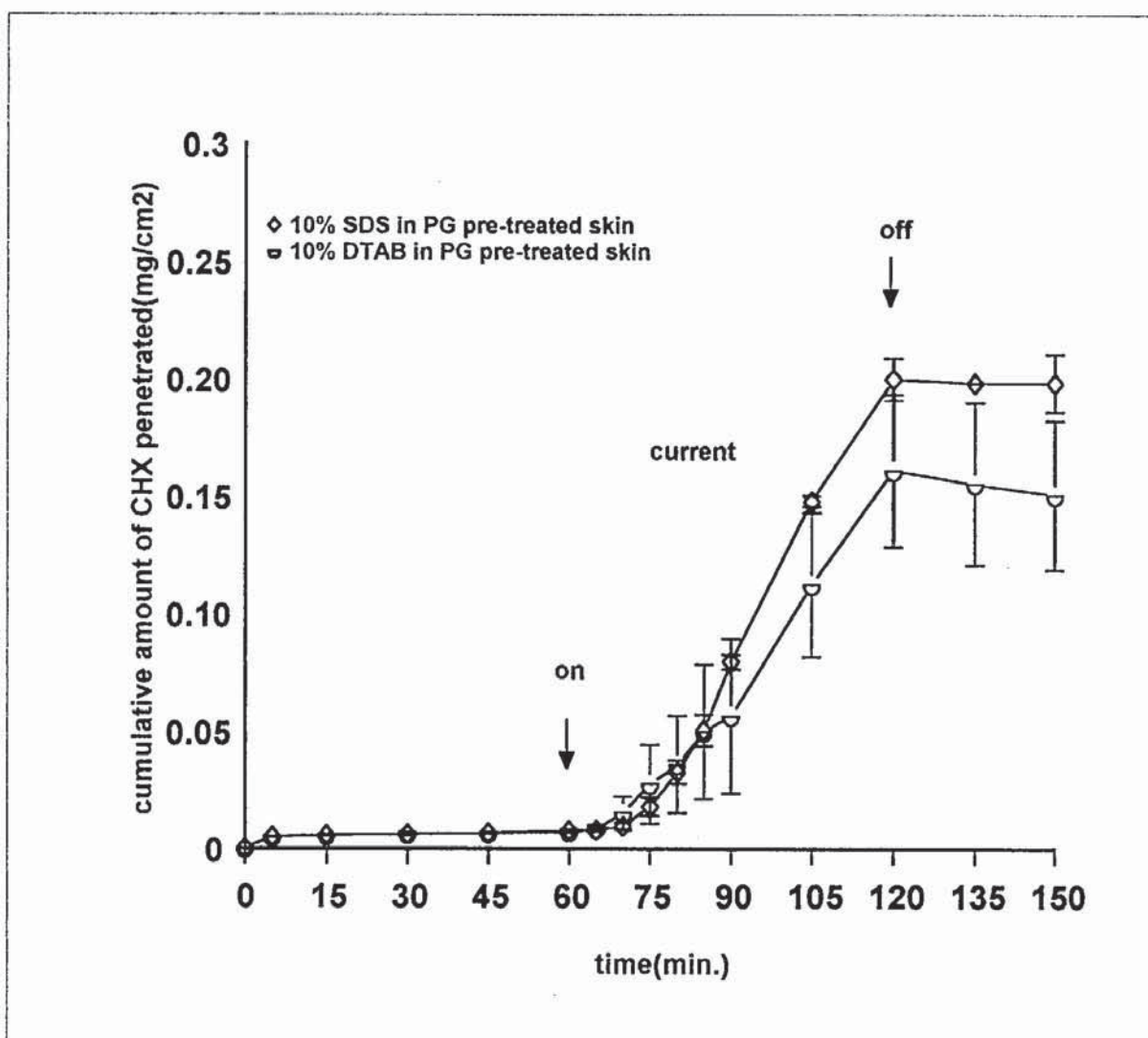


Figure 5.11 Effect of surfactants (enhancer) and iontophoresis on permeation of CHX across human breast skin (each point represents mean \pm s.d, n=4)

Pretreatment	Passive Q_{60} (mg cm ⁻²)	Iontophoretic Q_{60}^* (mg cm ⁻²)	ER ¹	ER ²
Control (no treatment)	6.5×10^{-4} (7.1×10^{-5})	6.9×10^{-2} (7.07×10^{-5})	-	-
10% OA/EtOH	5.55×10^{-3} (1.77×10^{-3})	8.13×10^{-2} (2.62×10^{-3})	1.18	125.08
10% Azone/EtOH	7.05×10^{-3} (7.07×10^{-5})	0.112 (4.94×10^{-2})	1.6	172.31
Neat Ethanol	3.64×10^{-3} (2.18×10^{-4})	0.101 (4.84×10^{-2})	1.46	155.4
10% OA/PG	6.85×10^{-3} (4.95×10^{-4})	0.25 (8.53×10^{-2})	3.62	384.62
10% Azone/PG	1.64×10^{-2} (4.88×10^{-3})	0.368 (8.25×10^{-2})	5.33	566.15
Neat PG	4.55×10^{-3} (2.12×10^{-4})	0.138 (2.26×10^{-2})	2	212.31
10% SDS/PG	7.7×10^{-3} (7.07×10^{-4})	0.192 (1.26×10^{-2})	2.78	295.38
10% DTAB/PG	6.95×10^{-3} (4.95×10^{-4})	0.154 (4.60×10^{-2})	2.23	236.92

Table 5.5 CHX transport data across pretreated human skin * corrected Q_{60} value (cumulative amount of CHX penetrated during passive diffusion has been deducted from the final value), ER¹ (enhancement ratio) = cumulative amount of CHX penetrated across enhancer pretreated skin (iontophoresis)/ cumulative amount of CHX penetrated across untreated skin (iontophoresis), ER² = cumulative amount of CHX penetrated across enhancer pretreated skin (iontophoresis) / cumulative amount of CHX penetrated across untreated skin (passive). Values in brackets represent the standard error.

Enhancer	Iontophoretic Flux ($\text{mg cm}^{-2} \text{ min}^{-1}$)	k_p ($\text{cm}^{-2} \text{ min}^{-1}$)	ER
Control (untreated skin)	1.77×10^{-3} (7.21×10^{-5})	2.50×10^{-3} (1.92×10^{-5})	-
10% OA/EtOH	1.56×10^{-3} (1.01×10^{-4})	2.2×10^{-3} (2.02×10^{-4})	-
10% Azone/EtOH	2.67×10^{-3} (1.40×10^{-4})	3.77×10^{-3} (3.15×10^{-4})	1.5
Neat EtOH	2.42×10^{-3} (2.57×10^{-4})	3.41×10^{-3} (4.69×10^{-4})	1.4
10% OA/PG	4.88×10^{-3} (7.53×10^{-5})	6.89×10^{-3} (1.68×10^{-5})	2.8
10% Azone/PG	8.25×10^{-3} (5.73×10^{-4})	0.0116 (1.05×10^{-4})	4.7
Neat PG	3.15×10^{-3} (4.36×10^{-4})	4.45×10^{-3} (6.80×10^{-4})	1.8
10% SDS/PG	4.21×10^{-3} (1.49×10^{-4})	5.95×10^{-3} (4.40×10^{-4})	2.4
10% DTAB/PG	2.95×10^{-3} (1.55×10^{-4})	4.17×10^{-3} (3.45×10^{-4})	1.7

Table 5.6 Effect of skin pretreatment on the iontophoretic flux of CHX, ER is the enhancement ratio calculated by dividing the iontophoretic flux across the pretreated skin by the iontophoretic flux across the untreated skin, values in brackets represent the standard error.

Looking at the enhancement ratios achieved with the enhancers combined with iontophoresis (table 5.5), it can be inferred that iontophoresis can be synergistic with enhancers to provide an additional driving force to maintain and control the target flux of CHX. 10% Azone/PG pretreatment resulted in the highest enhancement ratio (ER=4.77)

both during the passive diffusion studies and iontophoresis followed by 10% oleic acid/PG (ER=3.62).

To further increase the transport of CHX across human epidermis, and based on the results obtained with the saturated solution of CHX in aqueous ethanolic solutions (section 5.13.4), a saturated solution of CHX in 20% ethanol was prepared in the same manner as described in section 2.3.1 and was used as the donor solution. The permeation study was once more carried out on pretreated skin in the presence of a constant current of $0.5\text{mA}/\text{cm}^2$. 10% Azone/PG was chosen as the pretreatment regimen as this enhancer showed the highest synergistic effect with iontophoresis (table 5.6). The result is depicted in figure 5.12 and table 5.7. The cumulative amount of CHX penetrated (Q_{60}) across the untreated skin from a saturated solution of CHX in distilled water was to $6.5 \times 10^{-4} \pm 7.1 \times 10^{-5} \text{ mg cm}^{-2}$. Upon using the saturated solution of CHX in 20% ethanol, Q_{60} was increased to $0.0155 \pm 7.14 \times 10^{-3} \text{ mg cm}^{-2}$. Pretreatment of skin with 10% Azone/PG promoted this amount (Q_{60}) to $0.026 \pm 4.87 \times 10^{-3} \text{ mg cm}^{-2}$. As the current was applied to the system, the CHX amount was further increased to $1.00 \pm 0.0937 \text{ mg cm}^{-2}$, indicating an enhancement ratio of 4.66 compared to the iontophoretic diffusion of CHX across the untreated skin from a 20% ethanolic solution. Hence, it can be concluded that change of donor vehicle can further enhance the iontophoretic transport of CHX across pretreated human breast skin.

The observed result could be due to the higher concentration of CHX in 20% ethanol which causes an increase in the concentration gradient across the skin (CHX solubility = in 20% ethanol was $1.23 \pm 0.085 \text{ mg/ml}$). According to Fick's first law (equation 1.1), the flux is proportional to the concentration gradient therefore, by increasing the concentration gradient an enhanced diffusion would be expected. Another explanation for the increased flux of CHX could be the effect of ethanol on the barrier properties of the SC. It has been shown that ethanol can act as a penetration enhancer and thus, increase the penetration of chemicals across the skin (Yum *et al.*, 1994; Pershing *et al.*, 1990). The proposed mechanism of action was the ability of ethanol to alter the structure of the skin such as, extracting SC lipids and osmotic expansions, in ways that appreciably increase solute diffusivity.

Alteration of the SC resistance could be another explanation for the enhanced flux of CHX across the skin. Conaghey (1995) have shown that ethanol can lower the resistance of the SC and thus, increasing the current flow through the membrane. The enhanced current flow can result in a higher flux of the drug across the skin.

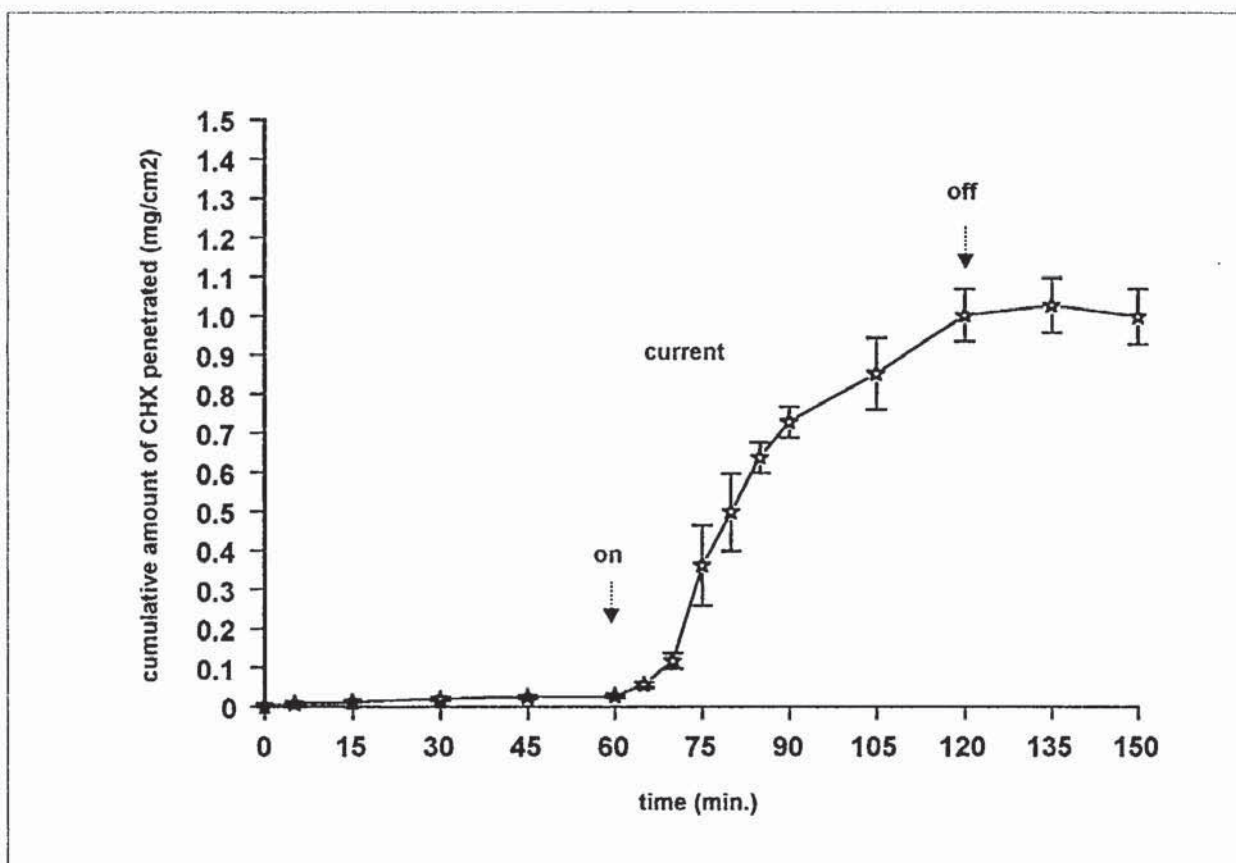


Figure 5.12 Effect of donor vehicle (20% ethanol), pretreatment of skin with 10% Azone/PG and iontophoresis on permeation of CHX across human breast skin (each point represents mean \pm s.d, n=4)

Enhancer	Iontophoretic Flux (mg cm ⁻² min ⁻¹)	k _p (cm ⁻² min ⁻¹)	ER
Control (no pretreatment)	6.44×10 ⁻³ (1.37×10 ⁻⁴)	5.24×10 ⁻³	-
10% Azone/PG	0.03 (3.13×10 ⁻³)	0.024	4.66

Table 5.7 Effect of vehicle and skin pretreatment on the iontophoretic flux of CHX, ER=enhancement ratio (CHX flux across pretreated skin/CHX flux across untreated skin), values in brackets present the standard error.

5.15 Summary

The results presented in this chapter provide evidence for the effectiveness of iontophoresis in enhancing the penetration of CHX across the human skin. The application of 0.5 mA/cm^2 resulted in a significant increase in the transport of CHX from a saturated solution in distilled water compared to the passive diffusion of CHX across the skin. The enhancement effect was further enhanced by introducing 10% and 20% ethanol in the system, respectively.

The possible contribution of electroosmosis to the iontophoretic flux of CHX was also studied using donor solutions with different pH (3-7). A decrease in both the passive and iontophoretic delivery of CHX was observed upon decreasing the donor pH. Since the iontophoretic delivery was affected to a higher extent compared to the passive diffusion, a suggestion was made that electrorepulsion can not be the sole mechanism of CHX iontophoretic flux and electroosmosis is also responsible for CHX transport. This suggestion was based on the report by Guy (1992), who reported that the membrane ionisation can be reduced by decreasing the vehicle pH and thus the iontophoretic delivery of a drug would suffer provided electrorepulsion was not the sole mechanism of iontophoretic transport.

The possibility of a synergistic effect of chemical penetration enhancers and iontophoresis on the permeation of CHX was also investigated by pretreating the skin with several enhancers prior to the iontophoretic studies. The pretreated membranes showed less resistance to the diffusion of CHX with the membrane pretreated with 10% Azone in PG showing the least resistance followed by the skin pretreated with 10% oleic acid in PG and 10% SDS in PG. The obtained results confirmed the existence of a synergistic effect.

CHAPTER SIX

ANTIMICROBIAL TESTING OF CHLORHEXIDINE DIHYDROCHLORIDE

6.1 Introduction

Most antiseptics are not simple solutions, but complex formulations of one or more antimicrobial agents, in combination with excipients. The method of formulation can profoundly affect the physical and biological properties of the active agent (Meakin, 1983). Any interactions between the active agent and excipients used in the formulation may affect the activity of the antimicrobial agent (Meakin, 1983). The presence of some compounds, such as organic chemicals, may reduce the effectiveness of the active agent by either adsorbing it and thus reducing the amount available for combining with the bacteria it is designed to kill or forming a protective coat around the bacteria, thereby preventing the penetration of the active agent to its site of action (Hugo and Russell, 1998). Therefore, it is not adequate to assume that an antiseptic formulation will be effective merely because of a specific amount of active agent has been included. Testing of antiseptics as, opposed to chemical assay, therefore, is of vital importance.

Various methods have been used to assess the microbiological activity of antimicrobial agents. Several researchers used a method involving the measurement of the minimum inhibitory concentration (MIC) of the antimicrobial agent (Muller and Sydel, 1993; Uekama *et al.*, 1980). MIC is the lowest concentration, expressed in micrograms per millilitre ($\mu\text{g/ml}$) or part per million (ppm), at which the antibacterial agent will inhibit the growth of a specific organism. Table 6.1 show the MIC values for chlorhexidine gluconate.

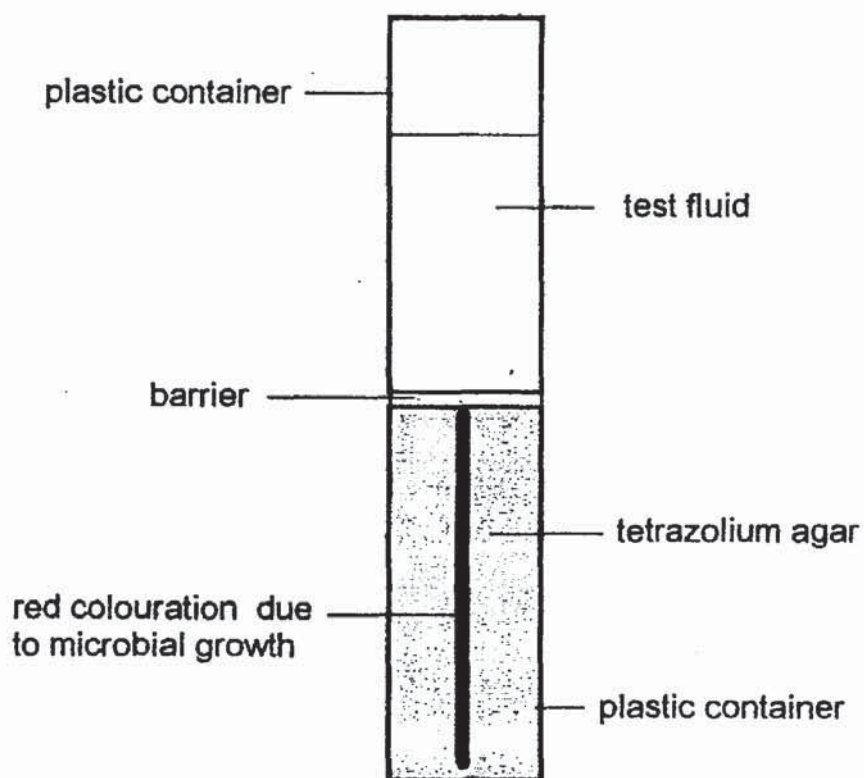
Test organism	No. of strains	MIC (mg/L)	
		Mean	Range
Gram positive cocci			
Staphylococcus aureus	16	1.6	1-4
Staph. epidermis	41	1.8	0.25-8
Gram positive bacilli			
Corneobacterium spp.	8	1.6	0.5-8
Propionibacterium acne	2	8	

Table 6.1 Bacteriostatic activities of chlorhexidine gluconate

Another is method to assess the activity of an antimicrobial agent the inhibition zone test (Atkins *et al.*, 1998). In this type of test, the antiseptic, or a dilution of it, is introduced into a well in an agar plate. It diffuses into the surrounding agar, which has been inoculated with the test organism. The growth of the organism is inhibited (if it is sensitive) and this is seen as zones of inhibition that are formed after an appropriate incubation period. This method is a qualitative, rather than a quantitative test since some compounds such as, cationic compounds, may interact with the growth medium which will result in a poor diffusibility. Hence, a compound giving a small zone of inhibition may not be intrinsically less active than a different type of chemical compound giving a large zone of inhibition when tested at the same concentration under similar conditions.

Another microbiological method is the bacterial viability assay in which either a known volume of the antimicrobial solution (Denton, 1991) or the solid antimicrobial agent (Pedersen *et al.*, 1993) is added to the broth culture of the test organism. At appropriate intervals, adequate intervals will be removed and transferred to a neutraliser medium (e.g. soya lecithin and 10% polysorbate 80). A viable count will be then performed on appropriate further dilutions and, by comparison with an untreated control, the efficacy of the antimicrobial agent will be investigated.

A further microbiological method developed in the Department of Clinical Microbiology at the Queen Elizabeth (QE) Hospital, Birmingham, as part of this study, assessed the effect of the antibacterial solutions. This method was based on the diffusion of antimicrobial agent across a membrane which had been mounted between two small containers (figure 6.1). The lower section was filled with a growth medium containing tetrazolium salt, which developed a red colour in the presence of any bacterial growth. The growth medium was stabbed by the appropriate organism. The solution under study was placed in the top section and the whole set up was then incubated at 37°C overnight. The set up was examined the following day for any effect of antimicrobial solution on the bacterial growth. The distance from fluid/agar interface to red coloration was then measured using a ruler. The shorter distance was an indication of a higher antibacterial effect. Basis of this model is illustrated in figure 6.2. This method examined the activity of antimicrobial agent penetrated across the skin.



The two halves are held together with elastic bands; the skin overlaps the container edge slightly so that a watertight seal is formed

Figure 6.1 Schematic diagram of model developed in Queen Elizabeth Hospital

1) no penetration



No penetration of antimicrobial into agar, so bacteria grow the entire length of the stab inoculation

2) some penetration

depth of penetration



Antimicrobial penetrates through barrier into the agar, inhibiting growth and therefore colour formation

3) greater penetration

depth of penetration



Greater penetration or antibacterial action inhibits growth further. Depth of penetration can be measured and is proportional to penetration and antimicrobial action

Figure 6.2 Basis of the model developed in QE Hospital

The aim of the present study was to investigate the effect of polymers used in the preparation of supersaturated solutions of CHX and chemical enhancers used for the pretreatment of the skin for the purpose of enhancing the permeation of CHX on the antimicrobial activity of CHX. Three methods were used for the microbiological testing during this study. The first method was the measurement of inhibition zone. This method was employed in the study of the effect of anti-nucleant polymer on antimicrobial activity of CHX. The effect of supersaturation on the antimicrobial properties of CHX was also studied using this method. The second method was based on the diffusion studies performed on the effect of pre-treatment on the permeation of CHX (section 3.5.8) and supersaturated solutions of CHX across human epidermis (section 4.4.6). The third method was similar to the method developed in Department of Clinical Microbiology at the QE Hospital, Birmingham which was used to study the antimicrobial activities of supersaturated solutions of CHX and the effect of skin pretreatment on the antimicrobial activity of CHX. The last two methods examined the activity of CHX penetrated across the skin and unlike the first method did not involve the direct contact of the solution with the bacteria.

6.2 Materials and Methods

6.2.1 Materials

Chlorhexidine dihydrochloride and Tetrazolium salt were purchased from Sigma Chemicals, UK. Brain Heart Infusion (BHI) agar, Brain Heart Infusion broth, Letheen broth and Iso-sensitive agar were purchased from Oxoid (Basingstoke, UK). Cellulose filters (0.2 μm , γ -irradiated) were purchased from Sigma Chemicals for sterilisation of test solutions.

6.2.2 Bacterial Suspension

Staphylococcus epidermis (NCTC 11047) was chosen as the test organism as it is one of the skin microflora and is reportedly responsible for the catheter-related infections (Elliot *et al.*, 1997). To prepare the bacterial suspension, one colony of *Staph. Epidermis* was placed in 20 ml sterile BHI broth using a sterile loop and left in the warm room (37°C) overnight while stirring. The number of organisms present in the suspension was calculated by performing a viable count by serially diluting the initial *Staph. epidermis* suspension (10^1 - 10^6 -fold). Triplicate nutrient agar (NA) spread plates were prepared for each dilution by spreading 100 μl aliquots of each dilution on BHI agar plates. The plates were incubated at 37°C overnight and the number of colonies was counted the following day.

6.2.3 Effect of polymers on the microbiological activity of CHX

To study the effect of polymers on the antimicrobial activity of CHX, the zone-inhibition measurement method was employed as follows:

10 ml of the prepared bacterial suspension (section 6.2.2) was dispersed by gentle shaking in 500 ml Iso-sensitive agar, which had been autoclaved and left to cool. The initial concentration of bacteria was $4.42 \times 10^8 \pm 1.1 \times 10^8$ CFUs. 100 ml of this medium was poured into three large square bioassay dishes fitted with lids and allowed to set in a cold room at 4°C for 30 minutes. Adequate numbers ($n=36$) of 3-mm diameter wells were cut into each plates using the end of a sterilised Pasteur pipette and a 40 μl aliquot

of each sample (distilled water, 10% w/v aqueous solution of PVP K30, 0.5% w/v aqueous solution of HPMC, CHX saturated solution and CHX saturated solutions with either 10% PVP K30 or 0.5% HPMC) was added to each well in a recorded but random sequence (n=5). The plates were left overnight at 37°C and the following day the diameter of the clearing zone around each well was measured using a ruler. Distilled water was used as control in this experiment. The distribution of the samples over the bioassay dishes are presented in figure 6.3.

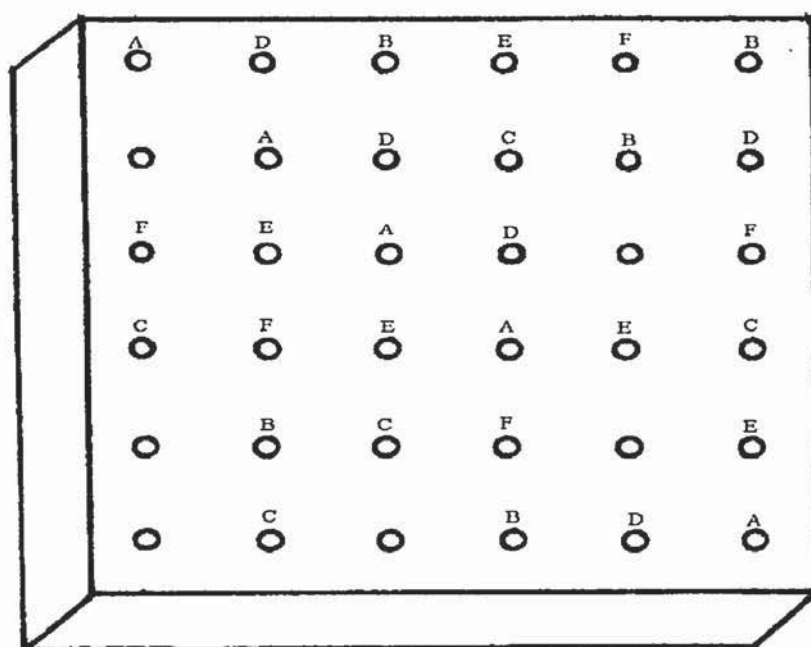


Figure 6.3 Distribution of samples over the bioassay plate, A = water, B = 10% w/v aqueous solution of PVP K30, C = 0.5% w/v aqueous solution of HPMC, D = CHX saturated solution, E = CHX saturated solution with 10% PVP K30, F = CHX saturated solution with 0.5% HPMC.

This method was also employed to study the antimicrobial activity of the supersaturated solution of CHX containing 0.3M NaCl and 10% PVP K30 as the antinucleant. The supersaturated system was prepared in the same manner as described in section 4.6.3. Bioassay dishes were prepared in the same manner as discussed before by pouring 100 ml of the medium (Iso-sensitive agar) containing 4.42×10^8 CFU/ml of bacteria. 40 μ l aliquots of the CHX supersaturated solution were placed randomly in six wells, cut in the plate in advance using the end of a sterilised Pasteur pipette. The plate was placed overnight at 37°C and the following day the diameter of the clearing zone around each well was measured using a ruler. As control, the same solution (distilled water, 10% PVP and 0.3 M NaCl) without CHX was used.

6.2.4 Microbial Activity of Saturated and Supersaturated Solutions of CHX Using Franz Diffusion Cells

Saturated and supersaturated solutions of CHX were prepared using the same procedure as described in section 2.3.1 and 4.6.3, respectively. Only the system with the highest permeation rate was used in this study (Saturated CHX + 10% PVP K30 + 0.3M NaCl). Franz diffusion cells, as previously described in section 2.2.2, were employed to study the antimicrobial activities of the supersaturated solutions over a 30-minute period. This experiment was based on the diffusion studies performed on the supersaturated systems (section 4.7.5). 10 ml of the bacterial suspension (section 6.2.2) with the initial bacterial count of $4.42 \times 10^8 \pm 1.1 \times 10^8$ CFUs was added to 100 ml sterile phosphate buffer (pH=6) and stirred gently for even distribution of the bacterial suspension within the solution. Franz diffusion cells were set up as described in section 2.2.2 except for the receptor compartment, which was filled with the bacterial phosphate solution. 1 ml of the test solutions (saturated CHX and supersaturated CHX) were then placed in the donor phase and at appropriate intervals, samples of the receptor (0.1ml) were removed with sterile syringes and serially diluted (10^1 to 10^5 -fold) with first Letheen Broth (9.9 ml), a neutralising medium, and then sterile phosphate buffer (9.9 ml). From each dilution, 100 μ l was spread onto three BHI agar plates and incubated at 37°C overnight. The colonies formed were then counted the following day. The experiment was performed in triplicate and as control the same solutions as donor phase but without CHX were used.

This procedure was repeated once again with the pretreated human skin as the mounted membrane and saturated solution of CHX in distilled water as the donor solution. Based on our findings during permeation studies of CHX across enhancer pretreated human skin (table 3.1), only 10% Azone/PG, 10% Oleic acid/PG and 10% SDS/PG were used for pre-treatment of the skin as these enhancers were more effective in enhancing the permeation of CHX across the skin compared to the other enhancers used in this study (section 3.6.3.5).

Donor and receptor compartments of diffusion cells the latter of which contained magnetic fleas were sterilised prior to use. All the CHX solutions were also sterilised using cellulose filters (0.2 μm , γ -irradiated).

6.2.5 Microbial Activity of Saturated and Supersaturated Solutions of CHX Using the Method Developed in QE Hospital

The method developed in QE Hospital (section 6.1) was employed to study the antimicrobial effect of supersaturated systems. For this purpose, supersaturated solutions of CHX with different degrees of saturation were prepared in the same manner as described in section 4.6.3. Iso-sensitive agar containing 0.01% tetrazolium salt was placed in the lower compartment of the set-up and was then stab inoculated with *Staph. epidermis*. The CHX solutions (saturated or supersaturated) were then placed in the top compartment and the whole set up was then left at 37°C overnight. The distance from fluid/agar interface to red coloration was then measured. The experiment was carried out in six replicates and skin used as the membrane was from the same source and area. As control, distilled water was used.

6.3 Results and Discussion

6.3.1 Effect of Polymers on Microbial Activities of CHX

Using the zone-inhibition measurement method, the effect of polymers on the bacterial growth was investigated. 40 μ l aliquots of the aqueous solutions of the polymers (10 % w/v PVP K30 and 0.5% w/v HPMC) were placed randomly in the wells, which were cut into the plate in advance. The effect of polymers on the antimicrobial activities of CHX was also studied. For this purpose, 10% PVP K30 and 0.5% HPMC were added to saturated solutions of CHX on separate occasions. 40 μ l aliquots of each system were also placed randomly in three wells of the plate as shown in figure 6.3. The plate was left overnight at 37°C and the inhibition zone was measured the following day using a ruler. No inhibition zone around the wells containing 40 μ l of the polymers was observed on the agar plate suggesting that the polymers were devoid of any antibacterial activities. Moreover, addition of 10% w/v PVP and 0.5% w/v HPMC did not induce any changes of antibacterial properties of CHX since the inhibition zone produced by saturated solutions of CHX (concentration of 0.78 ± 0.038 mg/ml) with and without polymers were exactly the same (zone diameter = 2 cm).

This method was also used to study the antimicrobial properties of the supersaturated solution of CHX. Based on the results obtained from the permeation studies carried out on the supersaturated solutions of CHX (section 4.6.3), only the system containing 0.3 M NaCl with 10% w/v PVP K30 (the anti-nucleating polymer) was used as this system showed a higher CHX flux compared to the other supersaturated systems. Surprisingly, the zone-inhibition method did not demonstrate any increase in the antimicrobial properties of the supersaturated system although an enhanced antimicrobial activity due to the potentially enhanced flux of supersaturated solutions was expected. Two explanations can be given for this failure, one is the fact that saturated and supersaturated solutions of CHX had the same amount of CHX hence, in the direct contact method they are bound to demonstrate the same antimicrobial activity. The second explanation could be the time needed in order to observe any antibacterial effect. The plates had to be left in the warm room overnight in order to demonstrate any

inhibitory effect on the growth of bacteria. Therefore, even if the supersaturated solution diffused faster throughout the agar during the first hour, we would not be able to observe this phenomenon. Hence, the adaptation of diffusion cells, in which the test solutions and the bacterial suspension were separated by the human skin seemed appropriate.

6.3.2 Antimicrobial Activity of Saturated Solution of CHX Using Franz Diffusion Cells

The results of the inhibition-zone test demonstrated the antimicrobial activity of CHX against *Staphylococcus epidermis* (NCTC 11047). However, in that experiment, the test solution was in direct contact with the bacteria while in an *in vivo* situation, CHX has to overcome the barrier properties of stratum corneum in order to remove the bacteria residing in the deeper layers of the skin. Previous permeation studies on saturated solution of CHX showed a poor transport rate of CHX across human epidermis (section 2.4.3). To investigate whether this low concentration of CHX penetrated has any effect on the inhibition of bacterial growth, Franz diffusion cells were adapted in this study. The phosphate buffer bacterial solution of *Staph. epidermis* prepared in the same procedure as described in section 6.2.2 was placed in the receptor compartment. A saturated solution of CHX (0.78 ± 0.038 mg/ml) was introduced to the donor and the effect of CHX permeated across the human abdominal epidermis on the viability of the bacteria was studied over 30 minutes. The 30-minute period was chosen as in practice, for skin sterilisation purposes, the antibacterial agent is only left on the skin for 2-3 minutes. A sample of 0.1 ml was withdrawn from the receiver every 1 minute for the first 10 minutes and then every 5 minutes for the rest of the study. Samples were all serially diluted 10^1 - 10^5 times with first sterile Letheen broth and then sterile phosphate buffer pH=6. The use of Letheen broth, a neutralising medium, was necessary as the presence of the antimicrobial agent in the sample might result in a lower colony count. Upon spreading 100 μ l of each dilution on three BHI agar plates and the overnight incubation at 37°C, the colony formed units (CFUs) were calculated. In the control cells, distilled water was placed in the donor department and the same procedure was performed.

The bacterial count performed on the samples withdrawn at time zero from the control cells was considered to be the initial viable bacterial count. The viable bacterial count of samples withdrawn after 30 minutes from the control cells, $(4.42 \pm 0.21) \times 10^6$ CFUs did not changed from the initial count, $(4.51 \pm 0.697) \times 10^6$ CFUs, significantly indicating no changes in the viability of *Staph. epidermis*. Over the 30-minute study, the bacterial count in the test cells did not change significantly, $(3.42 \pm 0.498) \times 10^6$ CFUs. Comparing the colony forming units (CFU) in the receptor phase for the CHX saturated solution with the CFUs in the control (distilled water) receiver compartment at each time point, we can see that the saturated solution of CHX showed no antibacterial activity in this system over a 30-minute study (figure 6.4). This was due to the low concentration of CHX in the receptor phase which itself was due to the poor permeation of CHX saturated solution across the skin.

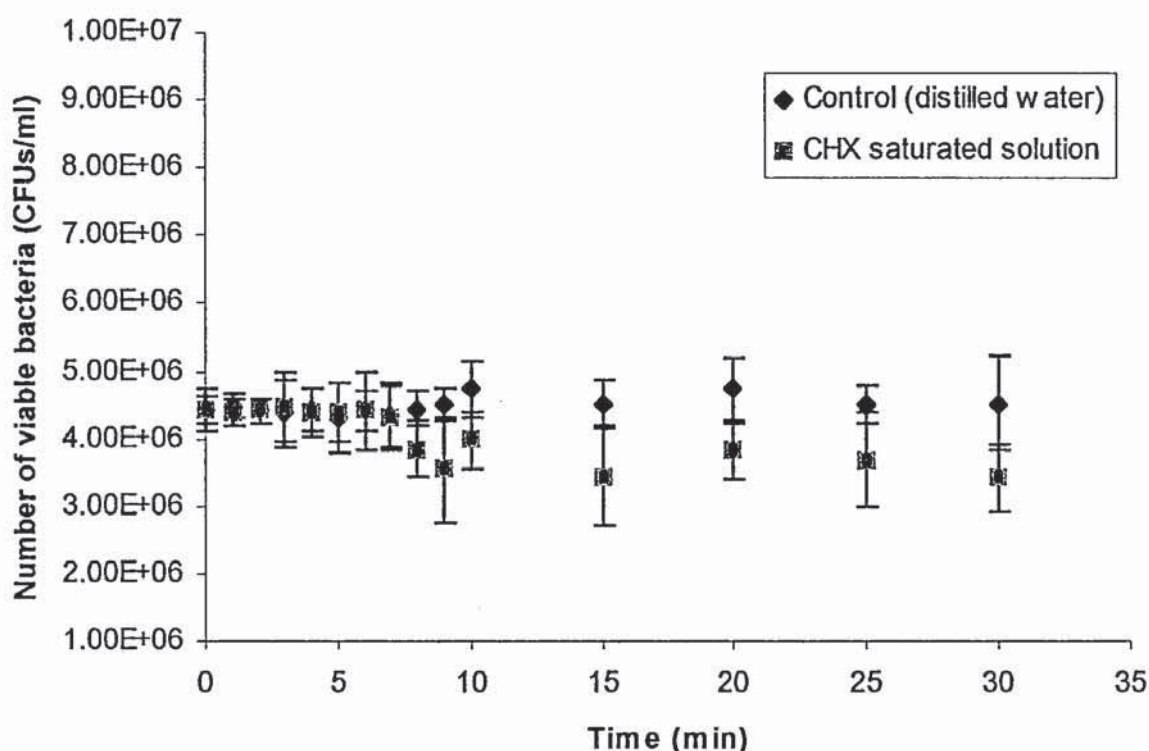


Figure 6.4 Antimicrobial Activity of Saturated Solution of CHX penetrated across the human abdominal epidermis. Points represents the mean \pm s.d., n=6

6.3.3 Antimicrobial Activity of Supersaturated Solution of CHX Using Franz Diffusion Cells

The poor permeation of saturated CHX across the skin could be the reason for its lack of ability to remove the residing bacteria in the deeper layers of the skin. Therefore, any improvement in its permeation would be beneficial. The enhanced permeation of CHX through employing supersaturated systems has been already demonstrated in section 4.7.5.2 (This supersaturated system resulted in a 7.65-fold increase in the flux of CHX across the skin compared to the saturated CHX solution). In order to see whether these systems demonstrate a greater antimicrobial activity, supersaturated solutions of CHX were used as the test solutions. Solution of 10% PVP (K30) with 0.3M NaCl in distilled water was used as the control. At each time point over a 30-minute study, the viable bacterial count in the receiver compartment for the test solution was compared to the viable bacterial count in the control receiver medium. Results are presented in figure 6.5 and show that after 30 minutes, the supersaturated solution of CHX can reduce the initial bacterial count from $(3.42 \pm 0.21) \times 10^6$ CFUs to $(1.01 \pm 0.498) \times 10^5$ CFUs in the receptor while saturated solutions of CHX did not reduce the number of viable colonies significantly (CFUs after 30 minutes = $3.51 \pm 0.697 \times 10^6$). The greater reduction observed in the number of bacteria was due to the higher permeation of CHX across the epidermis, which resulted in the higher concentration of antimicrobial agent in the receptor medium.

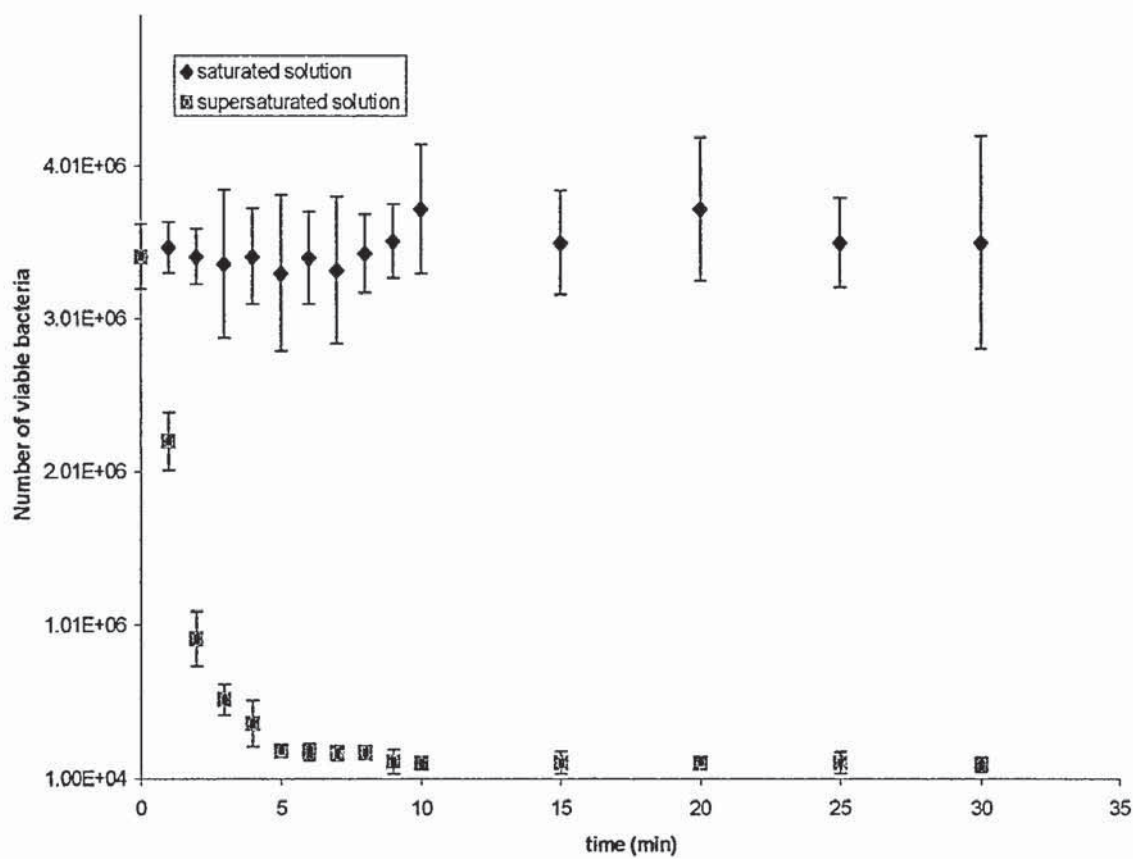


Figure 6.5 Antimicrobial activity of saturated and supersaturated solutions of CHX penetrated across the human abdominal epidermis. Points represents the mean \pm s.d., n=6

6.3.4 Effect of Pre-treatment of Skin on the Antibacterial Activities of the CHX Using Franz Diffusion Cells

The results of the permeation experiments (section 3.6.3) demonstrated the enhanced permeation of CHX across the enhancer pretreated skin. To assess the effect of these enhancers on the antimicrobial activity of the CHX, human epidermis was pretreated with 10% Azone/PG, 10% Oleic acid/PG and 10% SDS/PG separately and the diffusion studies were carried out once again as described in section 6.2.4. After 30 minutes, Azone and oleic acid pretreatment both resulted in lower bacterial population in the receptor medium. The viable bacterial count was reduced from $(3.51 \pm 0.498) \times 10^6$ CFUs to $(2.31 \pm 0.67) \times 10^3$ CFUs and $(2.68 \pm 0.432) \times 10^4$ after 10% Azone/PG and 10% oleic acid /PG pretreatment, respectively. This could be due to the higher concentration of CHX in the receptor medium. The higher amount of CHX was resulted from the effect of enhancers on the barrier properties of the SC. As discussed in chapter 3, Azone and oleic acid disrupt the SC structure by affecting the lipids in the SC. This would result in enhanced permeation of CHX across the skin and a consequent reduction in the bacterial count in the receptor. Results are depicted in figure 6.6.

However, although the permeation studies in section 3.6.3.6 showed a higher CHX flux from the skin pretreated with 10% SDS/PG, the number of viable counts in the receptor chambers of test diffusion cells after a 30-minute study was not changed significantly. The colony count after 30 minutes was $(3.11 \pm 0.697) \times 10^6$ CFUs which was not significantly different from the initial colony count of $(4.42 \pm 0.21) \times 10^6$ CFUs. This could be due to the neutralisation of CHX by the remaining SDS, an anionic surfactant, on the skin. This result is in agreement with the work of Jeffrey (1995), who also showed the incompatibility of CHX with anionic compounds. Results are illustrated in figure 6.7.

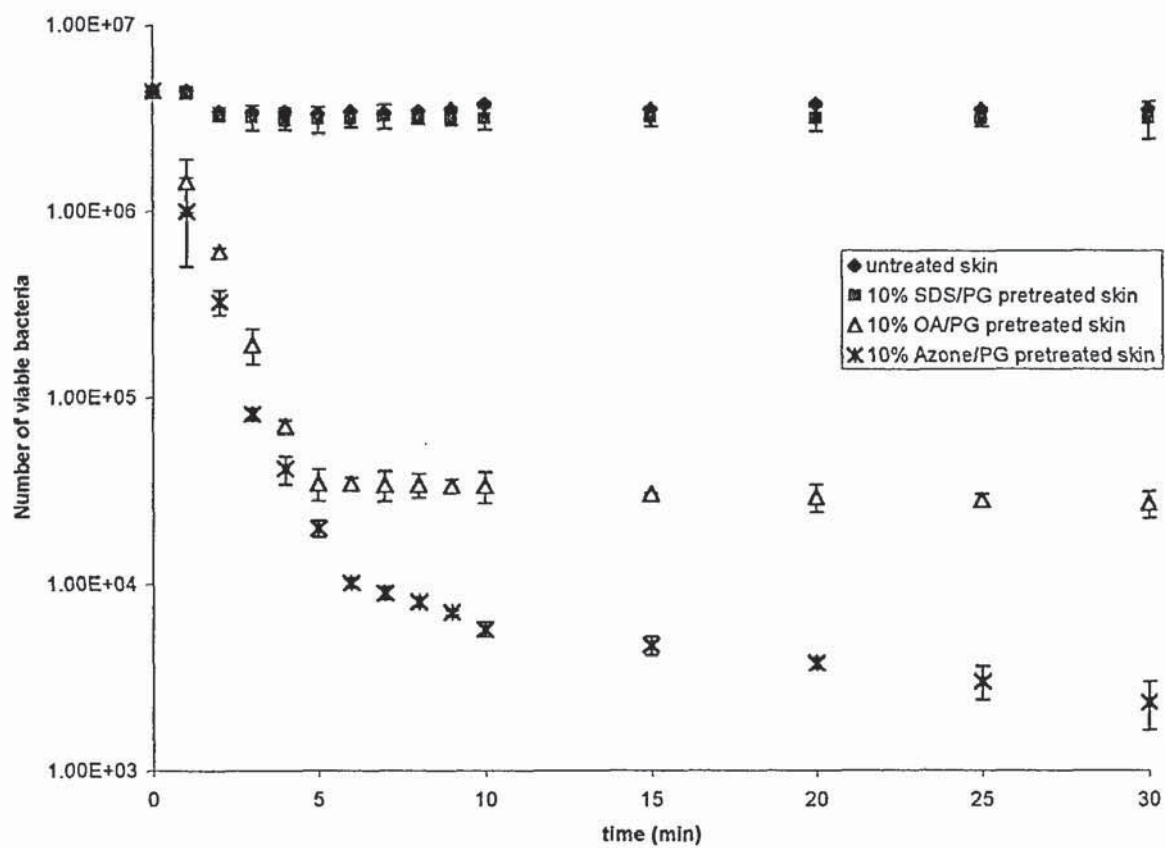


Figure 6.6 Effect of pretreatment on the antibacterial activity of CHX. Points represents the mean \pm s.d., n=6

6.3.5 Antimicrobial Activity of Supersaturated Solution of CHX Using the Model Developed in QE Hospital

Another method employed in this study to investigate the antibacterial activities of saturated and supersaturated solutions of CHX was the stab procedure developed in QE Hospital, Birmingham. Like the diffusion cell method, the test solution and the bacterial suspension are separated in this method by human skin thus mimicking the *in vivo* situation. As described in section 6.2.5, the system was set up by placing sterile Iso-sensitive agar containing 0.01% tetrazolium salt in the lower compartment of the set-up and then stab inoculating with *Staph. epidermis*. In the first set of experiments, saturated solution of CHX was placed in the upper container. Distilled water was used as control. After the overnight incubation, the distance from fluid/agar interface to red colouration was measured in both test and control set-ups. In the case of distilled water, a red line was observed right to the skin interface however, using the CHX saturated solution as the test solution, there was a 4-mm distance between the red line and the skin. This short distance indicates that the penetration rate of CHX saturated solution across the skin is low hence, only the growth of a small number of bacteria is inhibited.

Further studies were carried out on the supersaturated solutions using the same method. In this set of experiments, supersaturated solutions of CHX containing 0.3 M NaCl and 10% PVP K30 was placed in the upper part of the set-up. Saturated solution of CHX was used as control in this experiment. Using supersaturated solutions of CHX as the test solution and after the incubation time (over-night), the distance between the skin and red colour was measured. A 7-mm distance was measured showing a greater penetration or antibacterial action. This could be due to the higher flux of CHX from the supersaturated systems, which have a higher thermodynamic activity (section 4.7.5.2). The higher flux would result in higher concentration of the antimicrobial agent (CHX) and thus, more bacteria will be removed.

Employing this method, the effect of enhancer pre-treatment was studied once more. The experimental procedure was same as the one described in section 6.1, with the exception of the membrane which, in this case, was pretreated with enhancers. In the

case of Azone and oleic acid pretreatment, no red colour was observed showing the greater permeation and, consequently, antibacterial activity of CHX. However, SDS had a negative effect on the antimicrobial activity of CHX. The measured distance was 3 mm. Results supported the findings of permeation studies carried out using diffusion cells.

6.4 Summary

The results in this chapter show that the polymers used in the preparation of supersaturated solutions of CHX had no effect on the antibacterial properties of CHX. Furthermore, by using supersaturated systems or pretreatment of skin with enhancers, the bacteria in the skin can be targeted and removed and thus, the sterilisation of the skin can be achieved. However, the possible interaction between the active agent and the excipients used in the formulation or the enhancers used for the pretreatment of the skin should not be neglected as SDS, an anionic surfactant, showed to decrease the activity of CHX despite being able to enhance the permeation of CHX across the skin.

CHAPTER SEVEN

GENERAL SUMMARY

7.1 General Summary

Catheter-related infection has been recognised as a significant clinical problem. It is believed that the most likely source of contamination is the microorganisms on the patients' skin. Several approaches to prevent catheter-related infection have been developed. These include electrically charged catheters that repel organisms from the surface of the catheters, catheters containing antimicrobial agents, and the use of prophylactic antibiotics at the time of catheter insertion. Despite all the attempts, catheter-related infection is still a cause of morbidity and mortality in hospitalised patients. Care of the insertion site by using topical antiseptics seems more promising as this approach eliminates the cause of the infection rather than preventing the infection. An important consideration for the removal of bacteria that exist in the deeper layers of the skin is the diffusion of antimicrobial agent to the site of action. The aim of the present study was to enhance the transport of chlorhexidine, an effective antiseptic with the widest use, into the skin to eradicate skin bacteria residing in the underlying layers.

To enhance the permeation of chemicals, a variety of processes can be used. These include the modification of vehicle parameters such as solubility or pH, use of chemical enhancers that disrupt or modify the barrier properties of the skin, use of supersaturated systems and iontophoresis.

The permeation studies performed in chapter 2 showed that chlorhexidine, despite being an effective antiseptic has a poor permeation rate across the skin. Hence, the residing bacteria in the deeper layers of the skin can not be removed and, thus, they can act as the source of the contamination during the insertion of catheters. The poor permeation rate of CHX could be due to the fact that CHX exists as a dicationic species at the pH range of 4-7 and, according to the pH-partition hypothesis, flux is proportional to the concentration of the unionised species and the ionised species does not contribute to transdermal delivery.

To enhance the permeation of CHX across the skin, several chemical enhancers were employed and the membrane was pretreated prior to the permeation studies. 10% v/v

Azone in propylene glycol pretreatment regimen induced the highest enhancement effect on the diffusion of CHX across the skin followed by 10% v/v oleic acid in propylene glycol. Other chemical enhancers (ethanol, DMSO, SDS and DTAB) also showed accelerated transport of CHX to various degrees. To investigate the mechanism of action of these enhancers, DSC was employed to establish the mechanism of action of enhancers. The hydrated human stratum corneum showed a four-peak thermogram, one minor transition at around 35°C and three major transitions at around 68, 80, and 95°C. However, upon pretreatment of the skin with the enhancers, the SC thermogram was changed. In the case of 10% Azone/PG, all three transitions which were related to the SC lipids disappeared indicating that Azone/PG affects the lipid structure of the SC. The transition at 95 °C was also affected as a smaller peak at that temperature was observed compared to the thermogram of the untreated SC. This effect was related to propylene glycol. A similar thermogram was obtained for the SC pretreated with 10% oleic acid in PG indicating that this pretreatment regimen also affects the SC lipids and thus, disrupting the barrier properties of the SC.

It is well established that the principal driving force for diffusion across the skin is thermodynamic activity of the permeant in the donor vehicle. This activity is reflected by the concentration of the permeant in the donor vehicle as a function of its saturation solubility within that medium. The absorption of drugs can be accelerated by increasing the thermodynamic activity of the drug in the vehicle above the unity and this can be achieved through the use of supersaturated systems, which contain concentrations of solute in excess of the saturated solubility. In chapter four, supersaturated systems were prepared in order to promote the flux of CHX across the skin. A novel method based on the common-ion effect was employed for the preparation of supersaturated systems. Sodium chloride at different concentrations (0.1-0.3 M) was added to the saturated solution of CHX in distilled water and the supersaturated systems which were in the metastable state, were stabilised using anti-nucleating agents. The polymers used were PVP (two different molecular weight) and HPMC. HPMC showed a better ability in stabilising the supersaturated solutions; however, due to the increased viscosity of the final solution, the CHX permeation was not enhanced. PVP with the average molecular weight of 40000 was more effective compared to its lower molecular weight counterpart

and the supersaturated systems prepared with it showed an enhanced flux. The flux enhancement was also shown to be proportional to the degree of saturation.

Iontophoresis is a technique that has been used to enhance the delivery of drugs particularly charged and polar molecules through the skin. It is a process which causes an increased penetration of molecules into or through the skin by the application of an electric field. A number of experiments have strongly suggested that ionic flow occurs through pores, such as sweat, sebaceous glands, and hair follicles and since skin microflora are also distributed in these sites of the skin (Chapter 1, section 1.8), it seemed appropriate to employ iontophoresis to target the bacteria residing in the pores. In chapter five, iontophoresis was applied either alone or in conjunction with chemical enhancers to enhance the diffusion of CHX across the skin. While the separate application of iontophoresis and enhancers all improved the permeation of CHX, the efficiency of a combined chemical and iontophoretic approach was higher. The greatest flux across the epidermis occurred after the pretreatment of the membrane with 10% Azone in PG combined with the application of 0.5 mA/cm^2 constant current.

The effect of donor vehicle on the delivery of CHX across the skin was also studied. Incorporating 10-20% ethanol in the donor solution resulted in an increase in the solubility of CHX and an increase in the flux of CHX due to the increase of the concentration gradient across the membrane and the effect of ethanol itself on the structure of the SC. When used in combination with chemical enhancers (10% Azone/PG) and current (0.5 mA/cm^2), ethanolic solutions of CHX produced a greater flux compared to the saturated solution of CHX in distilled water.

Any interactions between the active agent and excipients used in the formulation may affect the activity of the antimicrobial agent. To investigate the effect of the polymers used in the preparation of supersaturated systems on the antibacterial properties of CHX, microbial tests were performed. Inhibition-zone measurement which is a method of assessment of the activity of an antimicrobial agent was performed in chapter six. In this test, solutions of CHX and the polymers were introduced into a well in an agar plate, which has been inoculated with the test organism. No interaction between CHX and the

polymers were seen as the inhibition zone produced was similar to the one observed with CHX solution alone. The effect of the skin pretreatment with enhancers on the antimicrobial effect of CHX was also studied using Franz-type diffusion cells. The number of viable colonies in the receptor chamber was reduced to a greater extent upon the pretreatment of skin with 10% azone/PG and 10% oleic acid/PG compared to the untreated skin. The enhanced antimicrobial effect was due to the higher concentration of CHX in the receptor phase. However, pretreatment of the skin with SDS, an anionic surfactant, did not enhance the antibacterial properties of CHX. This could be due to the inactivation of CHX, a cationic antiseptic, by SDS.

In conclusion, this study has presented a potential solution to the problem of efficient delivery of chlorhexidine to the bacterial sites in the skin for the prevention of the catheter-related infections. However, further studies need to be performed to formulate more stable supersaturated systems and to develop wearable electrical antimicrobial patches.

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APPENDIX 1

Phosphate Buffers (British Pharmacopoeia, 1999)

Phosphate Buffer pH 3.0

34 g potassium dihydrogen orthophosphate in 250 ml distilled water, pH adjustment with orthophosphoric acid.

Phosphate Buffer pH 4.9

40 g sodium dihydrogen orthophosphate and 1.3 g sodium hydroxide in sufficient distilled water to produce 100 ml. If necessary, pH can be adjusted with 1 m sulphuric acid or 1 m sodium hydroxide.

Phosphate Buffers pH 5.8, 6.4 and 7

These buffer solutions can be prepared by mixing 50 ml of 0.2 M potassium dihydrogen orthophosphate with the quantities of 0.2 M sodium hydroxide VS specified in the following table and diluting to 200 ml with distilled water.

pH	5.8	6.4	7.0
ml of 0.2 M sodium hydroxide VS	3.72	12.6	29.63