Understanding the *in vivo* dynamics of non-occlusive drug delivery systems

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Drug Delivery, Disposition and Dynamics

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ERRATA

Page i, line 21:	"to spread" is replaced with "for lateral diffusion"
Page 15, line 9:	"In particularly" should read "In particular"
Page 16, line 6:	"pharmaco-dynamic' should read 'pharmacodynamic"
Page 18, equation 1.2:	"=" is replaced with "α"
Page 28, line 8:	"deuterated compounds which is" should read "deuterated compounds which are"
Page 29, line 23:	"release" is replaced with "transport"
Page 63, line 2:	remove "calibration curve"
Page 74, line 9:	"it was probably" should read "it is probable"
Page 108, line 10:	"dermatopharmacology" is replaced with "dermatopharmacokinetics"
Page 127, line 11:	"sectiosn" is replaced with "section"
Page 161, Figure 5.7:	"IBU + OG + PEG 200 + OS" is replaced with "IBU + PG + PEG 200 + OS"
Page 165, line 21:	"radial" is replaced with "lateral"
Page 213, line 1:	"dermatogolgical" is replaced with "dermatological"
Page 220, line 7:	"1098-&" is replaced with "1098-1102"
Page 222, line 13:	"atr-ftir spectroscopy .1." should read "atr-ftir spectroscopy Part 1."
Page 222, line 16:	"atr-ftir spectroscopy .2." should read "atr-ftir spectroscopy Part 2."

ADDENDUM

Page 167:	General comment: The solubility of IBU in the residual phase, the interfacial tension between excipients and the skin surface, the pH of the excipients and any differences in the solubility parameter of excipients to that of the skin may also effect the lateral diffusion of drugs.
Page 206:	General comment: Recent reports by the FDA have warned of documented cases of adverse effects of secondary exposure of topical testosterone gel products (marketed as Testim 1% and Androgel 1%) to women and children who are not meant to receive testosterone treatment by contact with skin of patients using such products. It is feasible that drug may have spread across the skin surface outside the original area of application, making secondary exposure possible. However, as shown in this research, increasing the viscosity of the formulation may prevent the distance of lateral spread decrease secondary exposure.
Page 210:	General comment: While the studies present an understanding of the influence of lipophilicity and molecular weight on the lateral diffusion and/or penetration of topically-applied drugs, there is not enough evidence to conclude on the effect of other physicochemical properties (such as hydrogen bonding) on lateral diffusion.

Dedicated to my parents, Dun Poy Gee and Hui Ho

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ABSTRACT

Over the years, many studies have focused on characterising the penetration behaviour of drugs across the skin in order to develop methods to improve transdermal drug delivery. However, very few studies have been able to account for the fact that the process of drug penetration into the skin's major barrier, the stratum corneum (SC), occurs in tandem with the process of lateral diffusion across the skin surface. Furthermore, the exact distance that drugs spread radially from their application site, as well as the extent of their lateral diffusion within the SC bilayers, has been poorly defined.

Lateral diffusion from the application site increases the area of drug exposure and for potent drugs, this may increase the risk of secondary drug contact to third parties. Therefore, the aim of this thesis was to develop a method sensitive enough to determine both the lateral diffusion and penetration behaviour of topically applied drugs across the SC, as well as evaluate the impact of formulation excipients on the fate of drugs applied topically to humans.

Specially designed concentric adhesive tapes perforated into four sections of known diameter were developed to follow the distribution behaviour of small volumes of ethanolic solutions of drugs across the SC. Repeated stripping of the concentric tapes allowed for assessment of the three–dimensional distribution of three model compounds with different lipophilicities across human SC *in vivo*. Caffeine (CAF), a hydrophilic drug, formed a flat depot on the surface and in the uppermost regions of the SC. Hydrocortisone (HC), a relatively more lipophilic drug compared to CAF, exhibited a lower tendency to spread and instead, formed a narrow drug reservoir in the uppermost regions of the SC. Ibuprofen (IBU), a lipophilic drug, demonstrated greater drug penetration and lateral diffusion across the SC in comparison to CAF and HC. The SC is a heterogeneous system

with lipophilic and aqueous domains, and so, lipophilic drugs such as IBU are more soluble in the lipid medium and thus, the SC poses less resistance to their diffusion. In contrast, lateral diffusion across the skin will be less tortuous for hydrophilic drugs, such as CAF. For HC, however, lateral diffusion is reduced compared to CAF owing to its more lipophilic nature and its tendency to form drug reservoirs within the skin.

As IBU demonstrated the most significant penetration following topical application, the effect of excipients – propylene glycol (PG), polyethylene glycol 200 (PEG 200) and octisalate (OS), on the lateral diffusion and penetration behaviour of ethanolic solutions of IBU across human skin was also assessed. OS promoted lateral diffusion of IBU while the addition of PG and PEG 200 decreased lateral diffusion of drug and retained the majority of IBU within close proximity to the application area. As PG and PEG 200 are highly viscous materials compared to OS, it is likely that the movement of IBU across the skin surface is dependent on the flow of the vehicle in which it is dissolved. In addition, the larger contact angle formed between droplets of IBU and human skin when mixed with PG and PEG 200 (relative to IBU applied with OS alone) further highlights the resistance of viscous vehicles to spread.

After prolonged drug exposure, approximately 55% and 25% of IBU was recovered from the uppermost layers of the skin when applied alone and in the presence of PG, PEG 200 and OS, respectively. To determine whether this significantly lower recovery of IBU (when applied with excipients) was due to enhanced penetration, *in vitro* permeation studies were carried out to assess the percutaneous absorption of IBU with and without excipients. Following the onset of steady-state delivery, the flux of IBU increased by an average of 2-fold in the presence of PG, PEG 200 and OS compared to IBU applied alone. Therefore, it is likely that the significant loss of IBU from the uppermost region of the skin *in vivo* was due to enhanced permeation of IBU in the presence of PG, PEG 200 and OS.

The research and findings from this thesis demonstrate that following topical application, the competing processes of lateral diffusion and penetration of drug both contribute to the disposition of drugs within the SC. To an extent, drug lipophilicity may govern the distance of lateral diffusion across the SC and hence the level of secondary exposure. Furthermore, the ability to reduce lateral diffusion by increasing formulation viscosity may allow for the development of more safe and effective transdermal drug delivery systems.

STATEMENT OF ORIGINALITY

The Faculty Manager

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I hereby certify that the work presented in this thesis has not been submitted by me or by any other person for the award of a degree at Monash University or any other institution. To the best of my knowledge, this thesis does not contain material that has been published previously by another person except where due reference has been made.

Carol Maying Gee

October 2011

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To my parents, grandparents, brother and sister – I am sincerely grateful for your love, support, patience and care during my years of study. It'll always be treasured. Finally, many thanks go to Brent for the constant ray of sunshine, love and happiness that you bring to my life.

PUBLICATIONS

1. Gee CM, Nicolazzo JA, Watkinson AC, Finnin BC. 2011. Development of concentric adhesive tape for the assessment of lateral diffusion and penetration of topically applied drugs in man. Pharm Res (in preparation).
2. Gee CM, Nicolazzo JA, Watkinson AC, Finnin BC. 2011. Penetration and lateral diffusion of ibuprofen following topical application to humans. Pharm Res (in preparation).
3. Gee CM, Bunge AL, Nambukara C, Nicolazzo JA, Watkinson AC, Finnin BC. 2011. Mathematical model to predict lateral diffusion of drug following topical application in man. Pharm Res (in preparation).

COMMUNICATIONS

1. Gee CM, Nicolazzo JA, Watkinson AC, Finnin BC. 2010. Penetration and lateral diffusion of caffeine, hydrocortisone and ibuprofen following topical application of ethanolic solutions to humans. Proceedings of the Australasian Pharmaceutical Science Association Annual Conference, Brisbane, Australia.
2. Gee CM, Nicolazzo JA, Watkinson AC, Finnin BC. 2010. 2011. Penetration and lateral diffusion of ibuprofen following topical application to humans. Proceedings of the Barrier Function of Mammalian Skin, Gordon Research Conference, New Hampshire, USA.
3. Gee CM, Nicolazzo JA, Watkinson AC, Finnin BC. 2010. 2011. Penetration and lateral diffusion of ibuprofen following topical application to humans. Proceedings of the 6th Annual MIPS Research Symposium, Melbourne, Australia.*

*Award winning.

LIST OF ABBREVIATIONS

ACN	acetonitrile
ATR-FTIR	Attenuated total reflectance-fourier transform infrared
BSA	bovine serum albumin
CAF	caffeine
cm	centimetre
C_s	concentration gradient
Da	dalton
D_m	diffusion coefficient
ECETC	European Centre for Ecotoxicology and Toxicology of Chemicals Monograph
EtOH	ethanol (90% v/v)
g	gram
g/mol	gram per mole
GRAS	generally regarded as safe
H_2O	purified water
HC	hydrocortisone
HCl	hydrochloric acid (36% v/v)
HPLC	high-performance liquid chromatography

hr	hour
IBU	ibuprofen
IPA	isopropanol
J	steady-state flux
kGy	kilogray
K_p	permeability coefficient
L	thickness of membrane
LCMS	Laser-scanning confocal microscopy
logP	logarithm of the octanol-water partition coefficient
LOQ	limit of quantitation
LPP	Lipid-protein-partitioning
MDTS	Metered Dose Transdermal System
MeOH	methanol
µg	microgram
µL	microlitre
mg	milligram
mL	millilitre
min	minute
mPa.S	millipascal seconds

NaOH	sodium hydroxide
ng	nanogram
nm	nanometre
NSAID	non-steroidal anti-inflammatory drug
OECD	Organisation for Economic Co-operation and Development
OS	octisalate
PBS	isotonic phosphate buffer
PEG 200	polyethylene glycol 200
PG	propylene glycol
rpm	revolutions per minute
SC	stratum corneum
SCCP	Scientific Committee on Cosmetic Products
SCERH	Standing Committee on Ethics in Research involving Humans
sec	second
TDD	transdermal drug delivery
UV	ultraviolet
VOLPO N20	polyethylene glycol-20-oleyl ether

CHAPTER 1

INTRODUCTION

1 INTRODUCTION

1.1 STATEMENT OF THE PROBLEM

Over the years much research has focused on the use of skin as a route for drug delivery. Transdermal drug delivery is a method in which an active drug is applied onto the skin surface for the purpose of systemic and local treatments. However, the inherent properties of the skin limit the usefulness of this route of delivery. It is generally accepted that the bioavailability of topically applied drugs is in the order of 5 – 10%[1]. It is assumed that the non-absorbed drug is metabolized, shed via epidermal desquamation, washed off or spread across the skin surface. For potent drugs, the possibility of secondary exposure may cause adverse effects and thus, any unabsorbed drug may pose a safety risks to third parties as well as the immediate environment.

If the fate of the unabsorbed drug can be determined, then it may be possible to develop methods to reduce the amount of drug wasted in this way by better controlling drug application and disposition. In addition, an improved dose form may reduce the safety risk posed by the unabsorbed drug and decrease the exposure to third parties. Ultimately, an understanding of the fate of unabsorbed topically-applied drugs will allow for the development of techniques to increase the overall bioavailability of non-occlusive transdermal drug delivery systems.

To date, most transdermal studies have adopted *in vitro* methods to predict the absorption behaviour, permeability and penetration of topically-applied drugs. However, it cannot be assumed that the behaviour of drugs in *in vitro* models reflect the *in vivo* conditions. Large variations in *in vitro* studies have been reported, as excision, storage and experimental conditions may cause discrepancies in the penetration behaviour of drugs across human skin[2]. Furthermore, the absence of blood flow, metabolism and the usual

process of skin turnover may introduce a significant difference compared to *in vivo* studies. Therefore, to fully understand the behaviour, disposition and fate of drugs after application *in vivo* requires a model that is able to account for this drug loss as well as drug absorption. The model system needs to account for as close to 100% of the applied drug dose as possible.

Studies that focus on the extent of drug penetration into and through the skin often ignore the need to account for the unabsorbed drug. A drug's distribution within and through the skin is influenced by the formulation design, viscosity of the formulation, delivery method of the active agent, as well as its physicochemical properties of the drug itself. Nevertheless, very few studies have been able to recognize that the process of drug penetration occurs contemporaneously with the process of lateral diffusion across the skin surface. One can assume that the prevention of lateral spreading will enable greater local activity or absorption. Hence, capturing and quantifying drug loss through lateral diffusion in the upper layers of the stratum corneum will be this project's main area of focus.

1.2 DRUG DELIVERY THROUGH THE SKIN

1.2.1 Advantages of transdermal drug delivery (TDD)

The skin is the largest organ of the body and covers an area of 2 m^2 [3]. It is a multi-functional organ, with some of its main roles being to act as an interface between the body and the external environment, to prevent the ingress of foreign material and to prevent the loss of water. As it is the most easily accessible organ, delivering drugs for the purpose of local or systemic treatment is an appealing alternative to other routes[1].

TDD offers a more convenient and non-invasive method of delivery over the more conventional oral and intravenous routes. TDD bypasses first-pass metabolism that often limits the efficacy of many orally delivered drugs and also avoids the intolerability of

parental injections[4]. Furthermore, TDD offers direct access to diseased topical sites with local treatment[5]. For oral drugs with very short biological half-lives and high body clearance rates, TDD can provide a steady maintenance of blood level over a predictable period of time[4,6]. Additionally, patient compliance may be improved because of reduced frequency of administration and the ease of application and removal[7].

1.2.2 Disadvantages of transdermal drug delivery

As with other routes of drug delivery, the use of skin as a site of drug delivery is also associated with certain limitations. The main disadvantage of transdermal drug delivery is that the skin is a viable route for only a limited group of drugs due to the resistant barrier of the skin[4]. A number of physicochemical parameters have been identified that influence the diffusion process, and variations in permeation rates and time of onset of action can occur between individuals, different races and between the old and young[8]. Some drugs may also be metabolized in the superficial skin layers before reaching the cutaneous vasculature and therefore limit its therapeutic effect[9]. Furthermore, skin irritation and sensitization may be elicited by certain drugs[6].

1.3 STRUCTURE OF THE SKIN

1.3.1 Organisation of the skin

The structural composition of the skin (as shown in Figure 1.1) poses many limitations to the diffusivity of chemical across this membrane. The epidermis is responsible for the generation of the stratum corneum (SC), which forms the outermost layer of the skin and constitutes the skin's major barrier element due to its medium of keratinized cells (corneocytes) interdispersed within a highly ordered lipid rich matrix[10] as is discussed in further detail in Section 1.4.1. The viable epidermis, dermis and hypodermis

(subcutaneous tissue) all contribute to the overall characteristics and development of the SC.

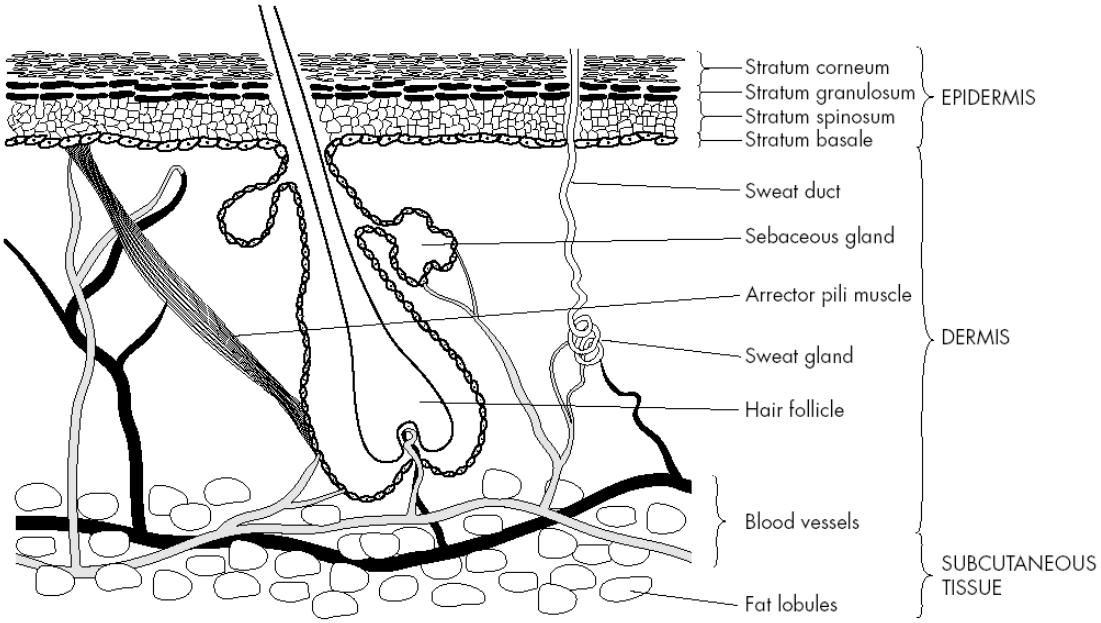


Figure 1.1 Schematic diagram of the structure of the skin. Adapted from Raton[11].

1.3.1.1 Viable epidermis

The cells in the viable epidermis undergo many morphological changes before forming the SC. Thus, the viable epidermis consists of several cell layers at varying levels of differentiation.

The stratum basale is the innermost part of the epidermis and consists of a continuous carpet of stem cells that reside on the basale lamina between the dermis and viable epidermis[12]. There are two types of stem cells that originate from here – the first type has the ability to divide and produce new cells (such as keratinocytes, melanocytes, dendrocytes and tactile cells which then proceed to produce the protein keratin, the skin-pigment melanin, white blood cells and sensory cells, respectively)[13]. The second type of cell remains stagnant and stays in the stratum basale to anchor the epidermis to the its

support matrix, the basement membrane[14]. The basement membrane is 50 – 70 μm thick and contains proteins that serve to maintain mechanical stability of the basement membrane, as well as ensuring adhesion between the basement membrane and basal keratinocytes[15].

The stratum spinosum is situated directly above the stratum basale and is given its name due to the spine-like appearance of desmosomes joining adjacent cells. The cells found in this layer are biochemically distinct and enclose a cytoplasm filled with numerous organelles and filaments[15].

The cell layer directly beneath the SC is the stratum granulosum, which contains electron-dense keratohyalin granules[16]. Within these granular cells are lamellar granule subunits which form the precursors of the intercellular lipid lamellae found in the SC as discussed in Section 1.4.2.3. In the uppermost layer of the stratum granulosum, the lamellar granules migrate to and fuse with the plasma membrane whereby they extrude all their cytoplasmic contents into the intercellular space that progresses to play a role in the intercellular lipid domains[14].

1.3.1.2 Dermis

The dermis sits beneath the viable epidermis and makes up the largest fraction of the skin, ranging from 0.1 – 0.5 cm in thickness, depending on the body site[15]. It plays a vital role in body regulation by controlling pressure, pain and temperature, as well as providing nutritive and immune response for the epidermis[16]. The main structural components of the dermis can be categorized into two layers – the papillary layer where collagen originates and the reticular layer that sits above the hypodermis (subcutaneous tissue)[14].

The dermis also consists of a matrix of connective tissue composed of collagen fibers that provide support and cushioning, and elastic connective tissues that provides elasticity,

interdispersed in a mucopolysaccharide matrix. Skin appendages such as sweat glands, pilosebaceous glands and hair follicles are also located here[17].

The main cell types of the dermis are the fibroblasts that produce: the connective tissue portion of the fibrous proteins – collagen, laminin, fibronectin and vitronectin; mast cells that contribute to the immune and inflammatory response; and melanocytes involved in the production of the pigment melanin. Furthermore, the dermis contains extensive blood vessels that provides nutrition, repair and immune response to the skin, as well as thermal regulation and immune protection to the rest of the body[14].

For systemic drug absorption, both the blood vessels and lymphatic system play a vital role by acting as sinks and thus keeping the drug concentration in the dermis low. However, because the blood vessels are positioned close to the interface between the epidermis and dermis, the dermis is not regarded as a major barrier to drug absorption *in vivo*[18].

1.3.1.3 Hypodermis

The hypodermis or the subcutaneous tissue layer is the innermost region of the skin and exists as a support between the upper skin regions and the internal structures such as bones and muscles. Other primary functions of the hypodermis are to act as a shock absorber, energy store, allow for skin mobility and to mold the body contours. Additionally, a layer of adipose cells arranged in lobules and linked to the dermis by collagen and elastin fibers provides insulation to the body[17].

1.4 SKIN AS SITE FOR DRUG DELIVERY

Drug transport across the skin is largely a passive process. While the dynamic nature of the skin is due to the differing functions of the various components of the skin, it is the outermost layer of the skin – the SC, which poses the greatest barrier to drug delivery

across the skin as discussed in Section 1.4.1. Once a compound has penetrated across the SC, it may penetrate through the viable epidermis with more ease and be eventually carried away by the dermal blood supply or transported to deeper tissues. Therefore, the dermis and hypodermis are not regarded as major barriers to drug absorption.

1.4.1 SC as the rate-limiting barrier

The application of topical products onto the skin has been used for thousands of years for the purpose of cosmetic or therapeutic effects and in the recent decades, a variety of topical formulations have been introduced as a remedy for both local and systemic treatment. However, it was not until 1944 that Winsor and Burch[19] showed that the flux of water through skin dramatically increased as the SC was ruptured with sandpaper. This finding was supported by Blank et al.[20] when they demonstrated via tape stripping that water loss through the skin was not significant until the majority of the SC was removed. Scheuplein et al.[21] extended this study by comparing the permeability of various solutes on stripped and unstripped skin and found that stripped skin significantly increased the permeability of solutes, thus strongly indicating that the SC forms the rate-limiting barrier to diffusion.

It is now generally accepted that the SC constitutes the skin's major barrier element due to its medium of keratinized cells interdispersed within a highly ordered lipid rich matrix[22], as detailed in Section 1.4.2 below.

1.4.2 Components of the SC

1.4.2.1 Corneocytes

The SC is only 10 – 20 μm thick (with exceptions of the soles of the feet and palms) and is generally comprised of 15 – 25 layers of flattened, stacked, hexagonal and cornified cells fixed within an intercellular lipid matrix[23]. Corneocytes, which are the cells that make

up the majority of the SC and the end product of the differentiation process of keratinocytes, provide structural support for the SC. Each cell is approximately 40 µm in diameter and 0.5 µm thick[15]. The interior of corneocytes is filled with bundles of keratin and proteins that are stabilized by the presence of disulfide bridges. These disulfide bridges cannot be dissolved without the presence of a reducing agent, which in turn contributes to maintaining the flat shape, toughness and flexibility of the SC[24]. The cohesiveness of corneocytes is maintained by desmosomes, which are responsible for interconnecting individual cell keratin cytoskeletal structures, thus establishing a tissue resistant to external shear force[25].

1.4.2.2 SC proteins

The majority of proteins in the SC are present on corneocyte cell envelopes and in the intracellular matrix[26,27]. By the final stage of differentiation, the main proteins present in the SC include the keratin filaments, involucrin, loricrin and profilaggrin[28-30]. Loricrin and involucrin are major components of the cornified cell envelope, whilst profilaggrin are present to ensure proper alignment of the keratin filaments and to provide flexibility to the epidermis[31]. Within corneocytes, keratin filaments are cross-linked to each other as well as to components on the corneocyte envelope by intermolecular disulfide bridges and it is this cross-linking that is responsible for the poor SC protein solubility[32].

1.4.2.3 Intercellular lamellae

The extracellular space in between the layers of corneocytes is filled with multiple lamellar lipid sheets. The composition of lipids vary with body site, but mainly includes cholesterol, ceramides and free fatty acids[22].

The role of cholesterol has been a subject of much debate. It has been suggested that cholesterol may be responsible for providing some fluidity to the gel phase of the skin

membrane which may otherwise be too rigid based on the fact that it is less fluid and less permeable than a typical liquid crystalline phospholipid-dominant biological membrane[33]. Others propose that cholesterol is used as an intercellular cement that reduces the mobility of the alkyl chains and need to be hydrolyzed prior to desquamation[34].

There are eight classes of ceramides present in the SC that are classified based on their polarity[35,36]. The function of each individual ceramide type is not fully understood. However, it has been accepted that the long hydrocarbon chains of ceramides allow for the tight lateral packing and the formation of highly ordered lipid bilayers. Such a formation could account for the water impermeability associated with the extracellular lamella arrangement of the lipids of the SC. Fatty acids function to maintain the homeostasis of the permeability barrier of the SC and a diminution of this lipid results in impairment in the barrier function of the SC[37].

All of these lipids exist as a continuous phase that surrounds the corneocytes. Thus the morphology of the SC is often likened to a 'brick and mortar' formation, with the corneocytes corresponding to the bricks embedded into the intercellular lipids analogous to the mortar[22], as shown in Figure 1.2. The structure of the SC, as well as the low surface area available for solute transport (solutes enter the body through less than 0.1 μm wide intercellular regions in between the corneocytes of the SC)[15], creates a highly tortuous path for solutes to travel and thus is successful in providing a barrier to water loss through the skin as well as to limit the penetration of topically-applied drugs across the skin.

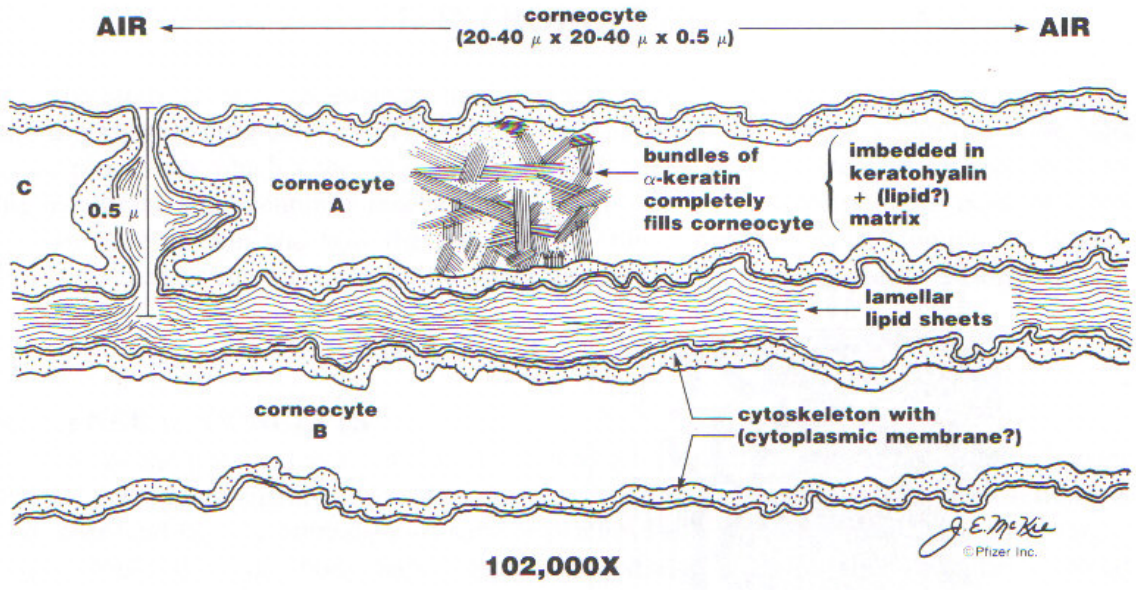


Figure 1.2 Schematic representation of the SC. Adapted from Kurihara-Bergstrom[38].

1.5 MECHANISMS OF DRUG TRANSPORT FOLLOWING TOPICAL APPLICATION

The aims of applying an active drug to the surface of the skin are as follows:

I. To remain on the skin surface to treat topical skin infections, act as insect repellents or for cosmetic reasons. These formulations are known as epidermal formulations.

II. To penetrate into the deeper regions of the skin, such as the viable epidermis and dermis to treat localized skin disorders. These are called endodermal or diadermal formulations.

III. To be absorbed into the blood stream for the purpose of systemic treatment. These are known as systemic formulations.

The clinical response following topical application however, is only initiated after a sequence of partitioning and diffusion processes. Following drug deposition onto the SC, the drug must initially partition out of its vehicle and diffuse through the living epidermis

where it may be subject to metabolism in the viable epidermis, then progress through the upper part of the papillary dermis where numerous permeable capillaries lie and enter the microcirculation, which leads to the activation of the desired pharmacological response.

As shown in Figure 1.3, it is generally accepted that the diffusing drug molecules have three potential routes of entry into the sub-epidermal tissue to initiate a pharmacological response – through the hair follicles with their associated sebaceous glands, known as the transappendageal route, or across the SC via the transcellular or intercellular route[16,39-41]. However, the lateral movement pathway of drugs across the SC is often ignored. Following topical application, the formidable barrier properties of the SC to drug penetration may encourage solutes to spread laterally on and within the SC. In addition, solutes may remain stagnant on the surface of the SC and be eventually removed from the skin via the natural process of desquamation.

1.5.1 Transcellular pathway

The most direct route for a drug to cross the skin is via the transcellular pathway. In this route, the drug has to cross the skin by directly passing through both the intercellular lipids of the SC and the cytoplasm of corneocytes. This is the shortest route (~20 µm), but the drug encounters significant resistance to permeation as it must cross 4 – 20 lipid lamellae between each corneocyte layer before crossing the hydrophilic corneocyte layers. However, this series of partitioning into and diffusing across both hydrophilic and hydrophobic domains is unfavorable for most drugs[40,42].

1.5.2 Intercellular pathway

The intercellular route is considered by many to be the major pathway for drug permeation. The permeant traverses the SC by passing through the intercellular lipid

domains between the corneocytes. This tortuous route is estimated to be as long as 500 µm[43].

1.5.3 Transappendageal route

Since the skin appendages occupy less than 0.1% of the total skin surface area, this route of penetration contributes very little to the overall absorption mechanism. However, any drug that does penetrate via this pelosebaceous unit is thought to be through the outer root sheath of the hair into the viable cells of the follicle, or through the sebaceous canal and into the sebaceous gland, which then progresses to the dense capillary networks that envelopes the glands[16].

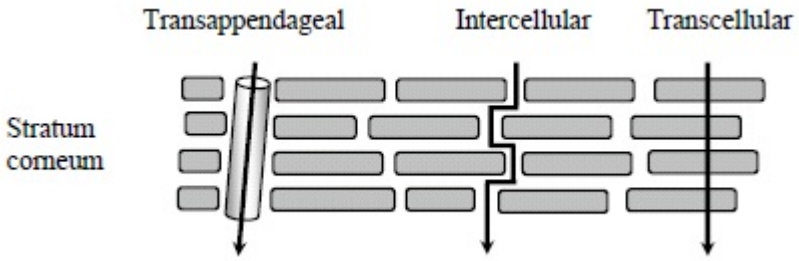


Figure 1.3 Potential routes of drug penetration across the SC. Adapted from Barry[44] and Roberts[45].

1.5.4 Skin metabolism

The epidermis and dermis are sites capable of high metabolic activity. The pharmacokinetics in the skin, the flux and distribution of drug within the skin may well be influenced by the presence of metabolic enzymes in the skin. Following drug deposition onto the skin, the drug substance can be subjected to two metabolic pathways which may degrade and remove its pharmacological effect before it reaches the target site to exert any effect: (i) the drug may be degraded by microflora, or (ii) the drug may be metabolized by various enzymes present within the tissue. In human cadaver skin, enzymes are present within 80 – 120 µm from the skin surface and corresponds to the layer superior to the

capillary vessels – thus, skin enzymes can successfully metabolize drugs before entering the systemic system[46]. However, unlike the liver in which enzymes are concentrated within a defined region of tissue space, skin enzymes are distributed over the nearly 2 m^2 of skin surface area. Thus, when drug is delivered over a small confined area of the skin, the impact of metabolism on total drug bioavailability may not be as significant[47].

1.5.5 Desquamation

The cells of the SC are constantly being shed from the surface via a process of desquamation and replaced through terminal differentiation in the viable epidermis so that the thickness of the SC is maintained. The total epidermal turnover time has been reported to range from 16 days to 48 days, depending on the experimental design[48-50] and the disease state of the skin. For example, there is increased cell proliferation of the epidermis for those with a skin condition known as psoriasis[51] and thus, a faster epidermal turnover time. As cells shed, they may separate as clumps, as single cells, as cell fragments, or as a mixture of these[49]. In addition, it has been estimated that humans with healthy skin shed between 2 x 10^8 and 10 x 10^{10} cells per day[52]. It is generally accepted that the desmosomes at the superficial layers of the SC are continuously degraded in the SC by proteases and this reduced cohesiveness encourages the sloughing off of surface skin cells. Therefore, topically-applied drugs associated to these shed skin cells (i.e. adsorbed, dissolved in and bound to) cannot be absorbed systemically and accordingly, it has been postulated that desquamation may significantly reduce and alter the systemic exposure of dermally-applied chemicals[53].

1.5.6 Lateral diffusion

Generally, transdermally-applied products have a bioavailability of less than 10%[1]. Thus any loss of drug from the surface of the treated site that did not reach the bloodstream may correspond to an increase in drug concentration in the adjacent and neighbouring

areas at the SC surface as a result of lateral diffusion, as well as the subsequent loss by either being rubbed off or shed via desquamation. Lateral diffusion occurs on the surface of the SC as well as in the upper layers of the SC and is facilitated by the simultaneous diffusion of drug within the lipid bilayers of the SC and redistribution from the blood into the skin[54], as shown in Figure 1.4. Lateral diffusion has also been shown to be a symmetrical process and there is little interindividual difference in the extent of lateral spread[55]. Nonetheless, the majority of transdermal studies performed to date have largely focused on developing a better understanding of the skin barrier properties and drug penetration, but drug spread across the SC has been given little attention. In particularly, the affect of the physicochemical properties of compounds on lateral diffusion, the affect of the addition of excipients on the lateral spreading of drugs as well as the exact distance of which drug spreads is poorly understood. Therefore, the development of a novel method to assess the lateral diffusion of drug across the SC will form part of the aim of this research project.

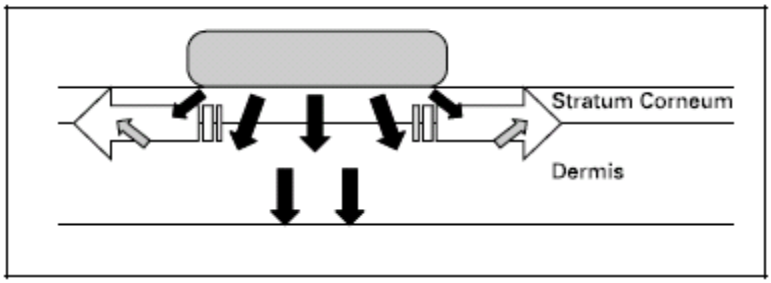


Figure 1.4 Diffusion into the skin as an unlimited compartment. Adapted from Schicksnus et al.[54].

1.6 FACTORS AFFECTING DRUG PENETRATION THROUGH THE SC

1.6.1 Physicochemical factors of permeant

The protective barrier function of the human skin imposes physicochemical limitations to the type of permeant that can traverse the barrier. Therefore, when selecting drug candidates for transdermal delivery, it must have the right physical chemistry and pharmaco-dynamic properties. For sufficient passive diffusion of drug into the blood stream following topical application, a drug should possess the following criteria:

- The therapeutic agent must be potent – the daily systemic dose should be ≤20 mg[56].
- Drug should have a molecular weight <500 Da to facilitate faster diffusion between cells[57].
- Permeant should have a melting point <200°C as permeants with a high melting point have been shown to have low solubility and therefore decreased flux through skin[57].
- The intercellular space within the SC contains multi-layers of lipids that provide both hydrophilic and lipophilic domains. Therefore, topically-applied drugs need to have a balance between oil and water solubility (octanol-water partition coefficient, also known as logP)[57]. Compounds with low logP exhibit lower partitioning into the skin lipids and those with high logP also show little permeability due to the inability to partition out of the SC. The optimum logP to achieve adequate partitioning is between 1 and 3[58].
- Maximum number of hydrogen bonding groups on a chemical structure should not exceed 2 to avoid interactions between the polar head groups of the intercellular lipids and the hydrogen bonding groups on the drug[58].

- Many permeants may be in the form of weak acids or bases. Their permeation will depend on their degree of ionization and how their state of ionization influences their solubility in the applied phase and ultimately, their partitioning into the SC. Under some circumstances, administering a drug in its ionized form may be valuable in that it provides higher solubility, compensating for lower permeability[42].

It should be acknowledged that the above factors are only general guidelines for selecting drug candidates for transdermal penetration. With this said however, little attention has been given to the effects of the above physicochemical properties on the lateral diffusion behaviour of drug across the skin. Lateral diffusion is a possible route of drug loss and thus, in order to gain a better understanding of the fate of unabsorbed drugs, the influence of the physicochemical properties on the simultaneous process of penetration in the upper layers of the SC and lateral spreading is required.

1.6.2 Fick's First Law of Diffusion

The simplest form of drug movement through and across the skin occurs by passive diffusion, whereby the molecules move from a region of high drug concentration on the surface of the SC to a region of low drug concentration within the strata. The most basic mathematical explanation of this diffusion process at steady state is defined by Fick's First Law of Diffusion, whereby the rate of transfer of diffusing substance through a unit area of a section is proportional to the concentration gradient through that section and can be presented as follows:

Equation 1.1

Where the flux (J) of permeant is the mass of drug penetrating through the membrane per unit area, per unit time; K_p is the permeability coefficient of the drug in the SC and C_s is the concentration gradient across the membrane.

K_p, is a function of the diffusion coefficient, D_m, and is inversely related to the length of the diffusion pathway, L, which is analogous to the thickness of the membrane.

$$\frac{\quad}{\quad}$$

Equation 1.2

D_m is a reflection of the inherent diffusibility and mobility of the molecule in the membrane. For molecules in solution, another factor also needs to be taken into consideration – the partition coefficient, K_m. The partition coefficient describes how many solute molecules are available for diffusion through a membrane, and is expressed as the ratio of solubility of the solute in the diffusing membrane and in its solvent. Therefore, the higher the K_m for a solute, the more ease at which it leaves its solvent. Hence, at steady-state conditions, the original equation 1.1 can be rewritten as[59]

$$\frac{\quad}{\quad}$$

Equation 1.3

From the above equations, it is evident that the primary driving force for diffusion is the activity gradient across the membrane. Since the thickness of the membrane is fixed, the molecular flux across the membrane can be manipulated by changing D_m (e.g. molecular size) and K_m (increasing drug partitioning into the membrane), as well as by increasing the solute concentration in the applied formulation.

However, as the skin is a stratified structure made up of a lipoidal domain in the SC supported by a dominantly aqueous epidermal layer, the physiological properties of the entire underlying structure will also influence the overall diffusion process. Therefore, the lipophilicity of a penetrant which governs K_m, must support the transfer into the SC, across the viable epidermis and into the dermis. Additionally, although the permeant must have appropriate solubility in its vehicle, this will likely have a negative effect on drug delivery as it will decrease the drug's leaving tendency from the vehicle, thereby decreasing drug transfer into the SC and ultimately drug partitioning into the SC. Therefore, the formulation requires a vehicle that facilitates drug transfer by having a sufficient 'holding capacity' for the therapeutic dose and the drug itself needs to have an adequate 'leaving tendency' from the vehicle[60].

The partitioning of a drug into the skin will also be influenced by its thermodynamic activity in the application vehicle. This can be improved by increasing the concentration of drug in the applied vehicle or by manipulating the vehicle to reduce drug solubility[58].

1.7 PASSIVE STRATEGIES TO ENHANCE TRANSDERMAL DRUG DELIVERY

The barrier properties of the SC limit the therapeutic efficacy of most drugs to be delivered through the skin[61]. Therefore, chemical approaches have been utilized to manipulate the SC barrier in order to improve the percutaneous absorption of poorly penetrating compounds, as summarized in Figure 1.5.

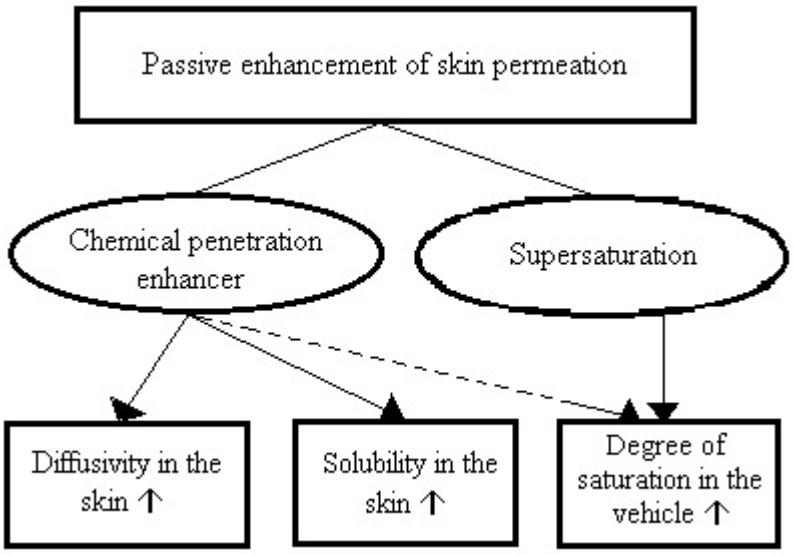


Figure 1.5 Strategies for enhancement of skin permeation according to Fick's First Law of Diffusion and the modes of action of chemical permeation enhancers and supersaturation. Adapted from Moser et al.[39].

1.7.1 Penetration enhancers

One commonly used approach to overcoming the barrier properties of the skin is by incorporating other vehicles or chemical compounds into the transdermal formulation to increase the diffusion of the solute. For poorly penetrating compounds, the addition of penetration enhancers can reversibly alter the skin barrier and thereby easing the penetrating pathway of compounds across the membrane to reach the systemic circulation. The solubility parameter is a numerical value which estimates the degree of interaction between materials[62]. Therefore, chemicals that are able to promote drug diffusion across the SC often have a solubility parameter similar to that of the skin and are known as skin penetration enhancers.

Barry et al.[63,64] devised the lipid-protein-partitioning (LPP) theory to describe the mechanisms by which enhancers affect skin permeability:

- Disruption of the intercellular bilayer lipid structure.
- Interaction with the intracellular proteins of the SC.
- Improvement of partitioning of a drug, coenhancer, or cosolvent into the SC.

Ideally, penetration enhancers should have the following properties[65]:

- Be both pharmacologically and chemically inert and chemically stable.
- Have a high degree of potency with specific activity and reversible effects on skin properties.
- Show compatibility with formulation and system components.
- Be non-irritant, non-sensitizing, non-phototoxic and non-comedogenic.
- Be odorless, tasteless, colourless and cosmetically acceptable.
- Have a solubility parameter approximating that of skin.

Permeants traversing the skin barrier are required to diffuse through the intercellular lipids regardless of which penetration pathway they take. Therefore, penetration enhancers that are thought to disturb the order of the highly arranged lipoidal intercellular structure will do so by decreasing the diffusional resistance of the SC to the incoming solutes. One possible mechanism by which enhancers are able to achieve this is through interaction of the enhancer with the polar head groups of the lipids. The lipid-lipid head group interactions and the packing order are thus disturbed and results in the facilitation of the diffusion of hydrophilic drugs[66]. This increased flow also encourages the flow of free water molecules between the bilayers and thus makes way for a larger area for polar molecules to diffuse. The use of water hydration as a penetration enhancer is a popular choice as it is safe and effective. Disturbing the lipid head groups with polar substances can also improve lipophilic drug penetration by simultaneously rearranging the lipid bilayer areas[67] as shown in Figure 1.6. Another possible mechanism of action of lipophilic penetration enhancers is

through interaction with the hydrocarbon chains in the lipid domains and thereby disturbing the organized packing order to increase lipid fluidization. This in turn, can also increase the penetration of hydrophilic drugs by concurrently influencing the order of the polar headgroups[68].

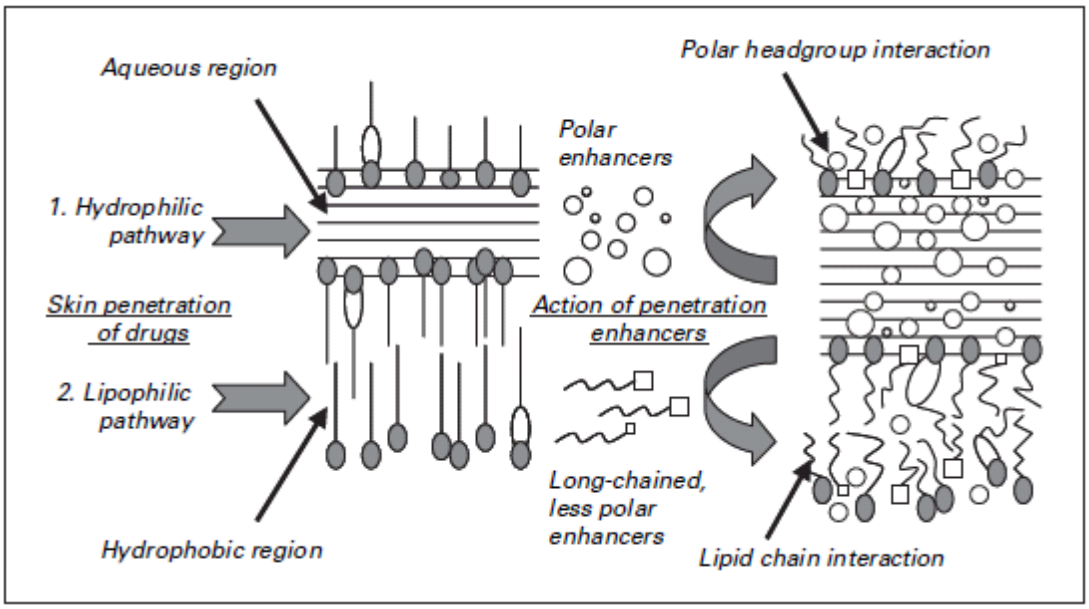


Figure 1.6 Hydrophilic and lipophilic pathways of drug penetration and mode of action of penetration enhancers. Adapted from Trommer et al.[41].

Unfortunately for many penetration enhancers used to date, in addition to increasing skin permeability they may also cause irritation, creating grounds for strict regulatory guidelines for their use. Octisalate (OS), however, is a chemical that is found in sunscreens and is identified as a 'generally regarded as safe' (GRAS) compound. Recent developments have revealed that OS is able to enhance drug permeation *in vivo* and *in vitro*[56,69,70] and this is of prime interest of our research group. It is predicted that the high lipophilicity of OS is capable of accumulating and forming a reservoir within the lipid phases of the SC and thus altering the pathway for penetrating solutes. OS itself however, is unlikely to permeate across the viable epidermis due to the hydrophilic nature of this

skin region[71]. Using high density differential scanning calorimetry, El Maghraby et al.[72] were able to determine that OS inserts itself within the lipid bilayers of the SC and thereby exerting a significant perturbation for the bilayer structure of the SC and rendering it disordered. Consequently, the membrane disrupting effect of OS allows it to act as a penetration enhancer of other solutes.

1.7.2 Cosolvents

To maximize delivery, the largest possible amount of drug should be dissolved in the formulation and become immediately bioavailable to the lipophilic SC[73]. For finite dose applications with a volatile component, drug may precipitate onto the SC surface and thereby impede the ability of drug into the SC. However, non-volatile components such as cosolvents may be incorporated into formulations to prevent drug from precipitating from solution as the volatile component evaporates. Furthermore, cosolvents may partition into the SC and disrupt the ordered intercellular lipids and enhance drug permeation[58]. Propylene glycol (PG) is one such chemical that is popularly used as a cosolvent constituent in dermatological formulations to enhance drug permeation through skin. It is a small molecule (molecular weight of 76.1 g/mol) that is known to penetrate through skin; induces a synergistic action when used together with penetration enhancers such as oleic acid; serves as a penetration enhancer in its own right; and also acts as a good solvent for permeants[40]. It is suggested that PG achieves its effect by altering the thermodynamic activity of the drug in the vehicle as it permeates through the skin, which then consequently changes the driving force for diffusion while partitioning into the tissue to facilitate uptake of the drug, as well as disturbing the intercellular lipid packing within the SC bilayers[74].

1.7.3 Supersaturation

In passive diffusion processes, 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. In formulations where the vehicle comprises of a volatile component, the drug flux across a membrane will change as the volatile component evaporates and the change in flux is expected to be proportional to both the concentration of the drug in the vehicle and its activity until saturation is reached and thermodynamic activity is at a maximum[75-77].

At a supersaturated state, the drug is at an exceedingly higher concentration than its equilibrium solubility limit. Thus, by this nature, the drug is physically unstable and is expected to precipitate out of solution while the flux remains constant as it is set by the driving force of the saturated solution. Flux levels above that of the saturated solution may also be achieved prior to termination of the supersaturated state. The presence of drug precipitation, however, may also counteract the enhancement effect and decrease drug flux. In such situations, the use of antinucleant polymers can either prevent or stall the crystallization process[75].

The enhancement effect of supersaturated systems was first demonstrated by Coldman et al.[78]. They studied the *in vitro* permeation of fluocinolone acetonide and its acetate ester from a mixture of volatile and non-volatile vehicle. After leaving their drug dose non-occluded, they found up to a 10-fold increase in flux compared to that of the non-supersaturated system. In addition, it has been found that regardless of its concentration, the flux of a drug across a membrane from a saturated solution should remain constant if no interactions between the membrane and other components of the formulation occur[79].

While the method of supersaturation as well as the addition of penetration enhancers and/or cosolvents has been reported to enhance transdermal penetration, their affect on the

lateral diffusion of topically-applied drugs have not been elucidated. The addition of these excipients may affect the extent of which drugs spread radially from the application site and therefore, the probability of secondary exposure of unabsorbed drug residing on the surface of the skin to the immediate environment. Thus, the effect of excipients on the penetration and lateral diffusion of drugs across the skin will also form part of this project.

1.8 METHODS USED TO ASSESS FATE OF TOPICALLY-APPLIED DRUGS

The ultimate goal of transdermal drug deliver is to ensure that compounds are delivered, preferably at a specific rate, to the systemic circulation[66]. Therefore, various methods have been developed over the years to assess the fate of topically-applied compounds, to measure the percutaneous absorption of transdermal formulations, to follow the penetration behaviour of solutes across the skin and to gain a better understanding of the structure and barrier properties of the skin.

1.8.1 *In vivo* methods

Percutaneous absorption *in vivo* is usually determined by the indirect method of measuring radioactivity after topical application of a radiolabelled compound and assessing the kinetics of skin permeation from the rate of drug excretion in the urine or blood. However, measuring *in vivo* bioavailability in man using this approach is often fraught with experimental and ethical difficulties. Instead, measuring the surface recovery of a material as it penetrates into the skin is a more non-invasive and popular approach. In surface recovery measurements, it is assumed that the difference between the applied dose and residual dose is the amount of drug absorbed.

1.8.1.1 Tape stripping

Tape stripping the skin surface is a common method in determining the concentration of chemicals in the SC. A chemical is applied to the skin for a fixed period of time after

which the SC is removed by successive tape application and removal. The tape strips are then assayed for chemical content either spectroscopically or using radioisotope techniques[80-83]. This technique was shown to be a reliable method in estimating percutaneous absorption as Rougier et al.[84] established a linear relationship between the SC reservoir content and percutaneous absorption using the urinary excretion method.

Although tape stripping has been extensively used to study the penetration behaviour of topically applied permeants[85-89], few studies have utilized tape stripping to follow the lateral diffusion behaviour of permeants across the skin surface. Furthermore, of the documented studies that has investigated the lateral spread of drug, the area or distance to which drugs spread laterally or transversely from the application site has been poorly defined[54,55,90]. Therefore, a more definitive measure of the extent and area of lateral spreading are required in order to better understand and quantify this phenomenon.

1.8.1.2 Quantifying tape strips

The total weight of drug removed per tape strip is also essential to gain a proper understanding of the concentration profile of drug across the skin. Several techniques to quantify drug removal such as solvent extraction[87] and direct ultraviolet (UV)-visible spectroscopic measurements[91] have been reported. All methods have quoted at least 90% recovery rate.

1.8.1.3 Determining SC concentration

In most tape stripping studies, assessment of the SC content is essential to gain insight into a drug's concentration profile within the skin. Various methods have been reported to quantify SC removal – the most common being gravimetric weighing[91], spectroscopy in the UV wavelength[55] or colorimetric protein counting[92]. Weighing each

individual tape strip before and after stripping is time consuming and subject to large variations and error due to the change in moisture content of the adhesive.

A modified Lowry assay developed by Dreher et al.[92] is a simple and quick method to account for SC removal in tape strips. This colorimetric method is carried out by the addition of sodium hydroxide to tape strips to extract the adhered SC protein cells. This solution is then mixed with hydrochloric acid and further treated with various reagents of a detergent compatible protein assay kit in a 96-well plate and finally read for absorbance in a spectrometer. Similarly, Breternitz et al.[93] adopted a modified Bradford assay using lyophilized bovine serum albumin as standards to evaluate the tape stripping effect using different tapes and it is this technique that will be adopted in this research.

1.8.2 *In vitro* methods

In vitro methods are valuable for screening procedures and for deducing permeability parameters such as flux, partition coefficients and diffusion coefficients of a permeating substance. The most common method for evaluating *in vitro* skin penetration is the use of diffusion cells, which can be in the form of either a two-compartment static diffusion cell[94] or a flow-through cell[95]. Excised skin is mounted in between a donor chamber and a receptor chamber, and the amount of compound permeating from the donor to the receptor side is determined as a function of time. The amount of permeant collected in the receptor fluid is analysed using a spectroscopic technique. Diffusion studies are a useful tool for the screening of percutaneous absorption of transdermally-applied drugs during the initial stages of formulation development. Furthermore, the experimental conditions of permeation studies using diffusion cells are easily controlled[96].

Tape stripping (as described above) is also commonly used *in vitro* to follow the distribution profile of permeants as well as to assess the percutaneous absorption of a

substance. In addition to determining the percutaneous absorption of drugs across the skin, efforts have also been made to determine the mechanism of action or transport pathways of penetrating substance. Attenuated total reflectance-fourier transform infrared (ATR-FTIR) spectroscopy has been used to study the mechanism of passive drug transport across the skin by measuring the change in infrared absorption associated with a penetrating substance[82]. ATR-FTIR has also been applied to the investigation of morphological differences or changes across the SC[97-99]. Although the ATR-FTIR can also be used to follow drug distribution across the SC, it is often limited to deuterated compounds which is often not readily available and expensive[42,100]. Autoradiography and laser-scanning confocal microscopy (LSCM) have also been used to visualize the penetration through and distribution of drug within skin but are unable to directly quantify drug concentration[101,102].

1.8.3 Correlation between *in vivo* and *in vitro* percutaneous absorption studies

During the development and optimization of transdermal dosage forms, it is important to understand the factors that influence a good *in vivo* performance. Of course, the most reliable skin absorption data are collected in *in vivo* human studies but such studies are generally not practical during the early developmental stages of new drug candidates. In addition, it is practically impossible to assess the skin permeability of materials using *in vivo* experiments alone[103]. Thus, *in vitro* models are often employed to screen the permeation profiles of a series of drug formulations, evaluate skin permeation enhancing properties and to determine the mechanism of action of potential carrier systems across the skin. However, the correlation of *in vitro* results to that of *in vivo* results has been a topic of much debate.

The most comprehensive study on the comparison of *in vitro* and *in vivo* absorption of a chemical compound was performed by Franz et al.[104]. Using drug residual and pharmacokinetic calculation methods, an excellent correlation was found between *in vitro*

human epidermal penetration flux and *in vivo* flux. Furthermore, the correlation was considerably better for compounds that exhibited greater penetration. For poorly permeating compounds (<1.0% absorption *in vivo*) however, the *in vitro* system significantly overestimated absorption. This observation was explained by the process of desquamation that occurs *in vivo*. On average, one cell layer of SC is lost via desquamation per day and so, for slow absorbing penetrants, a significant proportion of the applied dose of permeant will be lost in this process. However, since *in vitro* skin does not undergo cell turnover, a higher concentration of permeating compound will remain on the SC over time and be available for diffusion[105].

Scott et al.[106] also conducted an *in vivo* and *in vitro* comparison of the percutaneous absorption of eight pesticides of various molecular weights. In general, there was a good agreement between the amount of test chemical that could be removed from the surface of both *in vivo* and *in vitro* skin at the end of the experiment. Similar to the results from Franz et al.[105], the *in vitro* absorption values were usually higher than that of the *in vivo* results.

The effect of skin metabolism on the *in vivo* and *in vitro* recovery of a chemical compound from the skin surface was studied by Roberts et al.[107]. Here, the agreement between the two sets of data was not so impressive. Esterase activity *in vivo* resulted in almost complete first-pass metabolism of methyl salicylate in human skin, much greater than is seen *in vitro.*

From the above analysis, it is important to acknowledge the limitations of *in vitro* measurements and understand that data derived from such experiments should not be overintepreted. There is no doubt that the transdermal delivery of drugs *in vitro* can provide a constant rate of delivery. However, such a constant release rate will in most cases, not necessarily occur *in vivo* as factors such as time-dependent changes in first-pass

metabolism of drugs, or in the permeability characteristics of the percutaneous barrier could affect the absorption of the penetrant[108]. Furthermore, neither physiological nor pharmacodynamic responses occur *in vitro*; thus, a formulation applied to the skin *in vivo* may be affected by sweat or sebum secretion that cannot be mimicked *in vitro*. Similarly, a permeating compound that is able to induce vasodilation and thereby increase blood flow to increase clearance from the skin *in vivo*, will show no such effect *in vitro*[109].

Despite the varied correlation between *in vivo* and *in vitro* data, *in vitro* permeation experiments still provide and important tool for screening drug delivery systems and are a valuable method for predicting the behavior of drugs *in vivo*.

1.9 GAPS IN CURRENT LITERATURE

While the bioavailability of topically-applied drugs is accepted to be less than 10%[1], the need to establish the fate of the unabsorbed drug is often ignored. Unabsorbed drug residues may reside on the skin surface following lateral diffusion away from the application site. The larger skin area of drug exposure may in turn increase the risk of adverse effects to the immediate environment due to secondary exposure. Thus, a method needs to be developed to better understand the factors affecting drug lateral diffusion across the skin. More importantly, the impact of physicochemical properties of drugs and the addition of excipients on the lateral diffusion of drugs needs to be determined in order to obtain a better understanding of the fate of topically-applied drugs.

1.10 AIMS OF RESEARCH

The aim of the research studies in this thesis was to determine the lateral diffusion and penetration behaviour of topically applied formulations of three model compounds across the SC, as well as evaluate the impact of various formulation excipients on the fate of topically-applied compounds. Particular attention was focused on:

I. The development and optimization of a novel concentric adhesive tape design which could be applied to tape stripping humans for the simultaneous quantification of depth of penetration and lateral spread of model drugs (Chapter 2). The use of the Bradford assay to quantify the mass of SC removed per tape strip was also investigated. Furthermore, the optimization of the relevant experimental conditions to assess the lateral spreading and penetration of the model drugs was performed, with specific focus given to (i) determining the relevant dose of model compounds to apply, (ii) the total number of tape strips to remove, (iii) the experimental time points at which to tape strip human skin and (iv) the feasibility of the drug application method.

II. Utilization of the concentric adhesive tape design to assess the lateral diffusion and penetration behaviour of the model compounds across human skin *in vivo* (Chapter 3).

III. Utilization of *in vitro* flow-through diffusion cells to determine the extent of percutaneous absorption of the model compounds across human skin (Chapter 4).

IV. Utilization of the concentric adhesive tape design to determine the effect of excipients on the lateral diffusion and penetration of a model compound across the human skin *in vivo*. In addition, the effect of excipients on the percutaneous absorption a model compound across human skin *in vitro* was also assessed using flow-through diffusion cells (Chapter 5).

V. Utilization of the concentric adhesive tape design to determine the effect of excipients on the lateral diffusion and penetration behaviour of a model compound across the human skin *in vivo* over an extended exposure period. In addition, the effect of excipients on the percutaneous absorption a model compound across human skin *in vitro* was also assessed over an extended exposure time using flow-through diffusion cells (Chapter 6).

1.10.1 Choice of marker compounds

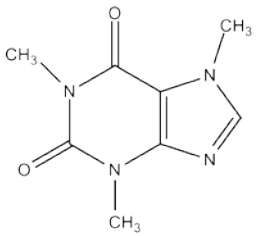
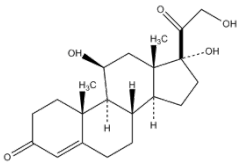
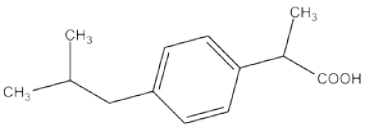
Some of the physicochemical properties of an ideal drug candidate for transdermal drug delivery are listed in Section 1.6.1. Ibuprofen (IBU) is a non-steroidal anti-inflammatory drug (NSAID) commonly used in the management of mild to moderate pain and inflammation conditions. It should be theoretically well absorbed through the skin as it is a relatively small molecule with an ideal logP of 3.51[110]. IBU was incorporated into formulations at a concentration of 5% w/v to match that of usual topical concentrations.

Caffeine (CAF) is normally used in cosmetics for its stimulating activity on fat metabolism and also in therapy for the treatment of headache and neonatal apnoea[111,112]. More recently, it has been suggested that topical application of CAF can inhibit carcinogenesis and increase apoptosis in UV-induced skin tumors in mice[113,114]. However, it is a hydrophilic drug with a logP of -0.07[115] and would thus be confronted with difficulty when attempting to permeate the skin barrier.

Hydrocortisone (HC) is a corticosteroid used in topical applications for the treatment of various skin disorders such as psoriasis[116] (increased cell proliferation[51]) and skin allergy reactions (also known as contact dermatitis)[117]. It is usually formulated in creams, ointments or lotions at concentrations ranging from 0.1 – 2.5% and absorption is found to be higher in denuded areas. HC was used as a marker compound in this project as it possesses physicochemical properties that are intermediate to those of CAF and IBU.

IBU, CAF and HC were selected as marker compounds in this research to allow for an assessment of the effect of drug lipophilicity and molecular weight (as listed in Table 1.1), on the process of lateral spreading. Additionally, the influence of a penetration enhancer, cosolvent and spreading agent on the penetration and lateral diffusion of these drugs was also assessed.

Table 1.1 Physicochemical properties of marker compounds.

	CAF	**HC**	**IBU**
Chemical structure			
Molecular weight (g/mol)	194.19	362.46	206.28
LogP	-0.07[115]	1.43[118]	3.51[110]

1.10.2 Choice of excipients

As mentioned in Section 1.4.1, the SC provides the primary barrier to transdermal drug delivery. Chemical penetration enhancers and cosolvents have thus been investigated and used to overcome this formidable barrier. An ideal skin penetration enhancer is effective, non-irritating and reversible[119]. OS can enhance skin penetration of drugs at least partly[72], due to the fact that it possesses a long alkyl chain that enables them to insert between the lipid bilayers and a polar head group that is capable of interacting with the lipid polar head groups, thereby reducing the conformational order of the SC lipids and increasing the permeation of drugs[58]. Moreover, OS and the SC both have similar solubility parameters. This allows OS to be readily dissolved in the SC and thus improve

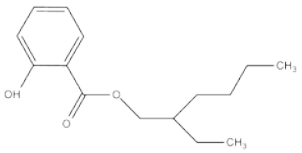
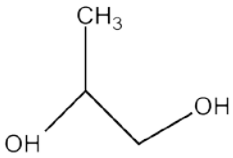
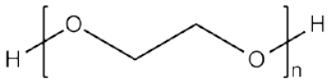
the partitioning properties of OS across the SC. Thus, OS will be included into some formulations to examine the effect on the percutaneous absorption and lateral diffusion of marker compounds through skin.

With this said, however, some chemical enhancers require the presence of a suitable solvent to carry them to the polar lipid domains of the SC to exert their function[120]. In studies that have been performed to date, PG is the most extensively researched cosolvent mainly due to its low molecular weight which makes it a good candidate for permeating through the skin. PG also exhibits good solvent properties as the permeation of PG drives the permeation of drugs[121]. For example Wotton et al.[122] showed that metronidazole had an enhanced permeability when the skin was pretreated with PG. Therefore, PG was included into some formulations in this research to evaluate its affect on the penetration and lateral spreading of the marker compounds.

Polyethylene glycol 200 (PEG 200) is widely used in cosmetics as surfactants, cleansing agents, emulsifiers, skin conditioners and humectants. It exists at room temperature as a clear viscous liquid and is often used in industry for its capacity to solubilise other substances in preparations. With respect to this project, PEG 200 is incorporated for several reasons: to serve as the non-volatile component while also potentially acting as a penetration enhancer by decreasing surface tension and conditioning the SC to increase diffusion of molecules through the skin[123]. With this said, however, some studies have reported that some PEGs have shown to effectively reduce skin penetration of certain compounds by raising the skin barrier function via the formation of complexes and/or by increasing viscosity[124-126]. Regardless, PEG 200 was incorporated into formulations studied in this research to determine what affect its viscosity had on the lateral spreading tendency of drugs on the skin surface.

The molecular structure of OS, PG and PEG 200 are shown in Table 1.2.

Table 1.2 Chemical structure of excipients incorporated into formulations.

	OS	**PG**	**PEG 200**
Chemical structure			

1.11 SIGNIFICANCE OF RESEARCH

If it can be determined that the residuals of unabsorbed drugs undergo lateral spreading on the surface of the SC as well as within the SC, then it is possible to develop methods to reduce the amount of drug wasted in this way and decrease the safety risk exposed to the surrounding environment. Fundamentally, this will lead to the development of methods to increase the overall bioavailability of transdermal drug delivery systems.

CHAPTER 2

DESIGN OF A NOVEL METHOD TO STUDY THE LATERAL DIFFUSION AND PENETRATION OF DRUGS *IN VIVO*

2 DESIGN OF A NOVEL METHOD TO STUDY THE LATERAL DIFFUSION AND PENETRATION OF DRUGS *IN VIVO*

2.1 INTRODUCTION

Over the years, reports have documented various methodologies to quantify and understand the penetration behaviour of topically-applied chemicals through the skin. The techniques include skin extraction measurements[127,128], removal of the hair follicles[129,130], quantitative autoradiography[131,132], spectroscopic methods[133-135] and the most commonly used method of horizontal sectioning – tape stripping[82,136-138]. Stripping the skin surface with adhesive tape is a minimally invasive technique that allows for sequential layers of the SC to be removed through repeated application and removal of adhesive tape. It is commonly used as a tool to investigate the localisation and distribution of topically applied substances within the SC. The drug removed with each tape strip is recovered via solvent extraction and quantified using common analytical methods. Tape stripping was successfully used, for example, by Trebilcock et al.[139] to assess the distribution and amount of fluazifop-butyl in the SC *in vivo* and *in vitro* and demonstrated that the amount of penetrant decreased with increasing depth into the SC. Furthermore, Trebilcock demonstrated good correlation between the concentration profiles of drug on human skin *in vivo* and *in vitro*.

The majority of studies utilise the method of tape stripping to follow the distribution of permeants across the SC at distances from the surface of the SC surface (i.e. downwards through the SC)[82,138-145]. However, very few studies have reported on the use of tape stripping to follow the lateral diffusion behaviour of drugs following application to the skin. Following transdermal drug application, permeants may also exhibit lateral diffusion on the SC surface as well as within the lipid bilayers of the SC. As Schicksnus et al.[54] revealed by extracting IBU from skin ring biopsies separated using punching tools, IBU

can spread up to 12 mm from the site of application soon after dosing and this distance can increase with time. Thus, lateral diffusion of permeants on and within the SC may occur simultaneously to the downward diffusion of drugs as shown in Figure 2.1.

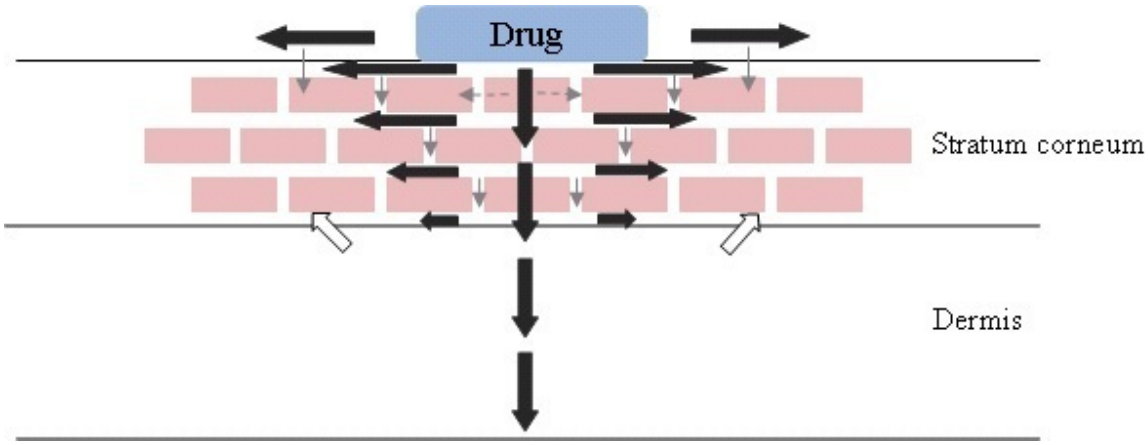


Figure 2.1 Routes of drug penetration and lateral diffusion across the SC.

Weigmann et al.[146] studied the influence of different formulations of clobetasol propionate on its penetration and lateral diffusion across the forearm SC using the tape stripping approach and successfully demonstrated that the application of clobestasol propionate in an emollient caused a pronounced lateral migration of the drug.

The process of lateral diffusion was further investigated by Jacobi et al.[55], by following the *in vivo* penetration and lateral spreading of UV filter substances across the forearm using tape stripping and UV-spectroscopic measurements. The degree of penetration and spreading was determined by tape stripping the application area, as well as the left and right regions adjacent to the application site. It was discovered that over time, the loss of material from the application area was reflected by the increase in concentration in the adjacent sites.

Thus, it is generally accepted that lateral spreading does occur when drugs are applied transdermally. However, the methods to examine lateral diffusion by tape stripping

that have been documented thus far only quantify the concentration of drug on either the left or right neighbouring sites to the application area and fail to account for drug loss via lateral spread to all neighbouring directions. Furthermore, the area or distance to which drugs spread laterally from the application site has been poorly defined. Thus, a more definitive measure of the extent and area of lateral spreading are required in order to better understand and quantify this phenomenon. As will become evident in the subsequent sections of this chapter, a modified method of Schicksnus et al.[54] use of concentric skin sampling was developed. Adhesive tapes were specially designed into concentrically perforated rings of 4 sections with known diameter (as shown in Figure 2.2) to allow for concurrent assessment of both the depth of drug penetration and the extent of lateral diffusion on the SC surface as well as within the SC layers.

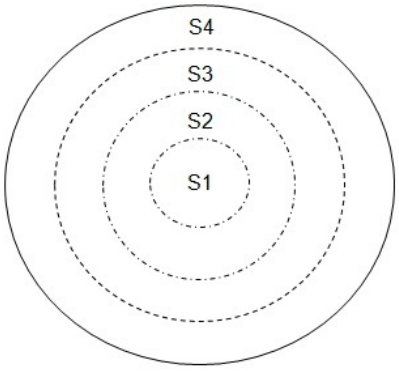


Figure 2.2 Schematic representation of specially designed concentric adhesive tape of 4 sections (S1, S2, S3 and S4, respectively).

To obtain the best estimate of the concentration-versus-depth profile of a drug across the SC, the amount of drug removed from each tape strip should be normalized with respect to the amount of tissue removed by each tape strip[86]. Various methods have been reported to quantify SC removal – the most common being by weight, which is time consuming and subject to large variations and error due to the change in moisture content

of the adhesive[136,147-151]. UV-spectroscopic methods[55] or colorimetric protein quantification[152] have also been used.

A modified Lowry assay developed by Dreher et al.[152] is a simple and quick colorimetric method to quantify SC protein removal in tape strips. This method is carried out by the addition of sodium hydroxide to tape strips to extract the adhered SC cells. The solution is then mixed with hydrochloric acid and further treated with various reagents of a detergent compatible protein assay kit in a 96-well plate and finally analysed for absorbance using spectroscopy. Similarly, Breternitz et al.[93] adopted a modified Bradford assay to quantify SC protein using lyophilized bovine serum albumin as an appropriate external standard. The Bradford assay relies on the direct binding of Coomassie brilliant blue G-250 dye with proteins to produce an absorbance maximum at 595 nm[153]. It is a simple, rapid, inexpensive and sensitive method. Thus, utilising the Bradford assay to quantify the mass of SC protein removed with each tape strip was the most appropriate approach to quantify the amount of SC protein removed per tape strip to allow for drug normalisation.

2.2 OBJECTIVES

Tape stripping is widely used to assess cutaneous drug or excipient levels in the skin after topical treatment, either in the removed tape strips, or directly in the tape stripped skin. Most studies that evaluate the penetration of permeant across the skin using the tape stripping method ignore the need to determine the lateral diffusion of the permeant. Conversely, some studies that have utilised tape stripping to study lateral diffusion have not specified the distance at which a drug may spread as well as simultaneously determining the depth of penetration or depth at which lateral diffusion may occur. Therefore, the objectives of this chapter was to:

- Develop and optimise a novel concentric adhesive tape design which can be applied to tape stripping humans for simultaneously quantifying depth of penetration and lateral spread of model compounds.
- To develop a Bradford assay to quantify the respective mass of SC protein removed by each tape strip to better define the local distribution of permeant within the SC
- To develop and optimise the relevant experimental conditions to allow assessment of the penetration and lateral diffusion of CAF, HC and IBU when applied to humans in subsequent chapters. Therefore, specific focus will be given to (i) determining of the relevant dose of CAF, HC and IBU to apply, (ii) the total number of tape strips to remove, (iii) the experimental time points at which to tape strip volunteers, and (iv) the feasibility of the drug application method.

2.3 MATERIALS

CAF, HC, IBU, octisalate (OS), propylene glycol (PG), bovine serum albumin (BSA) and Bradford reagent were purchased from Sigma-Aldrich (Castle Hill, New South Wales, Australia). Rainbow food colour dye (used to determine maximum distance of lateral spread) was manufactured by Queen Fine Foods Pty Ltd (Alderley, Queensland, Australia). Ethanol (90% v/v) (EtOH), isopropanol (IPA), methanol (MeOH), sodium hydroxide (NaOH), hydrochloric acid (36% v/v) (HCl), polyethylene glycol 200 (PEG 200) and phosphoric acid were obtained from Merck (Kilsyth, Victoria, Australia). Potassium dihydrogen orthophosphate was acquired from Univar (Ingleburn, New South Wales, Australia) and purified water (H_2O) was obtained from a Milli-Q™ water purification system (Millipore, Bedford, MA, USA).

2.4 METHODS

2.4.1 Preliminary *in vivo* study design

2.4.1.1 Drug application method – Metered Dose Transdermal System (MDTS)

The ideal transdermal formulation needs to deliver an adequate concentration of drug onto the skin surface that is able to be rapidly absorbed with minimum irritations to the surrounding area. The MDTS is one such system that is able to rapidly deliver a drug across the skin and encourage high drug diffusivity. The MDTS is a development from the Monash Institute of Pharmaceutical Sciences that is being commercialized by Acrux Ltd®. The technique involves the use of a combination of volatile solvents and non-volatile chemical components to modify the diffusion and partitioning behaviour of drugs. This formulation represents a micro-dose evaporative system that provides a passive and non-occlusive delivery[69].

The time course of events with the MDTS is summarized schematically in Figure 2.3. This evaporative system is sprayed onto the skin to produce a rapid-drying solution containing a volatile component that leads to an increase in surface drug concentration and thus, enhanced partitioning into the SC. The presence of the non-volatile component keeps the drug in solution to prevent precipitation or crystallization of the drug during evaporation of the volatile vehicle. It also functions to rapidly partition into the SC to disrupt the ordered arrangement of the intercellular lipids and thus, enhance the partitioning of the drug into the SC[58].

Overall, the MDTS leaves a transparent reservoir of drug on the surface of the skin surface that is slowly absorbed into our systemic circulation and it is this application method that this project aimed to replicate.

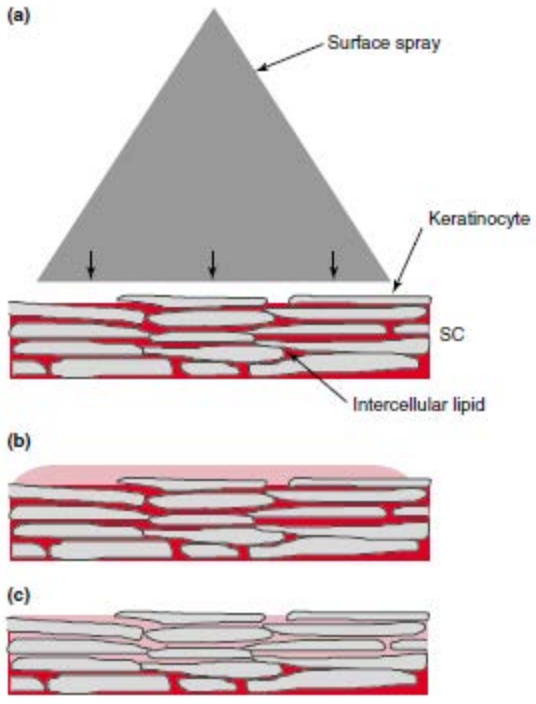


Figure 2.3 Schematic representation of time course of events following application of a drug using a MDTS. (a) Surface spray is applied to the SC. (b) The 'forced partitioning' concept, involving the rapid evaporation of the volatile vehicle and then the partitioning of

the drug and enhancer into the SC. (c) The drug and enhancer form a reservoir within the SC that is lipid in character and water resistant. Adapted from Thomas et al.[58].

2.4.1.2 Identification of appropriate tape stripping time points and total number of tape strips to collect

Tape stripping the volar forearm of participants was the major method employed in this research to follow the distribution of permeants within the skin. Therefore, it was necessary to determine the appropriate number of tape strips to apply and remove from participants in order to obtain sufficient information regarding the distribution profile of a permeant. Additionally, it was essential to determine the appropriate tape stripping time points to ensure that differences in distribution pattern may be observed.

One volunteer gave written consent to participate in the preliminary study which was approved by the Standing Committee on Ethics in Research involving Humans (SCERH), Monash University, Victoria, Australia (Project #CF08/1125 – 2008000555). The participant was 23 years of age and had no history of skin disease. The participant was asked to refrain from applying any topical applications to their left and right flexor forearms for at least 48 hrs prior to an experiment.

The volar forearm of a participant was wiped with Kimwipes™ saturated with EtOH to remove any sebaceous lipids or contaminants on the skin surface. Several tape stripping areas were clearly marked with permanent marker prior to dosing the skin to ensure each tape strip removed SC from the same site. The participant extended both forearms over a bench with the wrist facing upwards. A 25 µL aliquot of a solution containing either 1% w/v of CAF or IBU in IPA or 0.5% w/v of HC in IPA was pipetted onto the marked areas of the skin. These concentrations were chosen so that the amount of drug dosed in 25 µL would match that which would theoretically be delivered onto an area of 1 cm^2 using a

MDTS. The forearm remained flat and rested on the workbench for 30 secs or until all of the solvent had evaporated. The solution remained in contact with the skin for 30 mins, 2 hrs, 4 hrs and 8 hrs. These time points were selected as it allowed for screening of the most appropriate tape stripping time points at which a change in permeant distribution or recovery may be observed during the defined working day (thus minimizing potential interference of home-related activities on drug distribution). During the 8 hrs of which the solution was in contact with skin, the solution was left unoccluded and the participant was required to wear a T-shirt or have sleeves rolled up to avoid loss of drug via rubbing off onto clothing. In addition, they were required to prevent the skin site of interest from coming into contact with surrounding objects to avoid loss of drug.

At 30 mins, 2 hrs, 4 hrs and 8 hrs after application, the treated area was repeatedly tape stripped in order to remove sequential layers of the SC. Rectangular polyester adhesive tapes of 2.4 x 3 cm (3M, Product No 8440, ATA Distributors, Victoria, Australia) were applied onto the surface of the treated skin site. Each tape was folded onto itself at one end to produce a small overhang for easy handling purposes and to avoid contamination upon handling. After the tape was applied onto the marked treated site, a stainless steel slab weighing 1200 g and measuring 2.8 x 6 x 9 cm was placed over the tape for 3 secs to ensure even pressure was applied across the tape. The tape was then removed from the SC surface and placed into 10 mL of extraction solvent, as described in Section 2.4.4, and analysed for drug content by high-performance liquid chromatography (HPLC) as described in Section 2.4.5. 10 sequential tape strips were removed from each site as this has been previously reported to be adequate to remove sufficient amounts of the SC[154]. Tape stripping was performed on 3 sites of the forearm at each time point.

2.4.1.3 Determination of the extent of solvent spread on skin surface using MDTS application

As stated in the Section 2.4.1.1, finite doses of drugs were applied onto the skin surface using a method that replicates a MDTS and horizontal sections of the SC was removed over time using specially designed adhesive tapes to determine lateral diffusion. In order to ensure that a single piece of concentric adhesive tape encompassed the entire area over which a drug had spread, it was necessary to determine the maximum area that the solvent of choice would spread once applied to skin. This was carried out by spraying a MDTS filled with IPA into which no more than 10 drops of blue food dye had been dispersed.

The MDTS system is designed to deliver approximately 72 µL of solution onto the skin when sprayed to cover a circular area of approximately 20 cm^2. However, the area of application in the following studies were reduced by a factor of 40 to 0.5 cm^2, thus delivering a volume of 1.8 µL by inserting a plastic sheet into the cone of the device (as shown in Figure 2.4). This smaller application volume and area ensured that the solution was dosed on the flattest plane on the volar forearm, thus preventing the solution from spreading along the curvature of the forearm and making tape stripping difficult.

The volar forearm was wiped with a Kimwipes™ saturated with EtOH to remove any sebaceous lipids or contaminant that may impede with the lateral spreading of solution on the skin surface. The participant extended their left or right forearm at right angles to their torso such that the forearm was flat and facing upwards. A MDTS was placed over the forearm and sprayed once. The participant maintained the position of his/her forearm for another 30 secs to ensure that most of the solvent had evaporated. A blue circular depot was left on the skin surface which represented the extent of spread. After visual inspection, the diameter of the depot was measured with a ruler. These studies provided an indication

of the required diameter for the novel concentric die-cut adhesive tape, as the area of the tape could not be smaller than the area of the spray deposited by the MDTS.

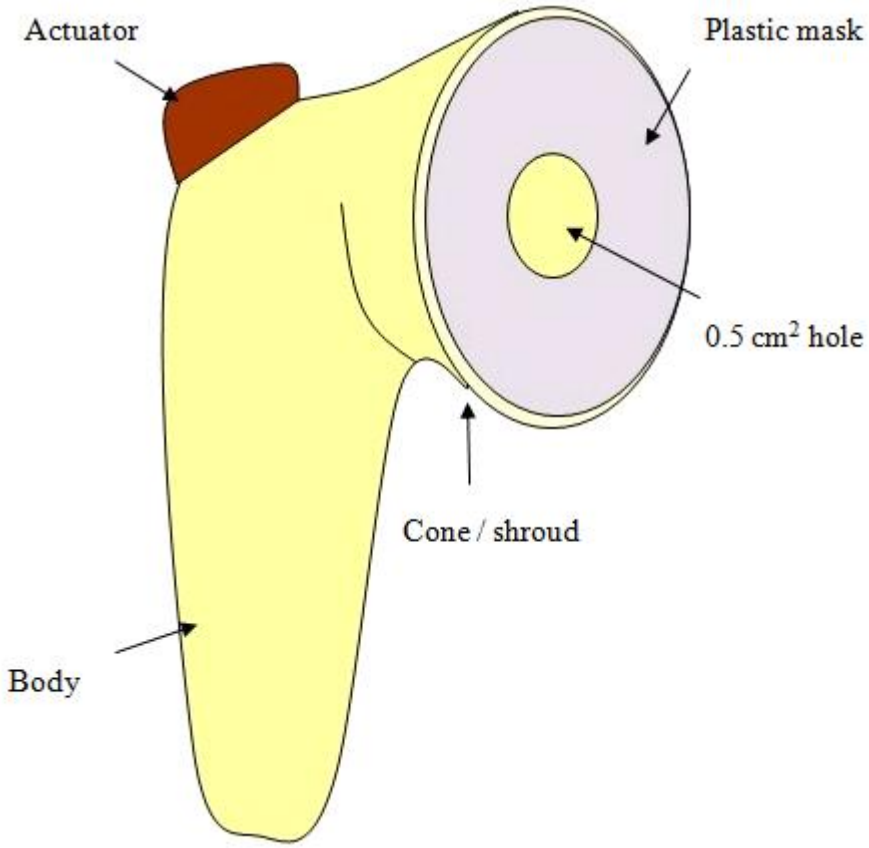


Figure 2.4 Schematic representation of a MDTS with inserted mask to reduce application area and amount of drug deposited per spray.

2.4.1.4 Reproducibility of drug release through MDTS mask

The MDTS mask was easily inserted and removed from the cone of the device. However, it was difficult to ensure that the mask was oriented in exactly the same position at each insertion. Thus, it was necessary to determine whether the amount of drug deposited on the skin by the MDTS was consistent and reproducible each time the mask was placed on the MDTS.

A MDTS unit was filled with 1% w/v CAF in EtOH. The MDTS shroud and mask was rinsed with EtOH and allowed to air dry. The MDTS unit was held in an upright

position and the actuator primed 6 times to ensure that the shroud and pump were adequately filled with solution. A circular piece of filter paper of 185 mm thickness (Advantec Ltd, California, USA) and measuring >20 cm^2 was attached to a clipboard in a vertical position. The MDTS was held against the filter paper and the actuator pumped once then removed to allow the solvent to evaporate. This was repeated 5 times to obtain an appropriate indication of reproducibility.

The amount of CAF sprayed onto the filter paper was determined via solvent extraction as detailed in Section 2.4.4 and quantified using HPLC as described in Section 2.4.5.

2.4.1.5 Determining spray pattern of MDTS deposit

As will be illustrated in Section 2.5.4.4, the amount of drug sprayed through the MDTS mask was variable. However, it was not known whether the variability was caused by the movement of the mask or the variability in spray pattern of the MDTS. For this reason, the spray pattern of the MDTS in the absence of a mask was investigated.

The perimeter of a MDTS cone was traced onto filter paper (Advantec Ltd, California, USA) and carefully divided into sections of ~0.5 cm^2 and labelled 1 – 53, as presented in Figure 2.5. The MDTS shroud, button and actuator were rinsed with EtOH and allowed to air dry. The MDTS unit was filled with 1% w/v CAF in EtOH and held in an upright position and the actuator primed 6 times. The piece of filter paper was attached to a clipboard in a vertical position. The MDTS was placed against the filter paper directly over the marked perimeter of the cone and the actuator pumped once then removed to allow the solvent to evaporate. This was performed on 3 different filter papers to determine reproducibility of the spray pattern. Each labelled section of the filter paper was carefully cut out and placed into individual 20 mL vials for extraction. The amount of CAF sprayed

onto each section was determined via solvent extraction as detailed in Section 2.4.4 and quantified using HPLC as described in Section 2.4.5.

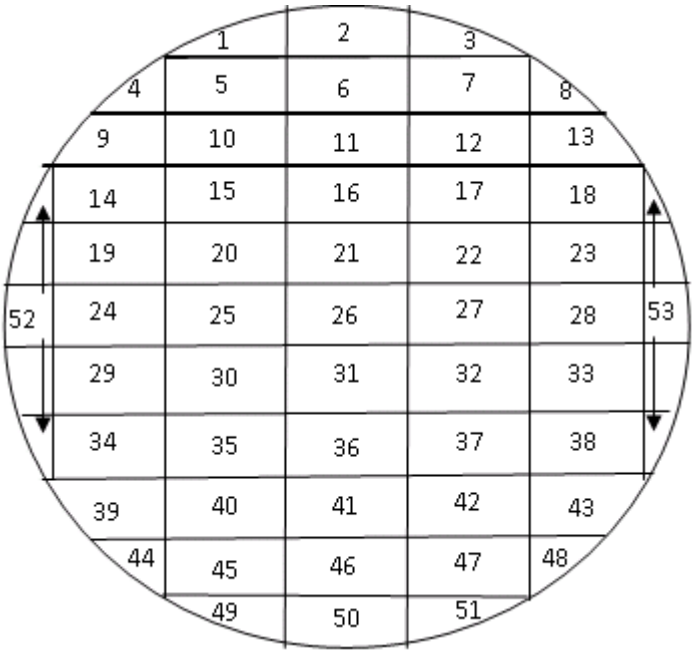


Figure 2.5 Division of filter paper to determine MDTS spray pattern.

2.4.1.6 Dosing accuracy and precision of HPLC manual injection syringe

As will be revealed in Section 2.5.4.4, inserting a mask into a MDTS device to dose drug over an area of 0.5 cm^2 was not feasible due to the inconsistency of spray pattern which in turn, caused large variability in the amount of drug sprayed through the mask. Therefore, a HPLC manual injection syringe (1 – 10 µL) was selected to dose the formulations onto test subjects as this application method is able to replicate the non-occlusive nature of the MDTS, without its inherent variability in spray pattern.

Although HPLC manual injection syringes are known to deliver acceptable volume accuracy, it was still essential to verify the accuracy and precision characteristics of the syringe to ensure minimal variability in doses applied onto human skin in subsequent studies (Chapter 3, 4, 5 and 6).

A 1.8 µL aliquot of ethanolic solutions of CAF 0.8% w/v, HC 2% w/v and IBU 5% w/v was dosed onto individual filter paper measuring 2 cm x 2 cm (n=8 replicates per drug). Following solvent evaporation, the filter papers were placed into individual 20 mL vials. The amount of drug deposited onto each filter paper was determined via solvent extraction as detailed in Section 2.4.4 and quantified using HPLC as described in Section 2.4.5. The accuracy and precision measurements were calculated from the following equations:

Equation 2.1

Equation 2.1

2.4.1.7 Uniformity of solvent spread following application with HPLC manual injection syringe

In addition to determining the reproducibility and accuracy of dosing with a HPLC manual injection syringe, it was also important to ensure that the spread of drug across the skin surface would be symmetrical following application. This was essential as each tape strip removed from the skin surface in the subsequent studies (Chapter 3, 4, 5 and 6) was split in half. One half of the concentric tapes were further split into individual sections to quantify for the amount of drug removed (while the other half of the concentric tape was quantified for the amount of SC protein removed using the Bradford assay as revealed in Section 2.5.4.2).

10 circles with a diameter of 3 cm^2 were traced onto a piece of filter paper. The centre of each circle was clearly marked which denoted the application point. A 1.8 µL

aliquot of ethanolic solution of either CAF 0.8% w/v, HC 2% w/v or IBU 5% w/v was dosed onto each individually marked circle. Following solvent evaporation, the filter papers were cut into two equal halves and placed into individual 20 mL vials. The amount of drug on each half of the filter paper was determined via solvent extraction as detailed in Section 2.4.4 and quantified using HPLC as described in Section 2.4.5. The accuracy and precision measurements were calculated from Equations 2.1 and 2.2.

2.4.2 Validation of the Bradford assay for quantifying SC protein

2.4.2.1 Bradford assay

It is important to normalize the amount of drug extracted from each tape strip to the corresponding mass of SC protein from the same tape strip in order to follow the drug-depth versus penetration behaviour of a permeant. The Bradford assay is considered to be an appropriate method to quantify the amount of protein removed by each tape strip.

S_1, S_2, S_3 and S_4 tape sections, as shown in Figure 2.2 were divided into 2 halves and 1 half of the tapes were individually placed into 6-well plates with the adhesive side facing down. A stock standard solution of 1 mg/mL of BSA was prepared in saline. The BSA stock standard solution was further diluted with NaOH to produce standard solutions between the ranges of 100 – 600 µg/mL. 50 µL of each standard were further diluted to 700 µL with 1 N NaOH in wells of 6-well plates. The plates were then incubated for 1 hr at 22°C on a horizontal plate using the Eppendorf Thermo-mixer Comfort (Eppendorf, New South Wales, Australia) at 300 rpm. Thereafter, a 50 µL aliquot of each well was transferred into a 96-well plate and 250 µL of Bradford reagent added. As an appropriate blank (required for blank subtraction), 50 µL of 1 N NaOH and 250 µL of Bradford reagent were added to 3 additional wells. The plates were then securely wrapped in aluminium foil to protect them from light and incubated at ambient conditions on the bench for 30 mins. Each plate was then placed in a FLUOstar Optima (BMG Lab Technologies,

Offenburg, Germany) where spectrophotometrical analysis was performed at 595 nm and the absorbance of each well recorded.

A linear calibration curve relating protein concentration (100 – 600 µg/mL) to absorbance at 595 nm was constructed. BSA concentrations of 100, 300 and 600 µg/mL (n=5) were used to determine the intra-day precision and accuracy and the inter-day precision and accuracy was assessed across 3 separate days.

2.4.2.2 Reproducibility of SC protein extraction using Bradford assay

Stripping the SC with adhesive tape was claimed long ago to remove a single sheet of horny layer[155]. However to assess the robustness of the Bradford assay, one tape strip was removed from various sites of the volar forearm to determine the reproducibility of the tape stripping method and consequently, the reproducibility of the Bradford assay.

The left volar forearm of a participant was wiped with Kimwipes™ saturated with EtOH to remove any sebaceous lipids or contaminants on the skin surface. Several tape stripping areas were clearly marked with permanent marker prior to ensure that each surface strip was removed from a different site. The participant extended the left forearm over a bench with the wrist facing upwards.

Polyester adhesive tapes of 2.4 x 1.5 cm (3M Product No 8440, ATA Distributors, Victoria, Australia) were applied onto each marked skin site. The tape was then removed from the SC surface and immersed in 1.5 mL of NaOH in a 6-well plate. 1 tape strip was removed from 6 separate sites on the forearm to test reproducibility. 10 tape strips were removed from a single site to observe whether the amount of SC removed decreased with an increase of tape strip number as have previously been reported[86,138]. This was performed in triplicate. The amount of SC protein removed by each tape strip was quantified using the Bradford assay as described in Section 2.4.2.1.

2.4.2.3 Effect of applied preparations on SC protein quantification of Bradford assay

As will become evident in Chapter 5, it was of interest to assess what affect the presence of vehicles containing – PG, PEG 200 and OS may have on the penetration and lateral diffusion of IBU. Therefore, it was necessary to ensure that the presence of these excipients when applied with IBU did not interfere with the Bradford assay in quantifying amount of protein.

Individual blank adhesive concentric tapes were immersed in 6-well plates containing 650 µL NaOH spiked with 50 µL of a BSA standard ranging from 100 – 600 µg/mL and 1.8 µL of ethanolic test vehicle of either (i) IBU 5% w/v (ii) IBU 5% w/v + PG 10% w/v + PEG 200 10% w/v (iii) IBU 5% w/v + OS 5% w/v (iv) IBU 5% w/v + PG 10% w/v + PEG 200 10% w/v + OS or (v) neat EtOH (control). The Bradford assay was performed as detailed in Section 2.4.2.1 to determine whether different vehicles affect the absorbance measurements SC proteins removed with adhesive tapes by comparing the absorbance of each formulation (with and without the addition of excipients) relative to the absorbance of adhesive tapes spiked with a known quantity of BSA.

2.4.3 Equilibrium saturated solubility of CAF and HC

Following development of the experimental design, it came to light that the need to split the concentric tapes in half following removal from the SC may render the amount of drug extracted from the adhesive tapes to be below the analytical detection limit. Hence, the maximum concentration of CAF, HC and IBU to be used was amended by the SCERH, Monash University, Victoria, Australia (Project #CF08/1125 – 2008000555) to match that of usual topical applications and to allow for a higher drug dose to be applied in 1.8 µL. However, concentrations of 3% w/v and 2.5% w/v of CAF and HC, respectively reached saturated solubility in both EtOH and IPA while 5% w/v of IBU in EtOH and IPA was below its saturated solubility. Therefore, a saturated solubility study of CAF and HC was

carried out in EtOH and IPA to determine the appropriate concentration of CAF and HC to test in the subsequent studies.

Excess CAF and HC were added separately to 5 mL of IPA or 5 mL of EtOH, respectively, in individual 10 mL centrifuge tubes. The suspensions were vortexed for 2 mins every hour for up to 3 hrs. At 3 hrs, the suspensions were centrifuged at 22°C in the Eppendorf Centrifuge 5804 R (Eppendorf, New South Wales, Australia) at a speed of 3500 rpm (1301.5 x *g*) for 15 mins. A 100 µL aliquot of supernatant was collected and diluted to 1 mL with EtOH or IPA. 25 µL of this diluted sample was then diluted further with 10 mL of the appropriate extraction solvent (MeOH:H_2O (50:50) for CAF and MeOH:H_2O (60:40) for HC). The concentration of CAF or HC in each diluted sample was then quantified using the HPLC analytical method described in Section 2.4.5. The saturated solubility of CAF and HC in each solvent was studied in triplicate and determined daily over 7 days.

2.4.4 Validation of CAF, HC and IBU extraction from adhesive tape

As the primary purpose of this research was to apply the tape stripping method to follow the distribution of drug across the SC, it was essential to adequately extract and quantify the amount of drug adhered to the applied tape strip. Therefore, the following paragraph outlines the validation of the extraction methods for CAF, HC and IBU from adhesive tapes.

Adhesive tape (3M Product #8440, ATA Distributors, Victoria, Australia) measuring 1.5 x 1.5 cm was laid flat with the adhesive side up and placed on a work bench. Ethanolic solutions of a low, medium and high concentration of CAF (equating to 0.01, 0.7 and 1.5 mg/mL), HC (0.0035, 0.07 and 0.35 mg/mL) and IBU (0.05, 2 and 5 mg/mL) were applied onto individual tape samples at a dose of 25 µL using a pipette. The adhesive tapes were left at ambient conditions on the workbench for 30 mins. This exposure time was

arbitrarily chosen to allow the EtOH to evaporate. At the end of the 30 min period, each tape strip was placed separately into 20 mL vials and submerged in 10 mL of the appropriate extraction solvent. The extraction solvent for CAF, HC and IBU consisted of MeOH:H_2O (50:50), MeOH:H_2O (60:40) and MeOH:HCl (90:10), respectively. The vials were securely sealed with a Teflon-lined lid and the enclosed tape strip and solvent was vortexed for 10 secs. The IBU samples were then placed in a shaking water bath (Ratek, Victoria, Australia) set to 80 strokes per min at 60°C for 30 mins. At the end of the 30 mins, the vials were removed from the water bath and left on the bench under ambient conditions overnight before being transferred to a sonicator (Unisonics, New South Wales, Australia) for 1 hr after which the mass of IBU removed by each adhesive tape was quantified by HPLC as described in Section 2.4.5. The CAF and HC samples were immediately placed in a sonicator for 1 hr after which the mass of CAF and HC removed by each adhesive tape was quantified using HPLC as detailed in Section 2.4.5. The recovery of CAF, HC and IBU from these tape samples were compared to that of the spiked solvent standards prepared in the same way as mentioned above, with the exception that the standards were applied directly into 20 mL vials.

2.4.5 Quantification of CAF, HC and IBU

2.4.5.1 Analytical methods

Unknown concentrations of CAF, HC and IBU extracted from adhesive tapes were determine using HPLC with a Water Symmetry® C_{18} column (5 µm particle size, 3.9 x 150 mm internal diameter) equipped with a Waters Symmetry® C_{18} guard column (3.9 x 20 mm) (Waters, Massachusetts, USA). The HPLC system used for CAF and HC quantification consisted of a Waters 2690 separations module, Waters 2487 dual wavelength absorbance detector, Waters 610 pump, Waters 600E system controller and a Waters 712 autosampler. The HPLC system used for IBU quantification consisted of a Prominence CBM-20A communications module equipped with a Prominence LC-20AD separations module, Shimadzu RU-10AXL fluorescence detector, Prominence SIL-20A HT autosampler and a Prominence CTO-20A column oven (Shimadzu Scientific Instruments Pty Ltd, Victoria, Australia). The chromatographic conditions for CAF, HC and IBU are outlined in Table 2.1.

Table 2.1 Chromatographic conditions used to quantify the amount of CAF, HC and IBU extracted from adhesive tape.

	Drug		
Condition	**CAF**	**HC**	**IBU**
Mobile phase	Solvent A: 0.057% phosphoric acid Solvent B: MeOH	Solvent A: MeOH Solvent B: H_2O	Solvent A: 5 mM phosphate buffer (pH 2.4[#]) Solvent B: MeOH
Flow	Gradient*	Gradient**	Gradient***
Flow rate	1 mL/min	1 mL/min	1 mL/min
Injection volume	50 µL	100 µL	10 µL
UV detection	273 nm	245 nm	-
Fluorescence detection	-	-	Excitation λ: 263 nm Emission λ: 288 nm
Column temperature	Ambient	Ambient	40°C
Run time	20 mins	17 mins	21 mins
Retention time	~6.6 mins	~3.9 mins	~8.7 mins
Concentration range	0.01 – 1.75 µg/mL	8.75 – 875 ng/mL	0.125 – 12.5 µg/mL

([#]) pH adjusted with 1M phosphoric acid.

(*) 0 – 9 mins, 22% B; 9 – 10 mins, 22 – 37% B; 10 – 17 mins, 37% B; 17 – 18 mins, 37 – 22% B; 18 – 20 mins, 22% B.

(**) 0 – 5 mins, 40% B; 5 – 6 mins, 40 – 10% B; 6 – 13 mins, 10% B; 13 – 14 mins, 10 – 40% B; 14 – 17 mins, 40% B.

(***) 0 – 10.5 mins, 75% B; 10.5 – 11.5 mins, 75 – 85% B; 11.5 – 17 mins, 85% B; 17 – 18 mins, 85 – 75% B; 18 – 21 mins, 75% B.

2.4.5.2 Preparation of CAF standard solutions

A stock standard solution of CAF was prepared at a concentration of 8 mg/mL in EtOH in a glass volumetric flask. Standard solutions were prepared by appropriate dilution of the stock solution in EtOH to give concentrations ranging from 0.0045 – 0.7 mg/mL. These standards were further diluted in MeOH:H_2O (50:50) to give final standard concentration ranging from 0.01 – 1.75 µg/mL.

2.4.5.3 Preparation of HC standard solutions

A stock standard solution of HC was prepared at a concentration of 20 mg/mL in EtOH in a glass volumetric flask. Standard solutions were prepared by appropriate dilution of the stock solution in EtOH to give concentrations ranging from 0.0015 – 2 mg/mL. These standards were further diluted in MeOH:H_2O (60:40) to give final standard concentrations ranging from 8.75 – 875 ng/mL.

2.4.5.4 Preparation of IBU standard solutions

A stock standard solution of IBU was prepared at a concentration of 5 mg/mL in EtOH in a glass volumetric flask. Standard solutions were prepared by appropriate dilution of the stock solution in EtOH to give concentrations ranging from 0.02 – 3 mg/mL. These standards were further diluted in MeOH:HCl (90:10) to give final standard concentrations ranging from 0.125 – 12.5 µg/mL.

2.4.5.5 HPLC assay validation

The method of linear regression (weighted by a factor of 1/x) which achieved a correlation coefficient (r^2) of at least 0.99 was used to determine the relationship between the mass of drug extracted and the peak area of drugs detected by HPLC quantification. Inter-day and intra-day precision and accuracy was determined from 5 replicates of

standard solutions at low, medium and high standard concentrations of CAF, HC and IBU on 3 different days.

2.4.6 Statistical analysis

Statistical analysis was carried out using SPSS Statistics 19 for Windows. In order to compare significant differences between the recovery of CAF, HC and IBU over time, a one-way repeated measures analysis of variance (ANOVA) test was used. A t-test was applied to determine whether significant differences existed between the solubility of CAF and HC in EtOH and IPA. A probability of $p<0.05$ was deemed significant. All results are presented as mean ± SEM, unless otherwise stated.

2.5 RESULTS

2.5.1 HPLC assay validation

2.5.1.1 CAF HPLC assay validation

A calibration curve relating CAF standards to peak area is shown in Figure 2.6. The intra-day precision and accuracy and inter-day precision for a low, mid and high concentration of CAF standards are reported in Table 2.2.

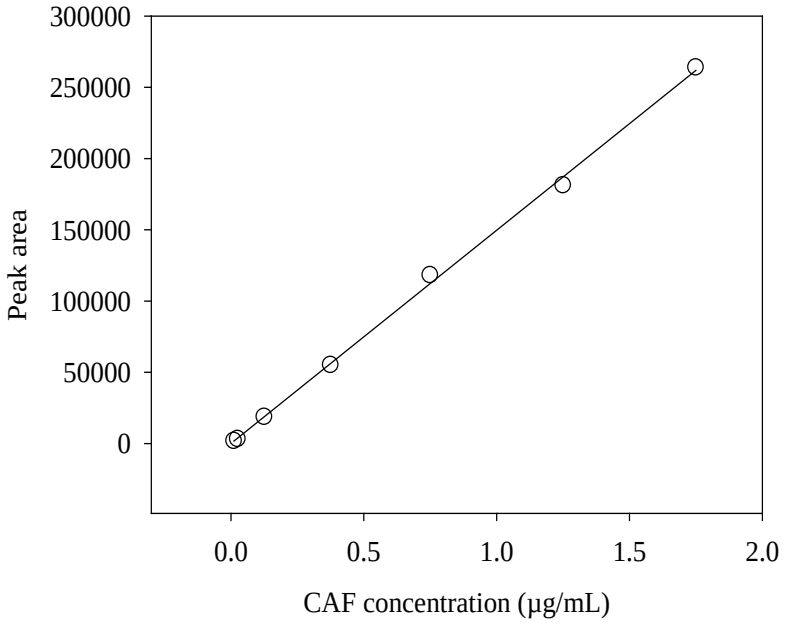


Figure 2.6 Calibration curve relating CAF concentration to peak area obtained by HPLC. Linear regression line was fitted with weighting (1/x) to give the following equation: Peak area = 146731.7 x CAF concentration – 105.8 (r^2=0.9923).

Table 2.2 Intra-day and inter-day precision and accuracy for HPLC assay validation for CAF quantification.

Concentration (µg/mL)	**Precision (%CV)**		**Accuracy (%) Mean ± SD (n=5)**
	Intra-day	**Inter-day**	
0.01	2.6	0.7	108.0 ± 2.6
0.375	4.2	4.3	105.7 ± 4.4
1.75	4.9	2.0	101.3 ± 4.9

2.5.1.2 HC HPLC assay validation

A calibration curve relating HC standards to peak area is shown in Figure 2.7. The intra-day precision and accuracy and inter-day precision for a low, mid and high concentration of HC standards are reported in Table 2.3.

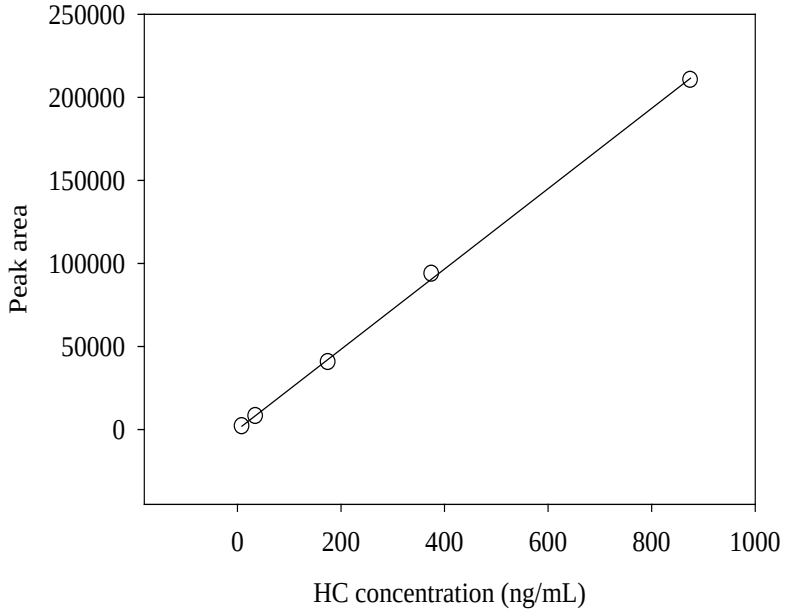


Figure 2.7 Calibration curve relating HC concentration to peak area obtained by HPLC. Linear regression line was fitted with weighting (1/x) to give the following equation: Peak area = 2436.7 x HC concentration – 772.8, (r^2=0.9995).

Table 2.3 Intra-day and inter-day precision and accuracy for HPLC assay validation for HC quantification.

Concentration (ng/mL)	**Precision (%CV)**		**Accuracy (%) Mean ± SD (n=5)**
	Intra-day	**Inter-day**	
8.75	7.6	4.8	107.4 ± 6.0
175	1.9	2.0	103.3 ± 1.9
875	2.0	0.4	101.3 ± 2.0

2.5.1.3 IBU HPLC assay validation

A calibration curve calibration curve relating IBU standards to peak area is shown in Figure 2.8. The intra-day precision and accuracy and inter-day precision for a low, mid and high concentration of IBU standards are reported in Table 2.4.

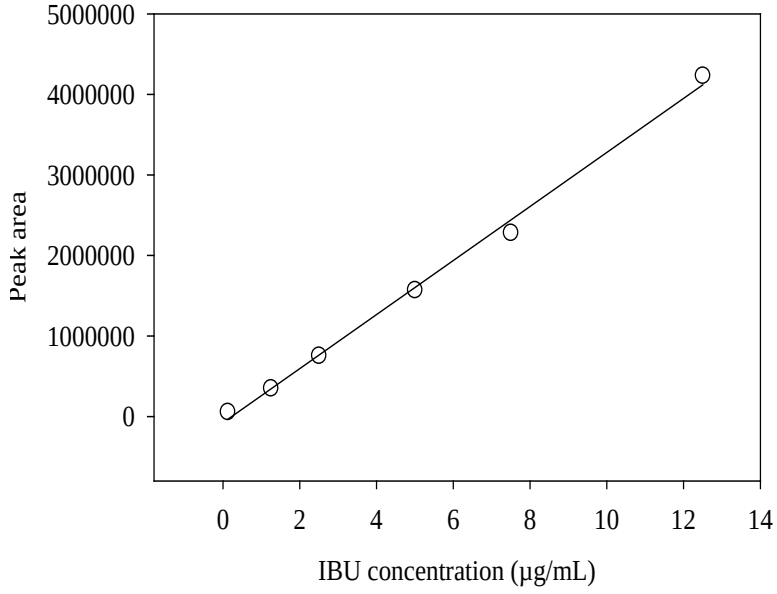


Figure 2.8 Calibration curve relating IBU concentration to peak area obtained by HPLC. Linear regression line was fitted with weighting (1/x) to give the following equation: Peak area = 31851.2 x IBU concentration + 6084.3, (r^2=0.9958).

Table 2.4 Intra-day and inter-day precision and accuracy for HPLC assay validation for IBU quantification.

Concentration (µg/mL)	**Precision (%CV)**		**Accuracy (%) Mean ± SD (n=5)**
	Intra-day	**Inter-day**	
0.125	4.4	1.5	96.4 ± 4.9
5	2.6	1.8	101.4 ± 2.7
12.5	2.9	4.2	100.7 ± 2.9

2.5.2 Validation of CAF, HC and IBU extraction from adhesive tape

The recovery and accuracy for the extraction of CAF, HC and IBU from adhesive tape are presented in Table 2.5 below. The percentage recovery of CAF, HC and IBU was within 100 ± 10% for all low, medium and high concentrations. The extraction methods used ensured high recovery of drugs as required by the guidelines (100 ± 10% for Organisation for Economic Co-operation and Development (OECD)[156,157] and 100 ± 15% for Scientific Committee on Cosmetic Products (SCCP)[158]). The accuracy of the extraction procedure was within the limits of <20% at all concentrations.

Table 2.5 Recovery and accuracy of CAF, HC and IBU from adhesive tape (mean ± SEM, n=5).

	Amount	**Spiked amount**	**Recovery (%)**	**Accuracy (%)**
CAF	0.25 µg	0.2 ± 0.0	103.4 ± 3.1	89.0 ± 2.7
	17.5 µg	17.4 ± 0.3	95.6 ± 2.6	93.5 ± 2.8
	37.5 µg	37.5 ± 0.4	94.0 ± 3.6	93.9 ± 3.6
HC	1.125 ng	1.3 ± 0.0	99.4 ± 1.7	120.0 ± 2.2
	17.5 ng	18.2 ± 0.1	94.0 ± 2.4	97.8 ± 2.5
	87.5 ng	88.1 ± 0.7	95.5 ± 3.1	96.1 ± 3.1
IBU	1.25 ng	1.4 ± 0.0	96.7 ± 1.2	107.8 ± 1.3
	50 ng	48.8 ± 0.8	91.5 ± 0.4	89.3 ± 0.4
	125 ng	121.7 ± 1.6	94.4 ± 0.7	92.0 ± 0.7

2.5.3 Validation of Bradford assay for quantifying SC protein

2.5.3.1 Bradford assay

A calibration curve relating BSA concentration to absorbance at 595 nm is shown in Figure 2.9. The intra-day precision and accuracy and inter-day precision for low, mid and high concentrations of the BSA standards are reported in Table 2.6.

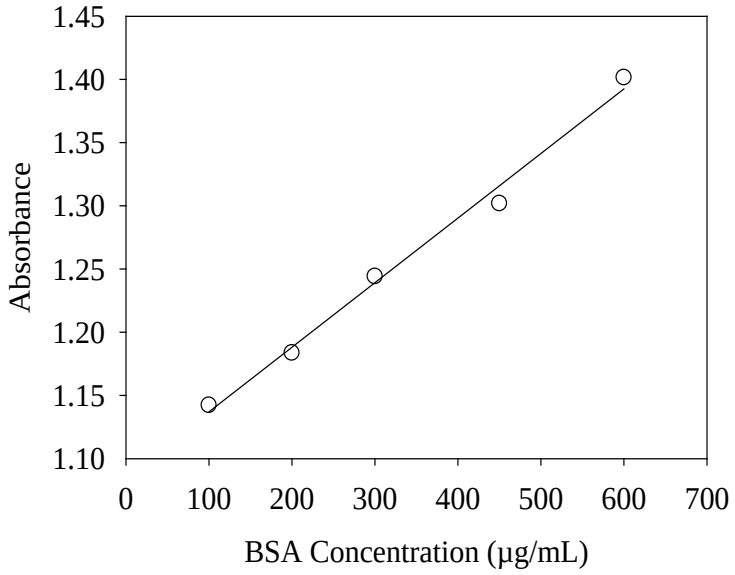


Figure 2.9 Calibration curve relating BSA concentration on tape strips to absorbance at 595 nm obtained by colourimetric protein assay. Linear regression line was fitted with weighting (1/x) to give the following equation: Peak area = 0.0005 x BSA concentration + 1.1, (r^2=0.9915).

Table 2.6 Intra-day and inter-day precision and accuracy of the Bradford assay for SC protein quantification.

Concentration (µg/mL)	Precision (%CV)		Accuracy (%) Mean ± SD (n=5)
	Intra-day	Inter-day	
100	0.5	2.4	103.0 ± 10.0
300	3.0	2.6	100.7 ± 11.1
600	3.3	2.2	97.2 ± 15.0

2.5.3.2 Reproducibility of SC protein extraction using Bradford assay

It has been claimed that a single horny layer of the SC is removed with each tape strip. Therefore, as an additional method to examine the feasibility of the Bradford assay to appropriately quantify SC protein, various sites of the uppermost layer of the SC were tape stripped to assess whether the claims put forward by Pinkus[155] and Van der Molen et al.[138] can be verified.

Figure 2.10 depicts the mass of protein removed from a single tape strip of the SC at 6 different sites of the volar forearm. An average mass of 94.38 ± 2.34 µg of SC protein was removed by a single adhesive tape strip. The good reproducibility of mass of SC protein removed from the SC surface is in agreement with claims by Pinkus[155] and Van Der Molen et al.[138] and further demonstrates that the Bradford assay is an appropriate method to quantify SC protein.

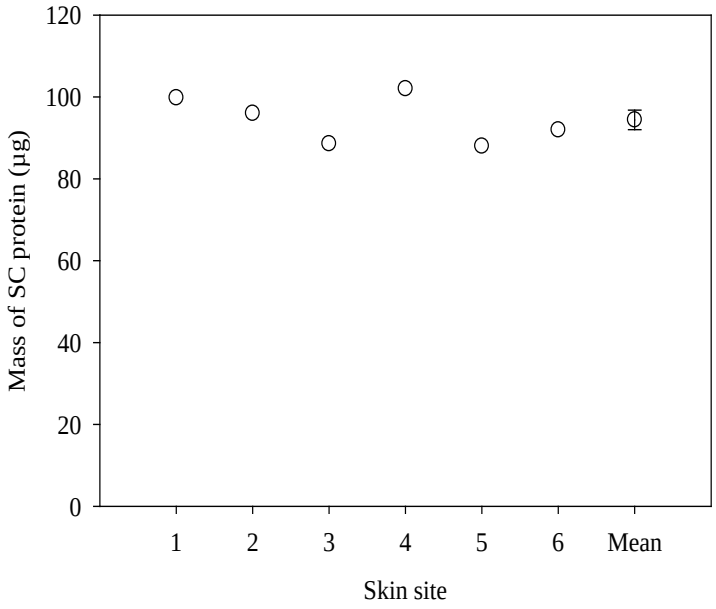


Figure 2.10 Mass of SC protein removed from a single tape strip from various sites of the volar forearm.

It is also generally accepted that the amount of SC removed via tape stripping decreases with an increase in depth. The mass of protein removed from 10 tape strips of the SC at 3 individual sites is shown in Figure 2.11. A larger weight of SC protein is removed from the first 3 tape strips. Less SC protein is removed with an increase in tape strip number and this is a reflection of the more compact and tightly bound corneocytes in the deeper layers of the SC as previously reported by Van der Molen et al.[138] and Pinkus[155].

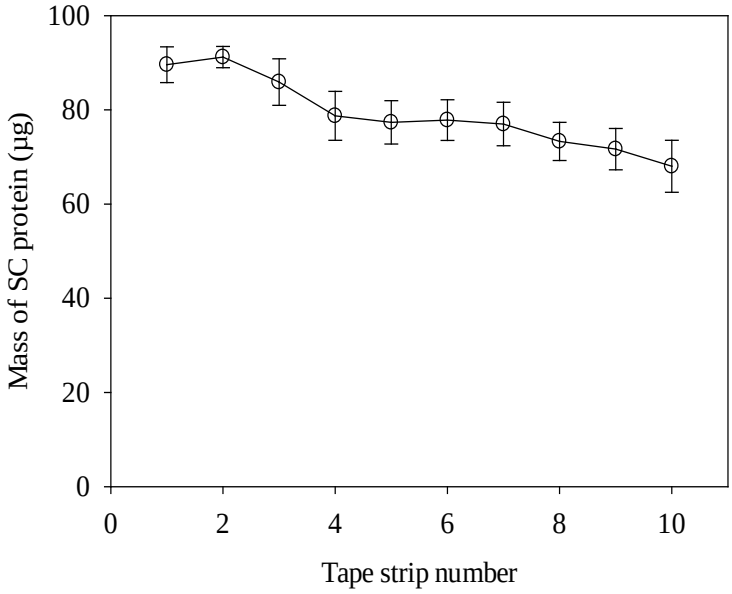


Figure 2.11 Trend of SC protein removal across SC with increase in tape strip number (mean ± SEM, n=3).

2.5.3.3 Effect of applied preparations on SC protein quantification of Bradford assay

As will become evident in Chapter 5, various excipients were applied onto the SC to evaluate their effect on the lateral diffusion and penetration of IBU. Therefore, it was important to make certain that the presence of various excipients on the SC surface did not interfere with the quantification of SC protein using the Bradford assay.

Table 2.7 shows the absorbance of protein standards in the presence of different excipients. Standards applied into wells with adhesive tape but no addition of any excipients act as the control. There was no significant difference in the absorbance reading of wells with excipients compared to that of the control. Thus, the presence of various excipients used in this research did not interfere with protein analysis.

Table 2.7 Absorbance of BSA standards in the presence of excipients at 595 nm (mean ± SD, n=5).

BSA Concentration (µg/mL)	Adhesive tape (control)	IBU	IBU + PG + PEG 200	IBU + OS	IBU + PG + PEG 200 + OS
100	1.1 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	0.9 ± 0.1	1.1 ± 0.1
200	1.2 ± 0.1	1.2 ± 0.0	1.2 ± 0.0	1.1 ± 0.1	1.1 ± 0.0
300	1.2 ± 0.0	1.2 ± 0.1	1.3 ± 0.1	1.1 ± 0.0	1.1 ± 0.0
450	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	1.2 ± 0.2
600	1.5 ± 0.2	1.4 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	1.2 ± 0.1

2.5.4 Preliminary *in vivo* study design

2.5.4.1 Identification of appropriate tape stripping time points and total number of tape strips to collect

One of the main objectives of this research project was to assess any changes in the lateral diffusion and penetration of various marker compounds across the SC with time using tape stripping. Therefore, it was necessary to carry out a preliminary *in vivo* tape stripping study to determine the appropriate time points at which to tape strip the forearm of volunteers following application of CAF, HC and IBU. This was required to observe any changes in drug distribution and recovery over time. Furthermore, it was essential to verify whether collecting 10 tape strips was sufficient to follow the penetration behaviour of permeants within the SC as was shown by Simonsen et al.[154].

The distribution profiles of CAF, HC and IBU across the SC after finite doses were applied onto the skin *in vivo* at 0.5, 2, 4 and 8 hrs are shown in Figure 2.12 – 2.14. The largest amount of CAF and HC are removed in the first tape strip across all exposure times,

after which, the mass of drug removed is considerably reduced and only very minute amounts are quantified from tape strip 4 onwards. The mass of IBU recovered, however, is gradually reduced with each subsequent tape strip and approaches zero at the tenth tape strip at 0.5, 2, 4 and 8 hrs. It should be noted that the amount of CAF, HC or IBU removed with each tape strip was not normalized against the amount of SC protein also removed by the corresponding tape strip. This was due to the fact that the main objective of this preliminary study was to determine the appropriate tape stripping time points and whether collecting 10 tape strips was sufficient assess the penetration behaviour of drugs across the outermost region of the SC.

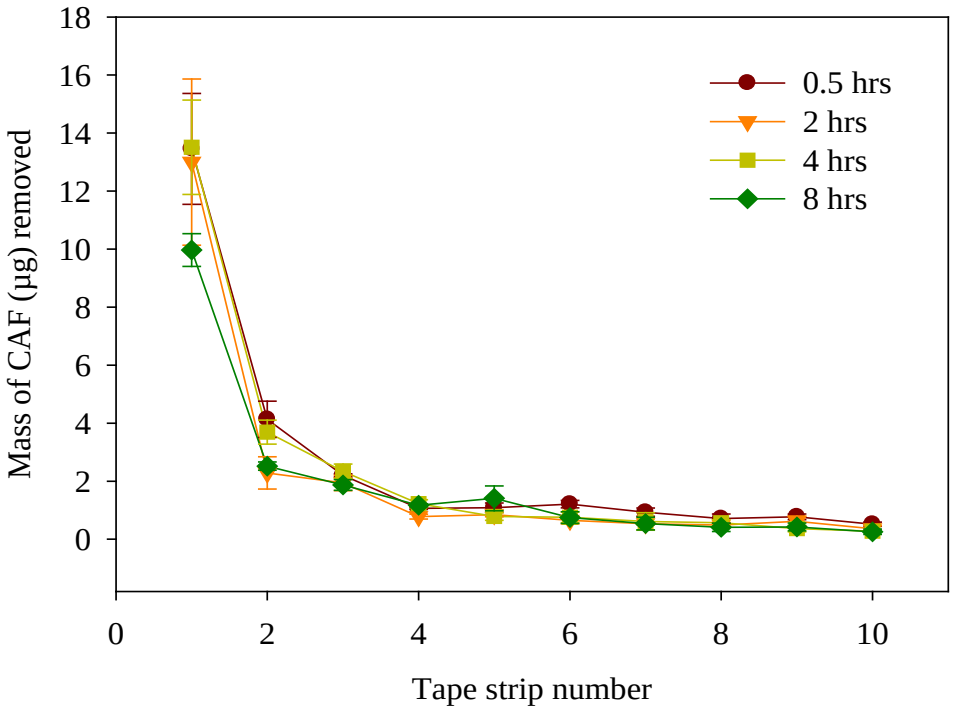


Figure 2.12 Distribution profile of CAF across the SC *in vivo* (mean ± SEM, n=3).

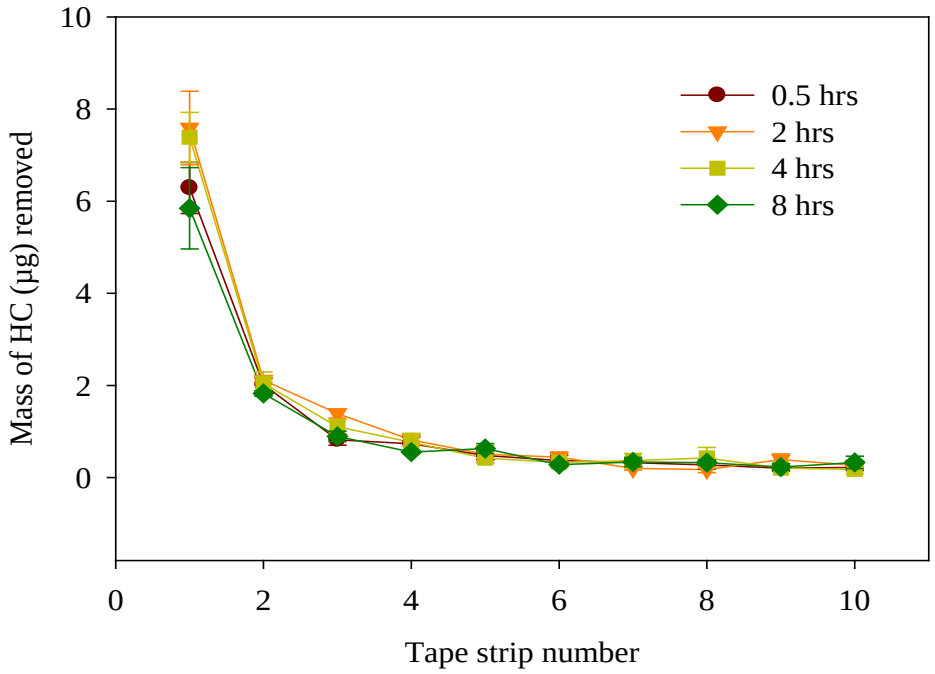


Figure 2.13 Distribution profile of HC across the SC *in vivo* (mean ± SEM, n=3).

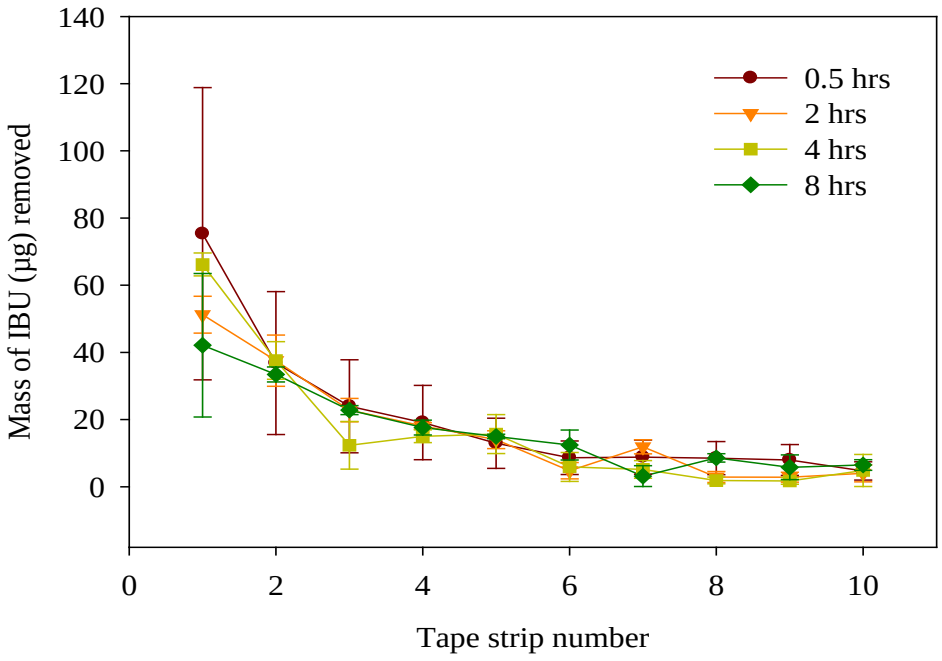


Figure 2.14 Distribution profile of IBU across the SC *in vivo* (mean ± SEM, n=3).

Table 2.8 shows the cumulative total percentage of CAF, HC and IBU recovered from the 10 tape strips at 0.5, 2, 4, and 8 hrs after application, relative to the applied dose. There was no significant difference in the percentage of drug recovered with prolonged exposure. CAF and HC showed similar recovery percentages at each tape stripping time point. However, the percentage of IBU recovered at 2, 4 and 8 hrs was significantly lower than that recovered of CAF and HC.

Table 2.8 Percentage of CAF, HC and IBU recovered from 10 tape strips after 0.5, 2, 4, and 8 hr exposure relative to the applied dose (mean ± SEM, n=3).

Time (hrs)	% CAF recovered	% HC recovered	% IBU recovered
0.5	69.6 ± 3.2	65.2 ± 5.3	55.6 ± 32.1
2	57.3 ± 10.7	77.2 ± 5.8	38.9 ± 22.5*
4	64.4 ± 6.6	73.5 ± 3.0	15.6 ± 9.0*
8	51.5 ± 3.0	62.5 ± 3.6	29.9 ± 17.2*

(*) indicates that the percentage of IBU recovered is significantly different from the percentage recovered of CAF and HC at the corresponding exposure time.

2.5.4.2 Determination of the extent of solvent spread on skin surface using MDTS application

Figure 2.15 shows the depot of blue dye left on the skin following application with a MDTS. The diameter of the MDTS mask is 8 mm and covers an area of 50 mm^2. Lateral spreading on the skin surface is obvious immediately after topical application as the blue dye (in IPA) spreads to a diameter of ~20 mm to cover an area of 314 mm^2.

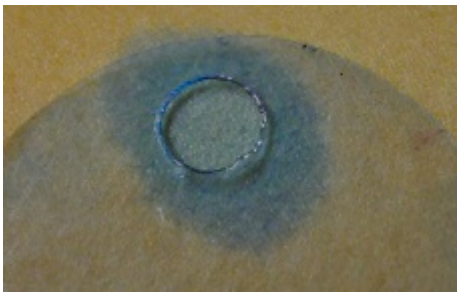
(a)  (b) 

Figure 2.15 Application of blue dye onto skin using MDTS to measure extent of lateral spreading (a) initial vision inspection of blue depot on skin ~30 secs after application and (b) plastic MDTS mask placed over depot demonstrating lateral spread.

This measured diameter denotes that the diameter of the concentric tapes to be designed must be at least equal to or larger than 20 mm to be able to account for all drug loss via lateral diffusion on the SC surface.

Schicksnus et al.[54] evaluated the process of lateral diffusion by extracting drug from concentric excised skin samples. A similar approach was adopted in this research. However, rather than using excised epidermis, *in vivo* tape stripping studies was performed on the forearm of human volunteers. Adhesive tapes that were specially designed and cut into concentric rings as shown in Figure 2.16 was used to determine spread. The innermost segment (section 1), is the initial application site and has a diameter of 8 mm. Section 2 forms the first ring around the application area and has a diameter of 16 mm. The third ring has a diameter of 24 mm and the largest ring covers a diameter of 32 mm. This larger diameter than that measured using the blue food dye ensured that any drug lost from the application area by lateral diffusion would be accounted for. Each circular section can be separated along the perforated lines into individual segments, allowing for the amount of drug and SC removed from each tape section to be quantified individually. In addition, the depth of drug penetration and the extent of lateral diffusion within the SC layers could also be determined concurrently by cutting the concentric tape into two equal halves as shown

in Figure 2.16. The backing material and adhesive of the concentric tapes are identical to the adhesive tapes used in the *in vivo* preliminary studies (3M, Product 8440, ATA Distributors, Victoria, Australia).

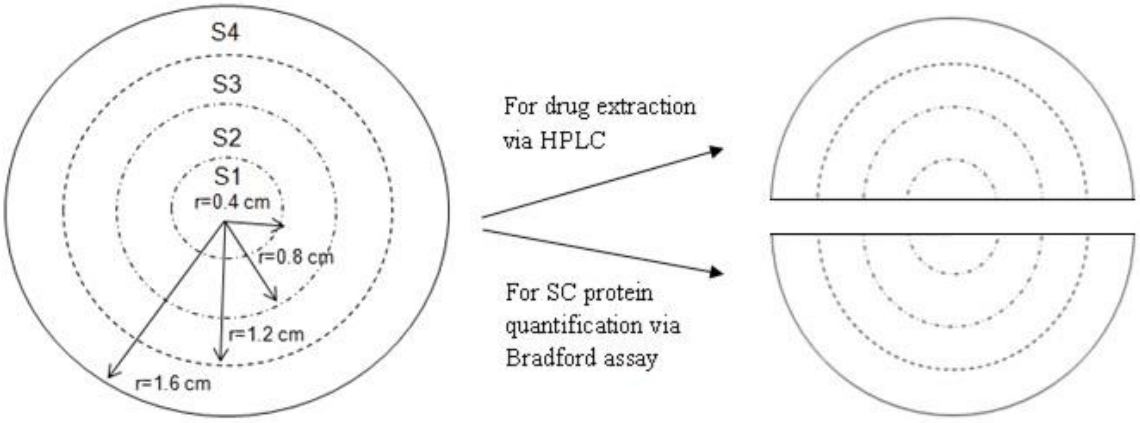


Figure 2.16 Illustration of specially designed concentric adhesive tape used to study the lateral diffusion and penetration of substances across the SC.

2.5.4.3 Equilibrium saturated solubility of CAF and HC

As the concentric adhesive tapes will be split in half following removal from the SC, it was probable that the amount of CAF and HC that may be removed by adhesive tape will be below the limit of detection if the maximum concentration of CAF, HC and IBU stipulated by the first revision of the SCERH, Monash University, Victoria, Australia (Project #CF08/1125 – 2008000555) was followed. Therefore, the maximum concentration of CAF, HC and IBU to be studied was permitted to be increased to match that of usual topical applications. However, a saturated solubility study was required to determine the concentration of CAF and HC to be used in the subsequent studies. The saturated solubility of IBU in IPA and EtOH was not tested as the mass of IBU added to 5 mL of both solvents exceeded the maximum concentration of IBU permitted in this research as stipulated by the SCERH, Monash University, Victoria, Australia.

The saturated solubility of CAF and HC in IPA and EtOH was achieved within 168 hrs and the solubility values shown in Table 2.9. Both CAF and HC showed noticeably higher solubility in EtOH than IPA. Therefore, EtOH was selected as the solvent of choice for the remainder of studies in this research as the higher drug solubility allowed for a higher concentration of CAF and HC to be dosed onto participants and consequently ensuring detectable amounts of drug in extraction samples.

Table 2.9 Saturated solubility of CAF and HC in EtOH and IPA over 196 hrs (mean ± SEM, n=3).

	CAF saturated solubility		**HC saturated solubility**	
	mg/mL	**% w/v**	**mg/mL**	**% w/v**
EtOH	9.0 ± 0.2	0.9 ± 0.0	25.0 ± 0.6	2.5 ± 0.1
IPA	2.7 ± 0.1	0.2 ± 0.0	8.5 ± 0.3	0.9 ± 0.0

2.5.4.4 Reproducibility of drug release through MDTS mask

The amount of CAF deposited onto filter paper following dosing through a mask enclosed in a MDTS unit is summarised in Table 2.10. Theoretically, 14.4 µg of CAF should be deposited per 0.5 cm^2. However, poor accuracy is shown as the MDTS delivers more than the theoretical weight of drug through the mask with each spray. In addition, the reproducibility of the MDTS to deliver a consistent weight of drug through the mask is poor, with a %CV of 38.36%. This suggested that the MDTS may not produce a uniform film of drug with each spray and for this reason, the spray pattern of the MDTS was determined, as shown in the Section 2.5.4.5.

Table 2.10 Mass of CAF delivered through mask of MDTS.

Sample number	Amount of CAF deposited onto filter paper (µg)
1	19.5
2	19.7
3	17.6
4	40.8
5	22.1
6	45.4
7	21.6
8	19.9
9	21.9
10	23.1
Mean ± SEM	25.2 ± 3.1
%CV	38.4

2.5.4.5 Determining spray pattern of MDTS deposit

Due to the poor precision of mass of CAF delivered through the mask a MDTS as outlined in Section 2.5.4.4, it was thought that the MDTS may not deliver a uniform layer of drug onto the skin. Therefore, it was essential to determine the spray pattern of a MDTS. Figure 2.17 shows the MDTS spray pattern of three separate deposits of CAF onto filter paper. Drug ejected from a MDTS unit is not evenly distributed, but rather deposits drug in a shape of a doughnut whereby 10 – 20 µg of CAF is found every 0.5 cm^2. The centre of the application area receives the lowest amount of drug. A dense area of drug is found in the lower half of the application area, which may be the result of gravity pull.

These contour plots demonstrate that although the MDTS spray pattern is reproducible, each spray does not deliver a uniform layer of drug. The amount of drug that

is dosed per spray is consistent and accurate (%CV < 5%), as shown in Table 2.11. However, the variability in the amount of drug sprayed per unit area and per repeated spray, is responsible for the inconsistency of drug deposited through a MDTS mask as shown in Section 2.5.4.4.

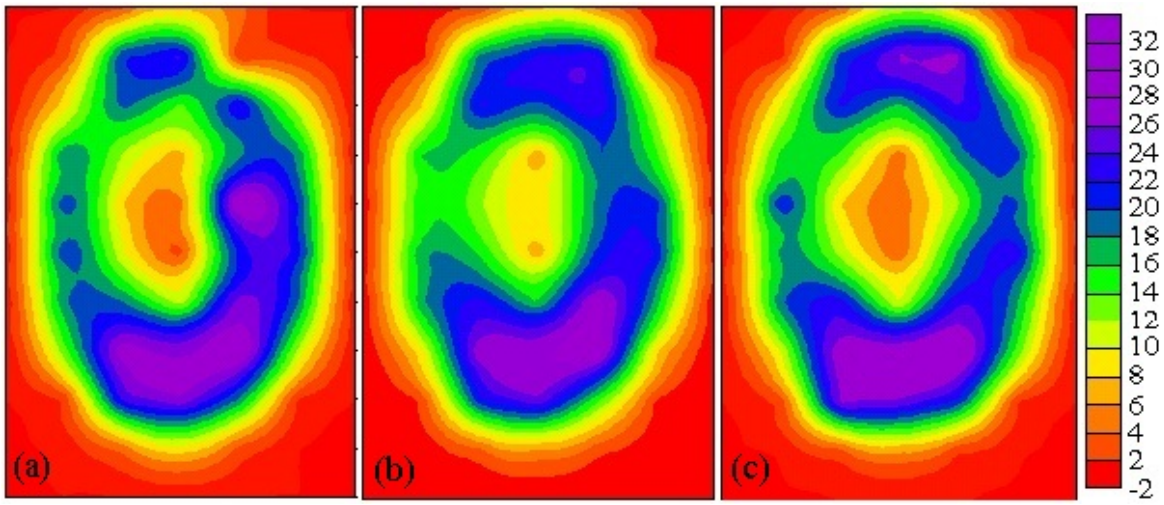


Figure 2.17 MDTS spray pattern of CAF (a) sample 1 (b) sample 2 (c) sample 3. Legend values are expressed as µg.

Table 2.11 Recovery of CAF deposit onto filter paper using MDTS.

	Sample 1	**Sample 2**	**Sample 3**
Total amount deposited (µg)	653.5	680.4	650.1
% Recovered*	90.8	94.5	90.3

(*) Percentage recovery of CAF relative to the theoretical application over 20 cm^2 (720 µg).

2.5.4.6 Dosing accuracy and precision of HPLC manual injection syringe

Due to the inconsistency of the MDTS to deliver a reproducible amount of drug through the MDTS mask per spray, a HPLC manual injection syringe was instead chosen as the drug application method in subsequent studies as this method is able to replicate the passive, non-occlusive enhancement design of the MDTS. The accuracy and precision of dosing 1.8 µL of ethanolic solutions of CAF, HC and IBU with a HPLC manual injection syringe is presented in Table 2.12. The syringe delivers a precise amount of drug as demonstrated by the %CV of less than 10% for each drug. It is also accurate as the measured amount of each drug deviated less than 5% from the expected dose. Therefore, a HPLC manual injection syringe was considered an appropriate substitute application method for the MDTS in the following studies.

Table 2.12 Accuracy and precision of dosing with HPLC manual injection syringe.

Parameter	CAF	HC	IBU
Theoretical dose (µg)	14.4	36.0	90.0
Dosed amount (µg) Mean ± SEM (n=8)	13.5 ± 0.4	37.5 ± 0.7	90.9 ± 2.9
% Accuracy Mean ± SEM (n=8)	93.8 ± 2.6	104.1 ± 2.0	101.0 ± 3.2
%CV	8.9	5.6	8.9

2.5.4.7 Uniformity of solvent spread following application with HPLC manual injection syringe

As previously outlined in Section 2.5.4.2, concentric adhesive tapes removed from the SC in the subsequent studies in this research were split into two equal halves. This allowed for the concurrent determination of the amount of drug removed per tape strip as well as the amount of SC protein removed by the same tape strip. Therefore, it was

necessary to establish that the manual injection syringe delivered a uniform amount of drug so that equal amounts of drug was removed from each half of the concentric tape strip.

The recovery of drug extracted from both halves of filter paper is shown in Table 2.13. Drug spread immediately after application with a HPLC manual injection syringe appeared to be symmetrical as the percentage of drug recovered from each half of filter paper was measured to be 50 ± 2% of the applied dose. Thus, this study shows that taking one half of a concentric ring following removal from the SC for drug quantification is a valid approach for quantifying total drug content removed in the whole ring.

Table 2.13 Evenness of drug spread following dosing with HPLC manual injection syringe (mean ± SEM, n=10)

	CAF		**HC**		**IBU**	
	Side A	Side B	Side A	Side B	Side A	Side B
% Recovered	49.3 ± 1.6	50.7 ± 1.6	51.4 ± 1.9	48.6 ± 1.9	49.9 ± 1.7	50.1 ± 1.7
%CV	10.2	9.9	11.9	12.6	10.7	10.6

2.6 DISCUSSION

Tape striping the skin with adhesive tape is a widely used method to examine the localization and distribution of substances within the SC[136,137,159,160]. It is a simple and non-invasive technique that is able to remove sequential SC layers *in vivo* and *in vitro* by repeated application of adhesive tapes[155]. For this reason, tape stripping was the first method of choice to use in the study of lateral diffusion and penetration of drugs in this project. However, it was necessary to design a novel shape of adhesive tape that enables both penetration and lateral spreading of the applied penetrant to be monitored.

The adhesive tapes developed were adapted from Schicksnus et al.[54] use of concentric skin samples to evaluate lateral spreading *in vitro*. Each adhesive tape is circular in shape and can easily be split into several concentric ring segments along the perforated divides as shown in Figure 2.16. The dimensions of the concentric rings were selected based on the visual inspection and measurement of the extent of spread of coloured food dye dispersed in IPA when applied onto the skin surface using a modified MDTS unit.

The layout of the adhesive tape is composed of a centre circle enclosed by three larger rings. The innermost circle is placed directly over the application area on test subjects and following tape removal, the concentric rings of 8, 16, 24 and 32 mm in diameter are separated. The amount of drug substance removed by the corresponding ring segment was then extracted and quantified by HPLC, and normalized to SC protein count. With repeated tape strips, this design allowed for the extent of lateral diffusion with depth of SC to be visualised three-dimensionally.

The correlation of the amount of drug extracted to it's location within the SC is required in assessing drug distribution within the SC[93,151,161]. The amount of SC removed by tape stripping is often not linearly proportional to the number of tapes removed[155],

therefore, the amount of SC protein removed by each tape segment is also of relevance in establishing the concentration profile, both laterally and perpendicular to the application area. The Bradford assay has been shown to be an effective method to quantify the mass of SC removed per tape strip. Blank adhesive tapes of identical areas were used to remove the uppermost layer of the SC from various sites of the forearm of one subject. The mass of SC protein extracted was consistent and reproducible as shown in Figure 2.10. Furthermore, the Bradford assay showed that the mass of SC protein removed decreases with an increase in tape strips layers, as shown in Figure 2.11. This is in agreement with the knowledge that corneocytes are more compact and tightly bound with increasing depth due to the presence of fully functional corneodesmosins that are responsible for corneocyte-to-corneocyte cohesion within the SC[162,163].

The effect of different vehicles on the efficacy of the Bradford assay was also evaluated and no significant differences in absorbance of protein standards in the presence of difference vehicles were observed. Undertaking these studies provides confidence in the use of the Bradford assay to quantify the mass of SC protein removed per adhesive tape in subsequent studies.

In order to obtain a concentration profile of drug within the SC, an adequate number of tape strips needs to be collected. The total number of tape strips required to remove most of the horny layer have varied in literature, from as few as 10 tape strips[154], 30 tape strips[155] or as many as 60 to 100 tape strips[164]. However, as this project is primarily focused on the possible routes of drug loss, removal of the entire SC with tape stripping was not necessary. Rather, the behaviour of drug at the uppermost region of the SC was of main interest. Figures 2.12 – 2.14 show the distribution profile of CAF, HC and IBU, respectively, across the SC after 10 tape strips. By the 10th tape strip, CAF and HC residues were not detected in the extraction solvent, while IBU appeared to have penetrated deeper

into the SC, as also shown by its lower recovery in Table 2.8. However, it is not known whether its lower recovery was due to lateral diffusion beyond the application site. Moreover, as the bioavailability of topically applied drugs is usually no more than 5 – 10%[1], it is unlikely that the unaccounted for drug will have penetrated much deeper. Thus, collecting 10 tape strips was sufficient to obtain a concentration profile of drugs within the SC.

The cumulative percentage of CAF, HC and IBU recovered from 10 tape strips of the forearm of a participant at 0.5, 2, 4 and 8 hrs are displayed in Table 2.8. At least 30% of drugs have either spread beyond the tape stripping area, been rubbed/washed off, metabolized or lost via desquamation only 0.5 hrs after application. Yet, the percentage of drug recovered does not change with time. Therefore, for the purpose of this project, tape stripping was performed at 3 mins, 3 hrs and 6 hrs after application in the subsequent studies (Chapter 3, 4 and 5). Tape stripping at 3 mins after application ensures complete evaporation of solvent and allowed for the lateral and depth distribution profile of drugs soon after application to be obtained. As the amount of drug recovered in this preliminary *in vivo* study did not appear to change between 0.5 and 8 hrs, tape stripping was to be collected at 3 and 6 hrs after application in the subsequent studies (Chapter 3, 4 and 5) to determine whether the lateral distribution of drug changes with time. Additionally, tape striping at 6 hrs rather than 8 hrs after application will allow for minimal inconvenience to volunteers.

The concentration of CAF, HC and IBU used in the preliminary *in vivo* studies to determine the distribution of drug across the SC was 1, 0.5 and 1%, respectively, in IPA. These concentrations were originally used as specified in SCERH, Monash University, Victoria, Australia (Project #CF08/1125 – 2008000555). However, following a conclusive study design whereby the removed concentric tape strips would be required to be split into

two halves to allow parallel measurements of the amount of drug and SC protein removed, it was ideal for the concentration of drugs to be increased to ensure detectable amounts of drug in samples. Thus, a saturated solubility study of CAF and HC in EtOH and IPA was carried out and results shown in Table 2.9. Both CAF and HC have noticeably higher solubility in EtOH than IPA. This is probably due to the formation of dipole-dipole interactions between EtOH and CAF or HC, which does not occur with IPA. Therefore, EtOH was chosen as the solvent of choice in these studies to allow for a concentration as close to that of usual topical concentrations to be applied for clinical relevance[165-167]. The maximum concentration of drugs to be applied as approved by SCREH, Monash University, Victoria, Australia (Project #CF08/1125 – 2008000555) were amended to allow for CAF 0.8% w/v and HC 2% w/v to be applied, which is equivalent to 80 – 85% of the calculated saturated solubility concentration. The concentration of IBU formulated was 5% w/v. The degree of saturation of IBU in EtOH, however, was not determined as there were no issues in dissolving IBU in either solvent to match that of topical products.

The feasibility of inserting a mask into the cone of a MDTS unit to deliver a smaller volume of drug to a defined area on the skin surface was assessed. Unfortunately, the non-uniform spray pattern of the MDTS as shown in Figure 2.17 resulted in inconsistent amounts of drug dosed onto the skin through the mask with each spray, as shown in Table 2.10. Therefore, the use of a HPLC manual injection syringe was selected as the alternative application method. This method is still able to replicate the passive, non-occlusive enhancement design of the MDTS and has been demonstrated to be very accurate and precise in its dosing volume, as seen in Table 2.12. The lateral diffusion of solvent away from the application site following dosing with the syringe is symmetrical. This will ensure that the amount of drug quantified in each half of the concentric adhesive tape will be

identical to the other, thus allowing for the adhesive tape to be evenly split after removal from the skin to allow for concurrent measurements of drug and SC removed.

2.7 CONCLUSION

The use of the tape stripping technique to study the distribution of drug across the SC was assessed. A new design of the adhesive tape layout was developed which allows for a three-dimensional analysis of the competitive pathway of penetration and lateral diffusion following drug application. The experimental design allows for the amount of drug removed be each tape strip with respect to the mass of SC protein removed by the same tape strip to be acquired simultaneously. While it would be ideal to use the MDTS (with mask) as the application method, the variability of spray pattern limits its use in these studies. A HPLC manual injection syringe will be used as an alternative method as it proved accurate and precise in delivering 1.8 µL aliquots of ethanolic solutions of CAF 0.8% w/v, HC 2% w/v and IBU 5% w/v onto test sites. 10 tape strips will be collected at 3 mins, 3 hrs and 6 hrs after application when assessing the impact of formulation excipients on the *in vivo* disposition of drugs. This will allow for the understanding of how the lateral distance and depth of drug from the application site changes with increased exposure.

CHAPTER 3

ASSESSING THE LATERAL DIFFUSION AND PENETRATION OF CAF, HC AND IBU *IN VIVO*

3 ASSESSING THE LATERAL DIFFUSION AND PENETRATION OF CAF, HC AND IBU *IN VIVO*

3.1 INTRODUCTION

Penetration of drugs through the skin is limited by the barrier properties of the SC[10,168]. For a topically applied drug to become bioavailable, it is subjected to a tortuous route of penetration around the dead corneocyte cells and through the interceulluar lipid domains, which are comprised mainly of ceramides, cholesterol and cholesterol derivatives[167]. The SC would therefore present a considerable barrier to the absorption of hydrophilic substances, while the viable epidermis, which is hydrophilic in nature, would act as a rate limiting step in the permeation of highly lipophilic substances[97]. Thus, for optimal percutaneous absorption, a diffusant should be reasonably soluble in both hydrophilic and hydrophobic conditions. In addition, the ease at which a drug permeates through the SC will also be influenced by other physicochemical properties, such as the drug's molecular weight, melting point, aqueous solubility and number of hydrogen bonds acceptors and donors[60]. The ideal properties of drug candidates for transdermal drug delivery are listed in Table 3.1.

Table 3.1 Physicochemical considerations of permeants for TDD.

Physicochemical property	Limits	Consideration
Molecular weight	<500 Da	The smaller the molecule, the faster its diffusion between cells[57].
LogP	1 – 3	Compounds with low logP exhibit lower partitioning into the skin lipids and those with high logP also show little permeability due to the inability to partition out of the SC. The optimum logP to achieve adequate partitioning is between 1 and 3[58].
Melting point	<200°C	Permeants with high melting points have shown low solubility and therefore decreased flux through skin[169,170].
H-polar head groups	<5	Presence of polar head groups on a molecule may impair its transepidermal transport since electrical charges on the diffusing molecule may interact with those along the diffusional pathway[59].

The physicochemical properties listed in Table 3.1 not only affect the penetration of a diffusing substance through the SC, but may also affect its lateral spreading behaviour on the skin. A drug that may not readily partition into and through the SC may be more inclined to spread radially across the upper most regions of the SC, while a drug that readily penetrates through the SC may retain the majority of drug within close radial proximity to the application site and therefore have a lower tendency to undergo lateral diffusion. Although to date, the effect of physicochemical properties on the penetration behaviour of drugs is widely understood, little attention has been given to their effect on the lateral diffusion behaviour across the skin. Lateral diffusion is a possible route of drug loss and thus, in order to gain a better understanding of the fate of unabsorbed drugs, the influence of the physicochemical properties on the simultaneous process of penetration in the upper layers of the SC and lateral spreading is required.

CAF, HC and IBU are three model compounds that have different physicochemical properties and therefore, are expected to exhibit different degrees of penetration and spread. Various studies have reported that the logP and molecular weight of a permeant are significant predicators of drug penetration across the skin[171-173]. CAF is very hydrophilic with a logP of -0.07[115]; IBU is deemed to have an ideal logP of 3.51[118] for skin penetration while HC is neither too hydrophilic nor lipophilic with a logP of 1.43[110]. CAF and IBU have comparable molecular weights of 194.19 g/mol and 206.28 g/mol, respectively, while HC has a larger a molecular weight of 362.46 g/mol which may impact the spreading and penetration behaviour of HC.

While it has been demonstrated that these three model compounds can penetrate the SC[174-179], literature relating to the competing processes of lateral diffusion and penetration of CAF, HC and IBU are scarce. It has been reported that the rate of lateral diffusion is considerably different from, and much faster (by up to nine orders of magnitude) than that of transbilayer transport over equivalent distances[180,181]. Following topical application, permeant can undergo (i) lateral diffusion on the SC surface, (ii) lateral diffusion within the SC bilayers, as well as (iii) diffusion through the lipid channels in between corneocytes (as shown in Figure 3.1). Thus, the process of lateral diffusion may play an important role in the mechanism of solute transport through the SC.

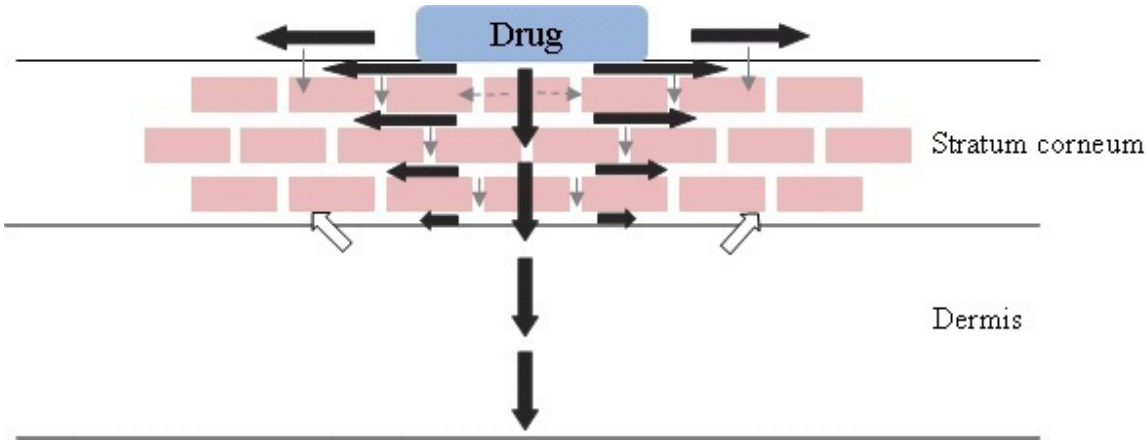


Figure 3.1 Routes of drug penetration and lateral diffusion across the SC.

3.2 OBJECTIVES

The few studies that have documented the process of lateral spread have been performed using *in vitro* methods[182,183]. While such studies are insightful, they may pose significant differences to *in vivo* conditions due to the absence of blood flow, metabolism and desquamation. Furthermore, the distance to which a penetrant spreads on the surface of the SC and within the SC bilayers following topical application has not been elucidated. Nonetheless, it is anticipated that a lipophilic compound will traverse the SC barrier with more ease and therefore have less lateral movement within the SC. A hydrophilic compound, on the other hand, is expected to have greater difficulty permeating through the SC and thus more prone to lateral diffusion across the skin surface. A better understanding of the effect of physicochemical properties on lateral diffusion may allow for the development of methods to prevent lateral spread and in turn, enable greater local activity and/or absorption.

Therefore, the purpose of the studies in this chapter was to:

- Determine the *in vivo* lateral diffusion and penetration behaviour of three model compounds – CAF, HC and IBU across the SC of humans by utilising the perforated concentric adhesive tapes (designed in Chapter 2).

3.3 MATERIALS

All chemicals used in the following studies were obtained from the same manufacturers and distributors as detailed in Section 2.3.

3.4 METHODS

3.4.1 Determination of lateral spreading and penetration of CAF, HC and IBU across human skin *in vivo*

3.4.1.1 Participants

Eight healthy volunteers (4 males and 4 females) provided written consent to participate in the study which was approved by the SCERH, Monash University, Victoria, Australia (Project #CF08/1125 – 2008000555). The participants were aged between 24 to 37 yrs of age and had no history of skin disease. Each participant was asked to refrain from applying any topical medicaments to their left and right flexor forearms at least 48 hrs prior to an experiment.

3.4.1.2 In vivo study design

The volar forearm of each participant was wiped with Kimwipes™ saturated with EtOH to remove any sebaceous lipids or contaminant on the skin surface. Circles of 0.5 cm^2 were marked on the flattest plane of the left and right volar forearms to clearly outline the application area. An area of 0.5 cm^2 was chosen to replicate the area of the innermost circle of the concentric adhesive tapes used in these studies. The sampling site was also

clearly marked with permanent marker prior to dosing the skin to ensure each tape strip removed SC from the same site in a manner that did not interfere with the lateral spreading. The participant extended both forearms over a work bench with the volar forearm facing upwards. A 1.8 µL aliquot of an ethanolic solution containing either 0.8% w/v of CAF, 2% w/v of HC, or 5% w/v of IBU was dosed onto individual marked areas of the skin. The forearm remained flat and rested on the bench for approximately 30 secs to allow for solvent evaporation. The solutions were allowed to remain in contact with the skin for 3 mins, 3 hrs and 6 hrs. During these sampling time points, the solutions were left unoccluded and the participant was required to wear a short-sleeved shirt or have their sleeves rolled up to avoid loss of drug by rubbing off onto clothing. The participants were allowed to resume their daily activities, although were advised to avoid activities that would cause perspiration as it may alter the spreading and/or penetration behaviour of the drugs applied.

3.4.1.3 Tape stripping procedure

The CAF, HC and IBU distribution profile across the SC at 3 mins, 3 hrs and 6 hrs was determined by sequential removal of the outer skin layers by tape-stripping with the specially designed concentric adhesive tapes (3M, Product No 8440CONRING32, ATA Distributors, Victoria, Australia), as outlined in Section 2.5.4.2 (see Figure 3.2). At the sampling time points, the concentric tapes were transferred to the marked skin area with tweezers, ensuring that section 1 of the concentric tapes was placed directly over the pre-marked application area. A stainless steel slab weighing 1200 g and measuring 2.8 x 6 x 9 cm was placed over the tape for 3 secs to ensure even pressure was applied across the tape. The tape was then removed from the SC surface and split into two equal halves. This enabled one half of the tape to be used to quantify the amount of extracted drug while the other half was used to quantify the amount of removed SC protein (as was validated in

Chapter 2). To quantify the amount of drug removed, the perforated ring segments from one half of the divided tape was further split into its individual sections, as shown in Figure 3.2 and submerged in individual vials containing 10 mL of the corresponding extraction solvent as detailed in Section 2.4.4. The amount of drug extracted from each section of each tape strip was analysed using validated HPLC methods as described in Section 2.4.5. The amount of SC removed by the remaining half of the divided tape was also split into its individual sections and immersed into 6-well plates containing 700 µL of NaOH in each well. The Bradford assay was then carried out as outlined in Section 2.4.2.1. 10 sequential tape strips were removed at each corresponding sample area at each time point.

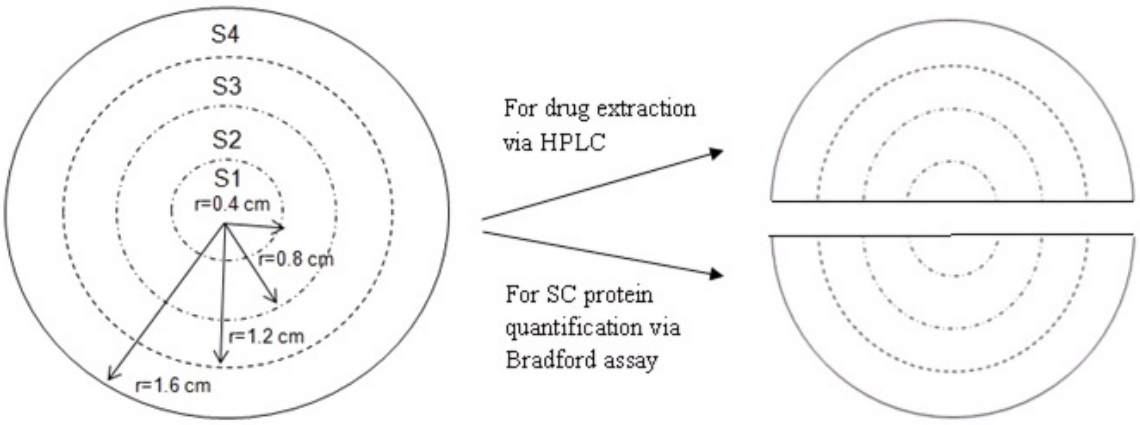


Figure 3.2 Schematic diagram of splitting of concentric tape for drug solvent extraction and SC protein quantification.

3.4.2 Data analysis

The amount of drug removed from each concentric tape section was normalised to the mass of SC protein removed by the same concentric tape section in order to eliminate the inter-subject variability in the total mass of SC protein removed per layer of tape strip. The normalised concentration of drug was plotted against the cumulative weight of SC protein per unit area. To allow for the easy visualisation of both lateral diffusion and

penetration across the SC, the normalised data was schematically shown on contour plots. The percentage recovery of CAF, HC and IBU at 3 mins, 3 hrs and 6 hrs relative to the applied dose was calculated in order to evaluate whether any difference in recovery with time may be a result of penetration into the skin. In the following sections, the distance of drug spread from the application site will be referenced as 'section 1', 'section 2', 'section 3' and 'section 4', which equates to distances of between 0 – 4 mm, 4 – 8 mm, 8 – 12 mm and 12 – 16 mm, respectively (as shown in Fig 3.2).

3.4.3 Statistical analysis

Using SPSS Statistics 19, a one-way repeated measures ANOVA test was applied to compare the percentage recovery of CAF, HC and IBU at each time point as well as to determine whether there was a significant difference in the percentage of each drug recovered per unit area in each concentric ring. A probability of $p<0.05$ was deemed significant. All CAF, HC and IBU recovery data are presented as mean ± SEM (n=8).

3.5 RESULTS

3.5.1 Lateral spreading and penetration of CAF, HC and IBU across human skin *in vivo*

Figure 3.3 displays the distance of spread of CAF, HC and IBU parallel to the skin surface against depth of penetration at 3 mins, 3 hrs and 6 hrs after application in the form of contour plots. A higher colour intensity indicates a higher drug concentration, according to the colour legend. However, it should be noted that given the differences in the maximum intensities for each drug (due to the different concentration of each drug dosed), absolute comparison in amount of drug that has spread or penetrated should not be made between CAF, HC and IBU.

At 3 mins after application, all marker compounds form a reservoir in the superficial layers of the skin as shown in Figure 3.3a, d and g. CAF forms a relatively flat reservoir that spreads over the upper skin layers, while HC forms a deep depot mainly concentrated within the boundaries of section 1 and IBU forms both a deeper and wider reservoir than that exhibited by CAF and HC. The recovery of CAF decreased over 6 hrs as demonstrated by the reduction in colour intensity of the contour plots shown in Figure 3.3a – c. The loss of CAF, however, is not reflected by further penetration into the underlying skin layers or increased lateral spread. Similar observations were detected for HC as seen in Figure 3.3d – f. On the contrary, the amount of IBU detected between 400 – 600 µg/area depth of the SC appeared to increase in section 3 between 3 hrs and 6 hrs, as shown in Figure 3.3h - i. However, it cannot be deduced whether this increase in IBU within the SC was due to lateral diffusion or penetration.

Upon closer inspection of Figure 3.3, it is also interesting to note that at sites directly beneath the application area, CAF, HC and IBU penetrated to differing depths of the SC. CAF could only be detected to a depth of approximately 600 µg/area (Figure 3.3a – c), while HC could be detected up to a depth of ~800 µg/area (Figure 3.3 d– f) and IBU penetrated the greatest distance to approximately 1000 µg/area (Figure 3.3g – i). These results may further attest the influence of drug lipophilicity on penetration as it was verified (in Section 3.5.2) that the type of drug applied does not affect the amount of SC protein removed by tape stripping.

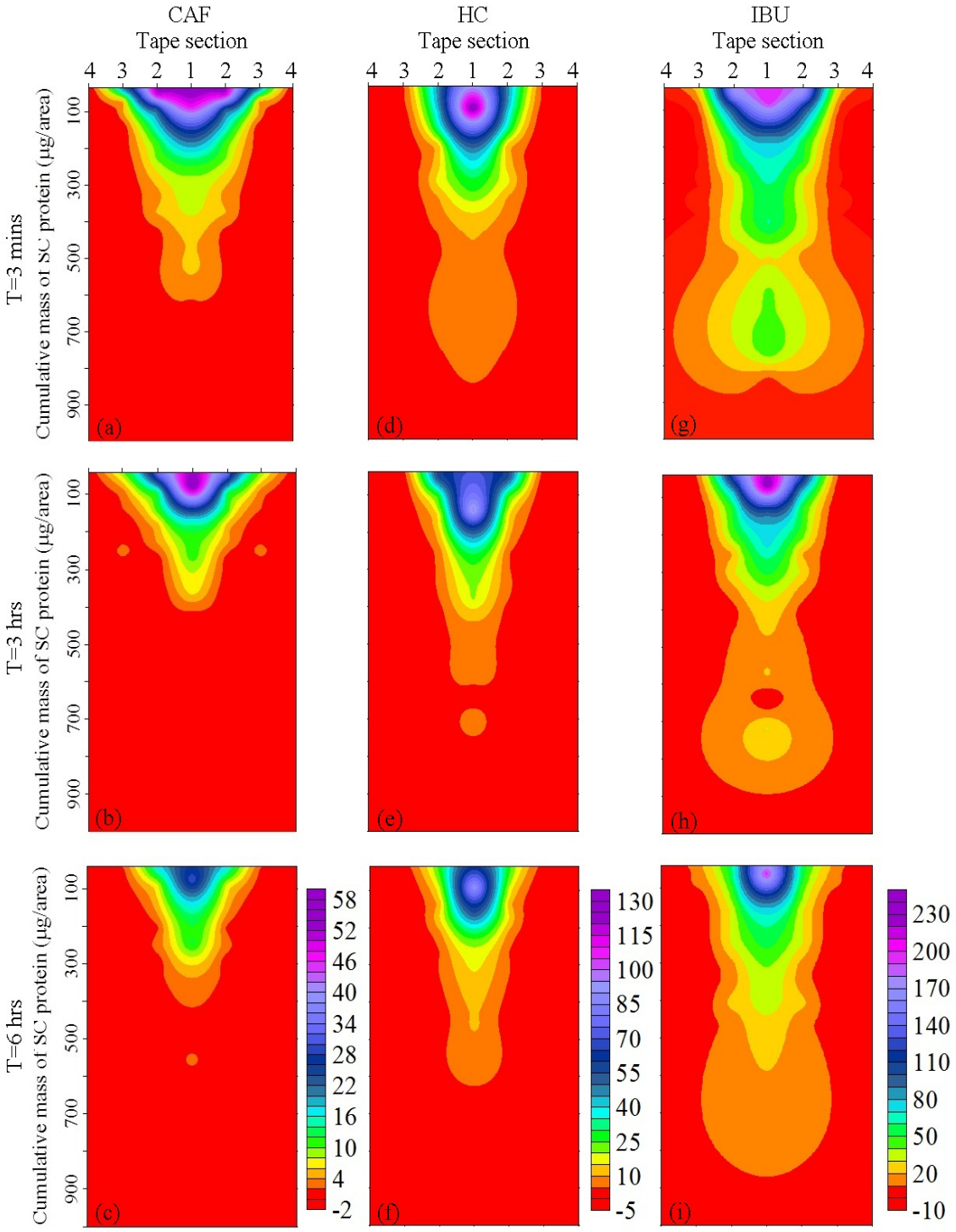


Figure 3.3 Contour plots displaying the extent of lateral spread and penetration of CAF, HC and IBU at 3 mins, 3 hrs and 6 hrs after application. Legend values are expressed as concentration of drug (ng/µg).

The lateral spreading behaviour of CAF, HC and IBU is further supported in Figures 3.4 – 3.6. At 3 mins, the normalized concentration of CAF (Figure 3.4) and IBU (Figure 3.5) in section 2 is similar to that detected in the application area (section 1), while the concentration of HC (Figure 3.6) in section 2 of each subsequent tape strip is less than half the concentration detected in section 1 of the corresponding tape strip. This suggests that CAF and IBU undergo a relatively rapid process of lateral diffusion and a higher tendency to spread than HC, further highlighting the tight reservoir effect of HC.

As can also be seen in Figures 3.4 – 3.6, all permeants show a common trend whereby the concentration of CAF, HC and IBU removed was highest in the first tape strip and progressively decreased with increased SC depth. Small amounts of CAF, HC and IBU can be detected in section 3 at 3 mins after application but negligible amounts detected in section 4.

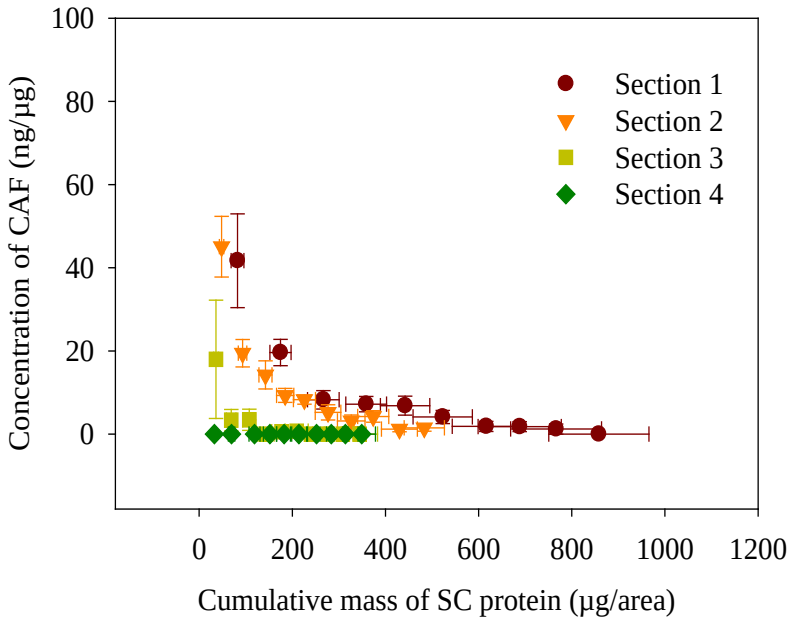


Figure 3.4 Distribution profile of CAF across the SC *in vivo* at 3 mins after application (mean ± SEM, n=8).

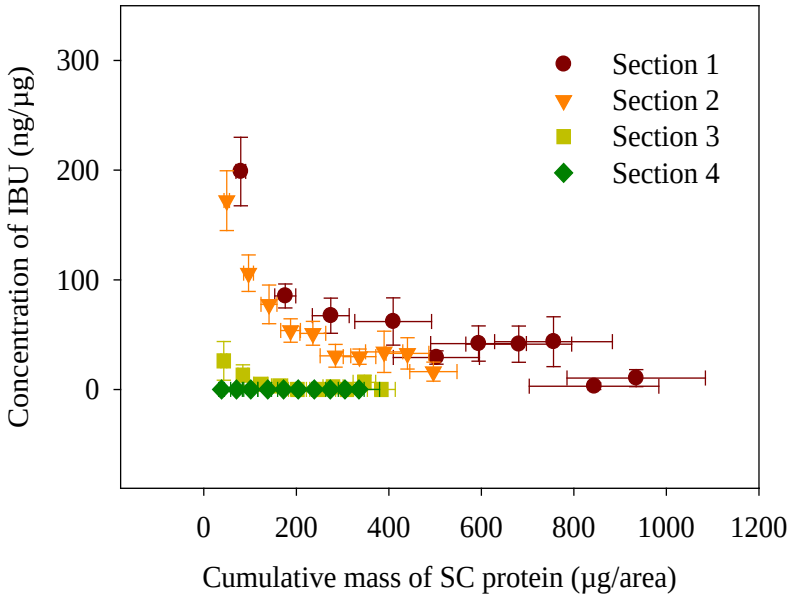


Figure 3.5 Distribution profile of IBU across the SC *in vivo* at 3 mins after application (mean ± SEM, n=8).

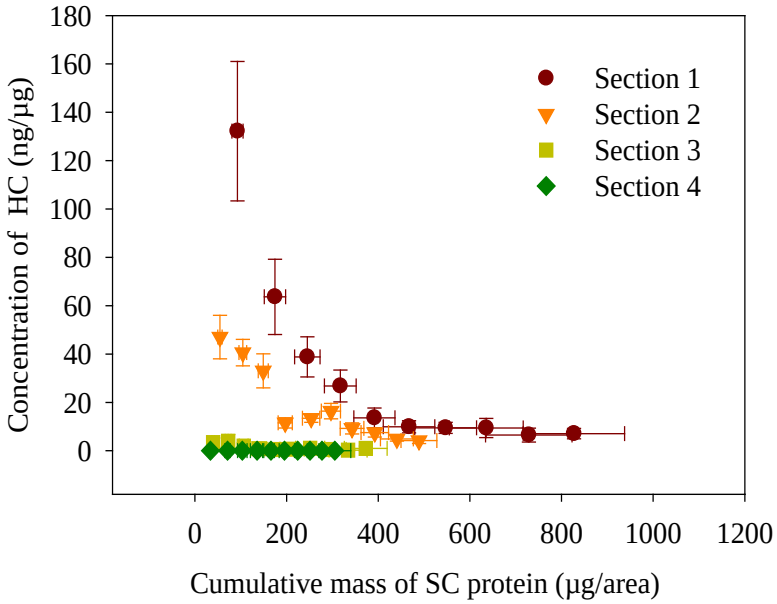
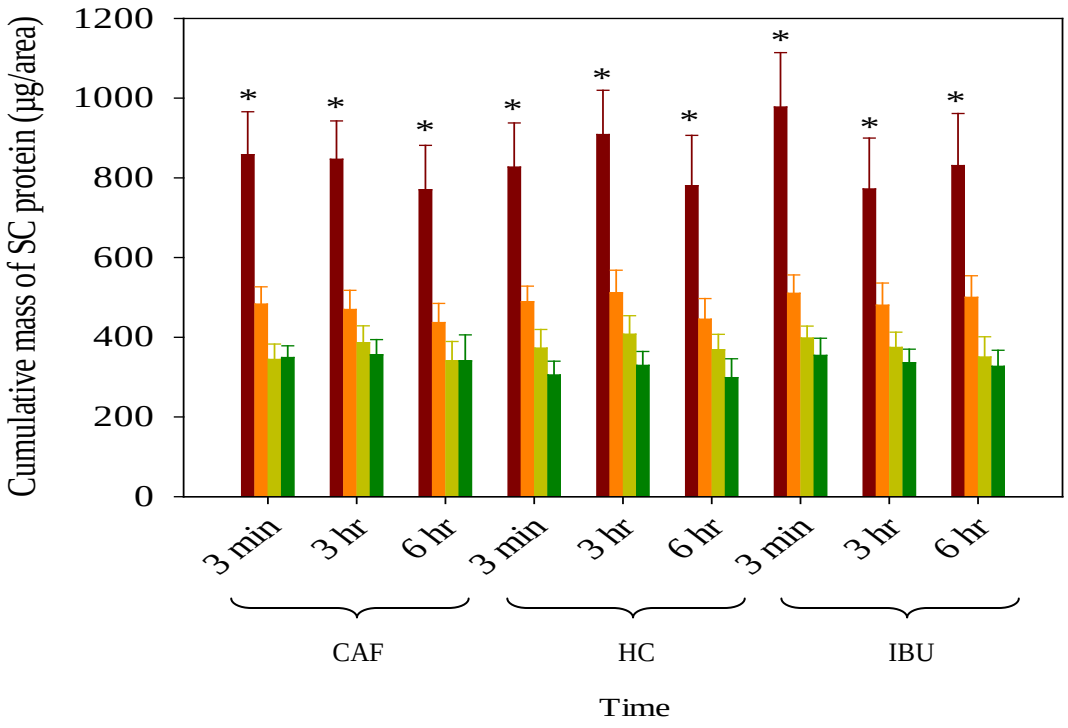


Figure 3.6 Distribution profile of HC across the SC *in vivo* at 3 mins after application (mean ± SEM, n=8).

3.5.2 Effect of drug on SC protein removal using adhesive tape

As the concentration of CAF, HC and IBU removed per tape strip was normalised against the mass of SC protein removed, it was of interest to assess whether the selection of drugs influenced the amount of SC protein that would be removed with adhesive tape and additionally, whether the duration of drug-to-skin contact affected SC protein removal. Figure 3.7 presents the cumulative mass of SC protein removed per unit area from skin sites under section 1 – 4 of the concentric adhesive tape over time when ethanolic solutions of CAF, HC and IBU were applied.

It is evident that neither drug type nor length of exposure time affects the reproducibility of SC protein removal with adhesive tape. However, the cumulative mass of SC protein removed from skin sites directly under section 1 is significantly higher than the cumulative amount removed at section 2, 3 and 4. The difference in cumulative mass of protein removed in section 2, 3 and 4 is not significant, despite a trend whereby the mass of cumulative protein removed decreases with increased distance from application site.



(*) denotes that the cumulative mass of SC protein removed from Section 1 is significantly different to the mass removed from sections 2, 3 and 4.

Figure 3.7 Cumulative mass of protein removed per unit area from combined 10 tape strips at site of CAF, HC and IBU application (section 1 (■), section 2 (■), section 3 (■) and section 4 (■)) at 3 mins, 3 hrs and 6 hrs after application (mean ± SEM, n=8).

3.5.3 Recovery of CAF, HC and IBU from adhesive tape *in vivo*

The recovery of CAF, HC and IBU extracted from all 4 sections of the concentric tapes of the combined 10 tape strips relatively to the applied dose was measured to assess whether any differences of recovery (between compounds) may be attributable to their different physicochemical properties. The percentage recovery of CAF, HC and IBU from the combined 10 tape strips at 3 mins, 3 hrs and 6 hrs relative to the applied dose is displayed in Table 3.2. What is evident from Table 3.2 is that while there is no significant difference between the recovery of CAF and HC between 3 hrs and 6 hrs, there is a

significant reduction of IBU recovered, suggesting a progressive depletion of IBU from the skin surface.

Table 3.2 % Recovery of CAF, HC and IBU at 3 mins, 3 hrs and 6 hrs after application relative to the applied dose (mean ± SEM, n=8).

	% Recovery		
Time	**CAF**	**HC**	**IBU**
3 mins	92.9 ± 5.6	83.0 ± 6.8	86.6 ± 5.6
3 hrs	64.5 ± 9.4*	66.9 ± 8.2*	66.2 ± 3.7*
6 hrs	53.2 ± 9.9*	64.2 ± 9.8*	47.7 ± 5.6**

(*) denotes significant difference to that recovered at 3 mins

(**) denotes significant difference to that recovered at 3 mins and 3 hrs

3.6 DISCUSSION

Tape stripping has commonly been used to follow the distribution profile of topical permeants across a membrane. However, the study of the penetration as well as lateral diffusion of permeants parallel to skin surface using concentric adhesive tapes to give a three-dimensional analysis of permeant behaviour upon skin contact is not well documented. Furthermore, though it is widely understood that drug lipophilicity greatly impacts skin penetration[60,172], its effect on the lateral diffusion of drug has not yet been elucidated. This study demonstrated that the logP of permeants also play a critical role in the distance which a drug spreads laterally away from its application site.

CAF, a hydrophilic drug with a logP of -0.07[115] formed a flat reservoir extending into section 3 of the SC surface at 3 mins after dosing (Figure 3.3a). Lateral diffusion appears to have occurred quite rapidly as the concentration of CAF found in section 2 is comparable to that detected in the same tape strips in section 1 as shown in Figure 3.4. The majority of drug resided close to the surface of the skin (within 600 µg/area) and depleted with time. On the other hand, HC, a relatively more lipophilic drug with a logP of 1.43[110] exhibited a lower tendency to spread and instead, the majority of drug was contained within the first two sections at 3 mins after application as seen in Figure 3.3d. Upon closer inspection of Figure 3.6, it is revealed that the concentration of HC in section 2 is half of that extracted in section 1 of the corresponding tape strips, thus giving rise to a deep and narrow drug depot on the skin surface. The depot formation of HC is in agreement with studies performed by Malkinson et al.[184], whereby HC penetrated the skin surface but did not readily permeate out of the SC. Instead, HC causes vasoconstriction of the local capillaries which slows down its removal rate from the SC and consequently instigates the formation of a drug reservoir or depot that releases drug over a period of days[185,186].

HC also penetrated a greater depth of SC compared to CAF – having reached 800 µg/area after 3 mins of application. Thus, it appears that a relationship exists between the initial contact area of hydrophilic solutes, such as HC, to skin and the depth of penetration as suggested by Karande and others[187]. Karande[187] assessed the dependence of skin permeability on reservoir size using an array of liquid and gel reservoirs with diameters ranging from 2 mm to 16 mm and observed that skin permeability significantly increased with a decrease in contact area. For small contact areas (<1 cm^2), the increased permeability was attributed to selective swelling of the skin which induced strains in the SC and thereby increased permeability. For large skin contact areas (>1 cm^2) however, swelling is unlikely to induce significant strains. This theory is supported by the behaviour of CAF and HC in the above results as the greater extent of lateral spreading of CAF gave rise to a higher contact area with skin, which in turn resulted in a wider and flatter drug reservoir at the skin surface and lower penetration. The higher spreadability of CAF on the SC surface may also be attributed to the formation of a layer of moisture on the skin surface[188]. As the presence of a moisture layer is aqueous in nature, this may facilitate the lateral movement of a water soluble compound such as CAF. HC, on the other hand, was concentrated within section 1 which covered a small contact area of 0.5 cm^2 and thus formed a deeper drug depot with drug penetrating further into the SC.

IBU has an ideal logP of 3.5[110] for skin penetration and is seen to form both a wide and deep drug reservoir soon after dosing, as seen in Figure 3.3g. IBU is detected to have spread into section 3 and reaches a SC depth of 1000 µg/area within 3 mins as seen in Figure 3.3g and Figure 3.5. However, it should be acknowledged that, as approximately 1000 µg/area of SC protein is removed from the combined 10 tape strips (regardless of drug applied or duration of contact), it is plausible to assume that some IBU may have penetrated beyond the SC depth which was sampled. The SC may be pictured as being

composed of protein bricks (keratin) in a continuous phase of lipophilic mortar, with paths through which hydrophilic and lipophilic substances can traverse[189]. As lipophilic drugs are soluble in the continuous lipid mortar, diffusion through the SC poses less resistance as they do so whilst staying in a favourable environment[190]. In addition, it is accepted that the increase in drug lipophilicity may increase their ability to disrupt the lipophilic domains of the SC and facilitate the diffusion of drug[110,191]. Thus, IBU is seen to penetrate deeper into the SC compared to CAF and HC.

The rapid rate of percutaneous penetration and lateral redistribution of lipophilic substances in *in vivo* animal models was also demonstrated by Simonsen et al.[154]. By collecting biopsies at distances of 1.3 and 2.9 cm from the application area, it was shown that a much higher concentration of butyl salicylate (logP of 4.63) was found at various distances from the application area in comparison to those of salicylic acid (logP 2.26). The higher degree of lateral distribution of butyl salicylate was explained by its lipophilicity which favoured partitioning into the SC and most probably lateral diffusion. However, it is unknown whether lateral diffusion occurred on the SC surface or within the SC bilayers as drug was extracted from skin biopsies with the entire SC intact.

Taking the above results into consideration, it can be speculated that diffusing in the lateral direction occurs faster than in the vertical direction of the SC – particularly for CAF and IBU. The faster rate of lateral diffusion compared to penetration was also observed by Jiang et al.[192] when assessing the distribution of drug in human sclera. It was suggested that diffusion parallel to the aqueous corneocytes might encounter less hindrance than traversing between the alternating lipophilic domain and aqueous proteins[190]. This may particularly hold true for hydrophilic compounds such as CAF whereby diffusion laterally across the skin will be less tortuous. In addition, lateral diffusion has been found to be strongly dependent on molecular size and pronounced for compounds with a molecular

size up to 300 g/mol[154]. This is in good agreement with our results as CAF and IBU, which have a molecular size of 194.46 and 206.28 g/mol, respectively, exhibited greater lateral diffusion than HC (with a molecular size of 362.46 g/mol).

A common trend observed between the distribution of CAF, HC and IBU from the above studies is that the concentration of drug was highest at the surface and then decreased with depth of SC and radial distance from the application site (Figure 3.3). This is a similar finding to that by Loden et al.[144] whereby the distribution profile of ketoprofen through the SC of human skin *in vivo* was studied. In addition, Loden et al.[144] also found that the concentration profile of ketoprofen did not show pronounced differences within 6 hrs of exposure. This is in accordance with the distribution profile of CAF, HC and IBU extracted from section 1 on the sampling sites. There was no significant difference in the distribution profile of permeants at sites directly below the application area over 6 hrs. This may be due to a higher drug loading at the initial site of contact which presents a steeper concentration gradient across the SC (at the application site) that provides the main driving force for the diffusion through skin.

Previous studies carried out by Rhodes et al.[193] suggests that only 50% of the applied formulation remains on the skin surface 8 hrs after application, with observed differences between formulation types[194]. This is in agreement with the results in the present study listed in Table 3.2 where between 47 – 65% of CAF, HC and IBU was recovered from the skin at 6 hrs after application. The reduced recovery of CAF and HC from the outermost regions of the SC at 3 hrs and 6 hrs is mainly due to depletion of drug from section 2 and 3 of the sampling sites as seen in Figure 3.3. No increase in CAF and HC penetration into the deeper skin regions or lateral diffusion was revealed with an increase of time. In contrast, the reduction of IBU detected in section 2 of the sampling site is reflected by an increase in lateral diffusion and/or penetration (from preceding SC region) into section 3 in the deeper

regions of the sampled SC at 6 hrs as demonstrated in Figure 3.3i. The majority of CAF, HC and IBU were lost between 3 mins and 6 hrs after application. The route of drug loss from the SC surface may be due to depletion via skin shedding, metabolism, gravity pull or absorption into the viable skin regions. Therefore, the proceeding chapter will assess how much of the drug lost from the SC *in vivo* may be attributed to further penetration into the viable skin (and made available for systemic absorption) using *in vitro* permeation studies.

In addition to following the distribution profile of permeants across and within the SC, this study also allowed us to determine whether the choice of drug and duration of drug-to-skin contact had an effect on the amount of SC protein removed via tape stripping as a function of distance from the application site. As shown in Figure 3.7, neither drug type nor length of skin contact influenced SC protein removal. However, it appears that the cumulative mass of SC protein removed from sites directly under the application area (section 1) was significantly higher than the cumulative mass of protein removed at distances further from the application area. This suggests that the amount of drug loading at a particular site may be indicative of the amount of protein removed as the results above show that higher drug content is found in section 1 and progressively decreases with distance. However, it should be acknowledged that although all efforts were made to ensure even pressure was applied to tape strips so as to remove a uniform weight of SC protein across the tape, it is still possible that (following splitting of the concentric tapes in half) one half of the concentric tape removed more SC protein than the other due to the presence of furrows[138]. Therefore, the cumulative mass of SC protein reported in this research may over estimate or under estimate the total mass of SC protein removed.

3.7 CONCLUSION

Tape stripping the volar forearm of participants with concentric adhesive tape was used to assess the lateral diffusion and penetration behaviour of CAF, HC and IBU across human skin. These studies demonstrated that:

- The lateral spreading behaviour of drugs appears to be dependent on the physicochemical properties of the compounds applied. CAF and IBU demonstrated a higher tendency to undergo lateral diffusion while HC formed a drug depot at the application site.
- IBU exhibited greater penetration through the SC while CAF and HC resided in the uppermost regions of the SC and this if most likely due to the differences in lipophilicity between drugs.
- At 6 hrs after application, there was a greater loss of IBU from the SC compared to CAF and HC. This is likely the result of the more ideal logP of IBU which facilitates its diffusion across the skin. This will be further investigated using *in vitro* permeation studies in the following chapter.

CHAPTER 4

ASSESSING THE PERMEATION OF CAF, HC AND IBU ACROSS HUMAN SKIN *IN VITRO*

4 ASSESSING THE PERMEATION OF CAF, HC AND IBU ACROSS HUMAN SKIN *IN VITRO*

4.1 INTRODUCTION

The main barrier to drug penetration through the skin is the SC due to its complex heterogeneous structure of keratinized cells embedded in a lipid-rich matrix. As the SC forms the rate-limiting barrier to percutaneous diffusion, it is of interest to determine the localisation and distribution of drug within the SC following topical application. In addition, the amount of drug that has penetrated into the viable epidermis is also important as it represents the amount of drug that may become systemically available.

Tape stripping is commonly used in dermatopharmacology to evaluate the local bioavailability of drugs that are designed to exert a local effect, as well as to follow the distribution behaviour of drugs (designed for local or systemic action) in the outermost layers of the skin[85,148]. It has been suggested that this technique is also useful for assessing the bioavailability of topically applied substances whose target site is the underlying viable epidermis[195]. Furthermore, it has also been postulated that when the SC is the rate-limiting barrier to drug penetration, drug levels in the SC should correlate with those absorbed systemically. Evidence to support this hypothesis was generated by Rougier et al.[84,196,197] by tape stripping the SC of animal and human models 30 mins following application of a range of compounds and correlating the amount of extracted compound to urinary excretion over a period of 4 days. A good correlation was achieved between the two measurements, thus indicating that tape stripping allows for the rapid and simple prediction of potential systemic exposure. As the amount of drug in the viable epidermis is considered available for systemic absorption[18], it is possible that drug levels in the SC also correlate to drug levels in the viable epidermis. However, although it is established that the SC concentration of drug often relates to that which diffuses into the viable epidermis, it

should be acknowledged that SC drug levels are more useful and relevant for assessing local, dermatological efficacy rather than plasma concentrations[149].

With respect to the results obtained previously in this research project (Chapter 3), it was found that at 6 hrs after topical application of ethanolic solutions of CAF, HC and IBU, between 47 – 65% of drug relative to the applied dose was recovered from the uppermost layers of the SC. While it has been found that the amount of drug in the SC is generally associated with the absorbed amount, it is highly unlikely that up to 50% of the applied dose would have been absorbed into the viable epidermis, as it is generally accepted that the bioavailability of topically applied substances is in the order of 5 – 10%[1]. One likely reason for this lack of ability to account for the unabsorbed drug is loss to the surrounding environment via desquamation or being rubbed/washed off, with only a small portion likely to have reached the systemic circulation.

Given the complexities associated with measuring the plasma concentration of topically applied drugs *in vivo,* the permeation of these drugs was assessed *in vitro* through the use of flow-through diffusion cells to measure their percutaneous absorption. Diffusion cells are commonly used to investigate the kinetics and flux of a compound across skin (of human and/or animal origin or synthetic membrane)[198]. They can also serve as a tool for the rapid screening of vehicle effects during the initial stages of formulation development. In addition, the experimental conditions of permeation studies utilising diffusion cells can be easily controlled[96].

In all *in vitro* flow-through diffusion systems, a membrane of choice is mounted as a barrier between a donor chamber and a receptor chamber, and the amount of compound permeating from the donor to the receptor chamber is measured as a function of time. A suitable receptor solution is continually pumped through the receptor chamber to maintain

sink conditions and the receptor fluid is collected in a vial[96,199]. The amount of drug quantified in the receptor fluid is representative of the systemic absorption *in vivo* as demonstrated by a task force appointed by the European Centre for Ecotoxicology and Toxicology of Chemicals Monograph (ECETC)[200].

The monograph set by the ECETC also highlights the importance of the receptor fluid to maintain skin integrity and to act as an appropriate sink for the penetrating compound. The ideal receptor phase provides an accurate simulation of the conditions pertaining to *in vivo* permeation of the test compound. Therefore, it is generally accepted that the concentration of the permeant in the receptor fluid should not exceed ~10% of the saturated solubility of the permeant[201,202] in order to maintain a favourable driving force for permeation. It has also been suggested that if a compound has an aqueous solubility of <10 µg/mL, then the addition of a solubilizer in the receptor fluid is necessary to improve solubility[203]. Therefore, a suitable receptor fluid for all permeants to be studied must be carefully chosen.

Isotonic phosphate buffer (PBS) pH 7.4 is a common receptor fluid of choice, although it is not always the most appropriate. For drugs with low water solubility, the addition of a non-ionic surfactant – polyethylene glycol-20-oleyl ether (VOLPO N20) is a popular choice of solubilizer as it has been found to not disrupt the barrier function of rat skin to compounds of low water solubility and has a negligible effect on the flux of compounds across skin[203].

4.2 OBJECTIVES

The objectives of the following studies were to:

- To measure the saturated solubility of CAF, HC and IBU in PBS pH 7.4 and/or 0.5% VOLPO N20 in water, as possible receptor solution for *in vitro* permeation studies.
- To determine the percutaneous absorption of CAF, HC and IBU (from ethanolic solutions) across human skin *in vitro* over 6 hrs to establish the possible fate of the un-recovered drugs observed in *in vivo* studies (refer to Chapter 3).
- To validate and carry out a mass balance analysis of CAF, HC and IBU in the *in vitro* set-up to account for all possible routes of drug loss *in vitro*.

4.3 MATERIALS

Sodium dihydrogen phosphate, disodium hydrogen phosphate and sodium chloride used to prepare isotonic solutions of phosphate buffer were purchased from Sigma-Aldrich (Castle Hill, New South Wales, Australia). Acetonitrile (ACN) was purchased from Merck (Kilsyth, Victoria, Australia). VOLPO N20 was purchased from Croda (Wetherill Park, New South Wales, Australia). All other chemicals used in the following studies were obtained from the same manufacturers and distributors as detailed in Section 2.3.

4.4 METHODS

4.4.1 Determination of CAF, HC and IBU saturated solubility in relevant receptor solutions

PBS pH 7.4 is often used as a receptor solution for CAF and IBU, while 0.5% w/v VOLPO N20 in water is used as a receptor solution for HC when conducting *in vitro* skin diffusion studies (personal communication through – Acrux Ltd). Although the receptor solution for CAF, HC and IBU had already been established by Acrux Ltd, it was necessary to perform a saturated solubility study of CAF, HC and IBU in their corresponding receptor solutions to ensure the absence of batch variability and to verify that sink conditions will be maintained in the subsequent *in vitro* permeation studies.

CAF and IBU were added in excess to 1.5 mL of PBS pH 7.4 while HC was added in excess to 1.5 mL of 0.5% w/v VOLPO N20 in water. The suspensions were vortexed for 30 secs and then placed on a rotating mixer maintained at 32°C for 72 hrs. At the end of this equilibration period, the samples were centrifuged for 15 mins at 13,500 rpm (19,357 x *g*) at 32°C. A 100 µL aliquot of each supernatant was then diluted with 10 mL of the corresponding extraction solvent (refer to Section 2.4.4) and assayed for CAF, HC or IBU by HPLC as described in Section 2.4.5. The saturated solubility of CAF, HC and IBU in each receptor solution was determined in triplicate.

4.4.2 Permeation of CAF, HC and IBU through human skin *in vitro*

4.4.2.1 Skin preparation

Surgically excised samples of skin from one male and one female were obtained after abdominoplastic surgery with informed consent and approval from the SCERH, Monash University, Victoria, Australia (Project #2006/565). The full-thickness skin (with subcutaneous fat attached) was immediately frozen and stored at -20°C for not more than 12 months. To prepare dermatomed skin, full-thickness skin was defrosted for 3 – 4 hrs

prior to being dermatomed. The skin was then rinsed by cleaning the surface with purified water and swabbing the surface with paper towels. Dermatomed skin was separated using a dermatome slicer set to cut at a thickness of 500 µm (Humeca, Enschede, Netherlands). The separated skin was then immersed in cold water for 30 mins and rinsed 3 times until the water appeared clear. The skin membranes were then transferred onto aluminium foil and stored in a freezer at -20°C until required.

4.4.2.2 In vitro diffusion studies

In vitro skin diffusion studies were carried out on stainless steel flow-through diffusion cells maintained at 32°C by the continuous pumping of thermostated water through hollow stainless steel bars which supported the cells. Prior to mounting the skin over the receptor chamber, the chamber was filled with receptor solution and a wire mesh was placed into the receptor chamber. The wire mesh was incorporated to promote turbulent flow of receptor solution through the receptor well, which alleviates the formation of unstirred, limiting boundary layers and thus aids the maintenance of sink conditions, as well as to prevent the formation of bubbles beneath the skin (refer to Figure 4.1).

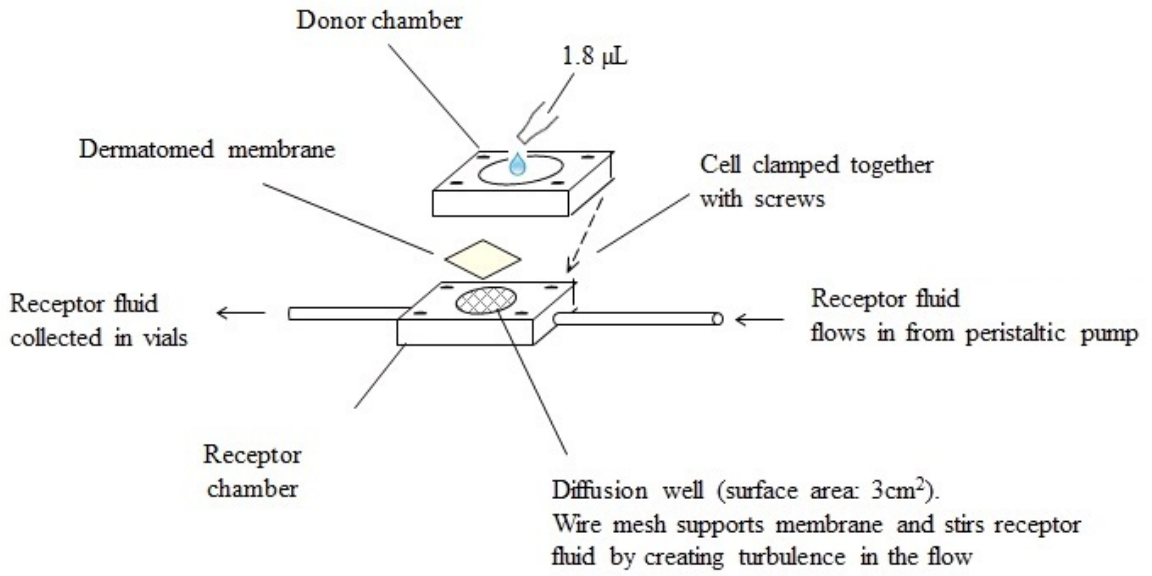


Figure 4.1 Schematic representation of a flow-through diffusion cell set-up.

Dermatomed skin (500 µm) that was previously cut into square pieces was mounted in diffusion cells with a donor chamber area of 3 cm^2. The donor chamber was then filled with approximately 2 mL of purified water or until a convex water meniscus was formed and left for 30 mins to assess skin integrity. Ensuring skin integrity was vital to prevent the skewed permeability data of the three model compounds. If the convex water meniscus at the end of the 30 min period appeared unchanged, the integrity of the skin was assumed intact and therefore suitable to be used in the permeation study. The water was removed from the donor chamber and the receptor solution was pumped through the receptor chamber for a further 10 mins at a flow rate of 0.5 mL/hr using a peristaltic pump (Watson Marlow Microcasette Pump, Cornwall, UK). In total, the skin was equilibrated with the receptor fluid for ~45 mins before a 1.8 µL dose of donor solution was applied onto the centre of the exposed membrane. The donor solutions were either CAF 0.8% w/v, HC 2 % w/v or IBU 5% w/v dissolved in EtOH. The diffusion experiments were carried for a period of 6 hrs, whereby the receptor fluid was collected into vials every 30 mins for the first 3 hrs, then a final sample collected at 6 hrs, using an automated fraction collector

(ISCO Retriever II, Teledyne ISCO, Nebraska, USA). The amount of drug permeated over time was quantified using HPLC assays that were previously validated by Acrux Ltd and detailed in Section 4.4.4. The permeation of each drug across human skin was studied across one female and one male donor, with four replicates conducted for each donor (n=8 in total for each formulation).

4.4.2.3 Mass balance drug analysis in vitro

A mass balance analysis of CAF, HC and IBU was performed *in vitro* immediately following diffusion sample collection at 6 hrs in order to be able to account for as close to 100% of the applied dose as possible. Drug was extracted from the uppermost layers of the SC (through tape stripping), in the remaining skin sample (post-tape stripping) and from the donor chamber as drug may have spread laterally across the SC surface and onto the chamber following dosing.

The skin samples used for the diffusion studies were tape stripped to obtain the distribution profile of drugs across the SC. This allowed for assessment of the amount of drug still residing in the upper layers of the SC at 6 hrs after application *in vitro*, which then consequently allowed for comparisons to be made to that recovered *in vivo* (from Chapter 3).

4.4.2.3.1 *Tape stripping procedure*

The CAF, HC and IBU distribution profile across the SC *in vitro* at 6 hrs was determined by sequential removal of the outer skin layers by tape stripping using specially designed concentric adhesive tapes (3M, Product No 8440CONRING32, ATA Distributors, Victoria, Australia), as outlined in Section 3.4.1.3. However, it should be noted that only section 1 and section 2 of the concentric rings were used in these *in vitro* studies due to the size constraint of the diffusion cells (3 cm^2). Following separation of the

donor and receptor chamber, the concentric tapes were transferred to the application area of dermatomed membrane with tweezers, ensuring that section 1 of the concentric tapes was placed directly over the pre-marked application area. A stainless steel slab weighing 1200 g and measuring 2.8 x 6 x 9 cm was placed over the tape for 3 secs to ensure even pressure was applied across the tape. The tape was then removed from the SC surface and split into two equal halves. This enabled one half of the tape to be used to quantify the amount of extracted drug while the other half was used to quantify the amount of removed SC protein. 8 sequential tape strips were removed from each donor membrane at 6 hrs. 8 tape strips were removed *in vitro* compared to 10 tape strips removed *in vivo* as it was discovered that by the 8^{th} tape strip, the entire epidermis had become detached from the rest of the skin sample.

To quantify the amount of drug removed, the perforated ring segments from one half of the divided tape were further split into their individual sections, as shown previously in Section 3.4.1.3, and submerged in individual vials containing 10 mL of the corresponding extraction solvent as detailed in Section 2.4.4. The amount of drug extracted from each section of each tape strip was analysed using validated HPLC methods as described in Section 2.4.5. The remaining half of the divided tape was also split into its individual sections and immersed into 6-well plates containing 700 µL of NaOH in each well. The amount of SC protein adhered to each tape section was then quantified using the Bradford assay as outlined in Section 2.4.2.1.

4.4.2.3.2 *Extraction of CAF, HC and IBU from donor chamber*

Immediately following the completion of the 6 hr diffusion study, each donor chamber was detached from the receiver chamber by unscrewing the bolts. The donor chambers were transferred into individual beakers containing 10 mL of the corresponding extraction solvent as detailed in Section 2.4.4. The extraction procedure also detailed in

Section 2.4.4 was followed and the amount of CAF, HC or IBU extracted from the donor chambers quantified using validated HPLC methods described in Section 2.4.5.

4.4.2.3.3 *Extraction of CAF, HC and IBU from dermatomed skin*

The amount of CAF, HC or IBU remaining in the viable epidermis or dermis was determined via solvent extraction. Following tape stripping, the remaining viable skin was transferred into 20 mL vials and the extraction procedure as detailed in Section 2.4.4 was followed. The amount of CAF, HC or IBU extracted from the skin membranes were quantified using the validated HPLC methods as described in Section 2.4.5.

4.4.3 Validation of CAF, HC and IBU extraction from donor chamber and dermatomed skin

Donor chambers were rinsed with purified water and EtOH and left to dry under ambient conditions overnight. Ethanolic solutions of a low, medium and high concentration of CAF (equating to 0.0045, 0.15 and 0.7 mg/mL), HC (0.0035, 0.07 and 2 mg/mL) and IBU (0.02, 1 and 5 mg/mL) were applied to individual donor chambers at a dose of 25 µL in order to deposit 0.11, 3.75 and 17.50 µg of CAF, 0.08, 1.75 and 50 µg of HC and 0.50, 25 and 125 µg of IBU, respectively. These masses were chosen as they represent the range of mass that were present in the skin obtained from *in vivo* studies (performed in Chapter 3) and thus may be present in the diffusion studies. The donor chambers were left at ambient conditions on the workbench for 30 mins. This exposure time was arbitrarily chosen to allow the EtOH to evaporate. At the end of the 30 min period, the donor chambers were transferred into 250 mL beakers and CAF, HC and IBU was extracted from the donor chambers in the same manner that the drugs were extracted from tape strip samples as described in Section 2.4.4 and analysed using the HPLC methods as detailed in Section 2.4.5.

The validation of CAF, HC and IBU extraction from dermatomed skin was carried out using the same procedure as detailed above. However, ethanolic solutions of a low, medium and high concentration of CAF, HC an IBU were dosed onto the SC of dermatomed skin sections measuring 1.5 x 1.5 cm and transferred into 20 mL vials following solvent evaporation.

Recovery was determined by comparing the peak areas of CAF, HC and IBU extracted from the donor chamber and dermatomed skin samples to those obtained from the solvent standards (prepared as detailed above with the exception that the standards were applied directly into 20 mL vials). The accuracy was determined by repeating the extraction of CAF, HC and IBU at low, medium and high concentrations and comparing the recovered amount to that of the theoretical.

4.4.4 Quantification of amount of CAF, HC and IBU diffused through dermatomed skin

4.4.4.1 Preparation of CAF standard solutions

A stock standard solution of CAF was prepared at a concentration of 100 µg/mL in EtOH in a glass volumetric flask. Standard solutions were prepared by appropriate dilution of the stock solution in PBS pH 7.4 to give concentrations ranging from 0.1 – 50 µg/mL.

4.4.4.2 Preparation of HC standard solutions

A stock standard solution of HC was prepared at a concentration of 500 µg/mL in MeOH in a glass volumetric flask. Standard solutions were prepared by appropriate dilution of the stock solution in 0.5% w/v VOLPO N20 in water to give concentrations ranging from 0.03 - 20 µg/mL.

4.4.4.3 Preparation of IBU standard solutions

A stock standard solution of IBU was prepared at a concentration of 1000 µg/mL in EtOH in a glass volumetric flask. Standard solutions were prepared by appropriate dilution of the stock solution in PBS pH 7.4 to give concentrations ranging from 0.02 – 5 µg/mL.

4.4.4.4 Analytical methods

The concentration of CAF, HC and IBU permeated across dermatomed skin *in vitro* was determined using pre-validated methods already existing at Acrux Ltd. Quantification of CAF, HC and IBU were measured using a reversed phase Waters Symmetry C_{18} 5 µm (3.9 x 150 mm) column equipped with a Waters Symmetry C_{18} 5 µm (3.9 x 20 mm) guard column (Waters, Massachusetts, USA). The HPLC system consisted of a Waters 2690 Separations Module HPLC system, Waters 2487 dual wavelength absorbance detector, a Waters 610 pump, Waters 600E system controller and a Waters 712 autosampler. The chromatographic conditions for CAF, HC and IBU quantification are detailed in Table 4.1.

Table 4.1 Chromatographic conditions used to quantify the amount of CAF, HC and IBU permeated across dermatomed skin *in vitro*.

	Drug		
Condition	**CAF**	**HC**	**IBU**
Mobile phase	0.05% TFA in 10% ACN:MeOH (85:15)	H_2O:MeOH:ACN (58:27:15)	Line A: H_2O, pH 2.5* Line B: ACN Gradient**
Flow rate	1.1 mL/min	1.0 mL/min	1.0 mL/min
Injection volume	30 µL	100 µL	100 µL
UV detection	272 nm	245 nm	219 nm
Column temperature	35°C	40°C	30°C
Run time	6 mins	9 mins	9 mins
Retention time	~4 mins	~5 – 6 mins	~3.3 mins
Linear concentration range (µg/mL)	0.1 – 50	0.03 – 20	0.02 – 5

(*) pH adjusted to with 1M phosphoric acid

(**) Gradient run: 0 – 0.5 mins, 40% B; 0.5 – 2 mins, 40 – 90% B; 2 – 3 mins, 90% B; 3 – 4 mins, 90 – 40% B; 4 – 9 mins, 40% B

4.4.5 Data analysis

The permeation of CAF, HC and IBU across human skin *in vitro* was measured as the mass of drug (μg) permeated per unit area. The amount of drug removed from each concentric tape section was normalised to the mass of SC protein removed by the same concentric tape section in order to eliminate the inter-subject variability in the total mass of protein removed per layer of tape strip. The normalised concentration of drug was plotted against the cumulative weight of SC protein per unit area. The distance of drug spread will be referenced as 'section 1' and 'section 2', which equates to distances of between 0 – 4 mm and 4 – 8 mm from the application area, respectively.

4.4.6 Statistical analysis

A one-way repeated measures ANOVA test was applied to compare the percentage recovery of CAF, HC and IBU at 6 hrs in the donor chamber, viable skin, tape strips and receptor fluid, relative to the applied dose. In addition, a one-way repeated measures ANOVA test was also used to investigate whether there was a significant difference in the percentage of each drug recovered per unit area in each concentric ring. A probability of $p<0.05$ was deemed significant. All statistical measurements were carried out using SPSS Statistics 19 for Windows. All data are presented as mean ± SEM (n=8), unless stated otherwise.

4.5 RESULTS

4.5.1 Determination of CAF, HC and IBU saturated solubility in relevant receptor solutions

The saturated solubility values of CAF, HC and IBU in the relevant receptor solution media at 72 hrs are shown in Table 4.2. To ensure that sink conditions were maintained in the subsequent permeation experiments, the maximum concentration of drug appearing in the receptor chamber was maintained below 10% of the experimentally-determined saturated solubility.

Table 4.2 Saturated solubility of CAF, HC and IBU in PBS pH 7.4 and/or 0.5% VOLPO 20 in water after 72 hrs (mean ± SEM, n=3).

Saturated solubility (mg/mL)		
CAF in PBS pH 7.4	**HC in 0.5% VOLPO N20**	**IBU in PBS pH 7.4**
35.60 ± 0.20	0.60 ± 0.01	5.50 ± 0.01

4.5.2 Validation of CAF, HC and IBU extraction from donor chamber and dermatomed skin

Recovery and accuracy for the extraction of CAF, HC and IBU from the donor chamber and dermatomed skin are presented in Table 4.3 and 4.4, respectively. The recovery of CAF, HC and IBU from the donor chamber and dermatomed skin samples was within 100 ± 20 % for all concentrations. The accuracy of the donor chamber and dermatomed skin extraction procedure was also within accepted limits (<20%) at all concentrations.

Table 4.3 Recovery and accuracy of CAF, HC and IBU from donor chamber post-diffusion study (mean ± SD, n=5).

	Amount (µg)	**Spiked amount (µg)**	**Recovery (%)**	**Accuracy (%)**
CAF	0.1	0.1 ± 0.0	103.3 ± 17.5	106.2 ± 17.9
	3.75	4.0 ± 0.2	109.0 ± 4.3	106.0 ± 4.6
	17.5	16.7 ± 0.9	103.4 ± 10.2	95.4 ± 9.4
HC	0.1	0.1± 0.0	88.4 ± 7.2	114.2 ± 9.3
	1.75	1.7 ± 0.7	113.4 ± 4.2	108.2 ± 4.0
	50	51.8 ± 1.2	105.9 ± 4.1	109.8 ± 4.3
IBU	0.5	0.5 ± 0.1	108.1 ± 16.9	115.0 ± 20
	25	28.9 ± 0.5	100.4 ± 4.5	116.0 ± 5.0
	125	129.0 ± 4.4	107.6 ± 4.3	111.0 ± 4.3

Table 4.4 Recovery and accuracy of CAF, HC and IBU from dermatomed skin post-diffusion study (mean ± SD, n=5).

	Amount (µg)	Spiked amount (µg)	Recovery (%)	Accuracy (%)
CAF	0.1	0.1 ± 0.0	119.0 ± 11.4	115.1 ± 11.0
	3.75	3.6 ± 0.1	116.3 ± 5.5	112.1 ± 5.4
	17.5	16.8 ± 0.6	98.9 ± 6.2	95.1 ± 5.9
HC	0.1	0.1 ± 0.1	93.2 ± 4.7	110.3 ± 5.5
	1.75	1.7 ± 0.7	102.9 ± 7.8	100.5 ± 7.6
	50.0	51.7 ± 1.1	102.7 ± 7.1	106.2 ± 7.4
IBU	0.5	0.6 ± 0.1	88.3 ± 16.8	105.3 ± 20.0
	25	29.4 ± 0.6	95.4 ± 5.5	112.7 ± 6.4
	125	132.9 ± 4.7	96.9 ± 7.8	102.9 ± 8.3

4.5.3 *In vitro* diffusion study

The permeability profile of IBU through dermatomed human skin is shown in Figure 4.2. A total of 1.4 ± 0.3 µg of IBU was detected in the receptor solution after 6 hrs, equating to 1.6 ± 0.3% of the applied dose. No CAF or HC could be detected in the receptor fluid after 6 hrs, using the validated assay.

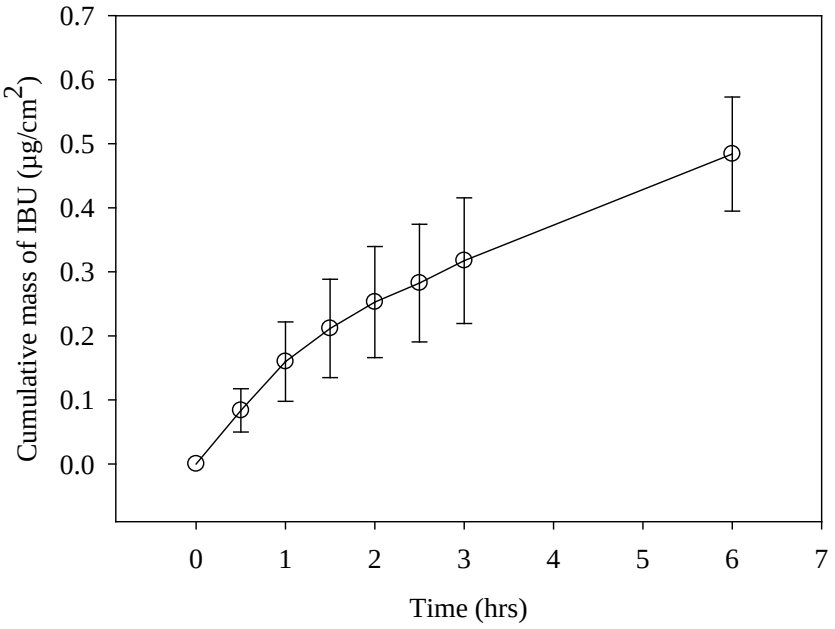


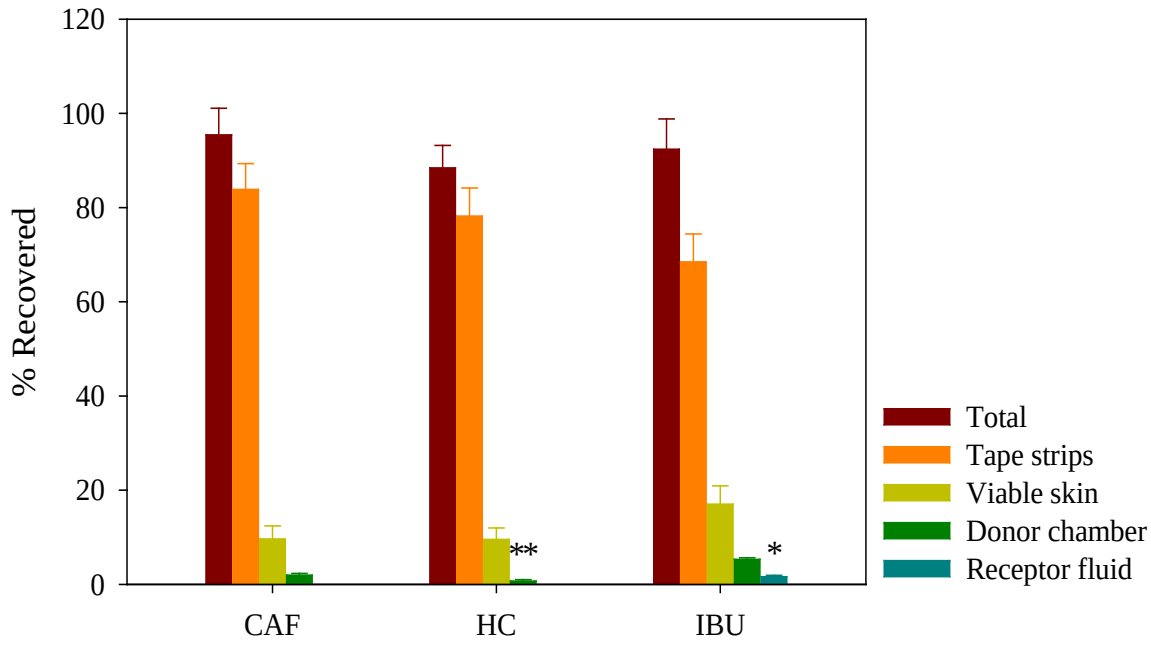
Figure 4.2 *In vitro* permeation profile of IBU across dermatomed human skin (mean ± SEM, n=8).

4.5.4 Mass balance drug analysis *in vitro*

A mass balance analysis of CAF, HC and IBU was performed whereby the amount of drug residing in the uppermost layers of the SC, the viable epidermal and dermal skin section, the donor chamber of the diffusion cell set-up, as well as the amount of drug permeated through the dermatomed membrane into the receptor solution was determined. The percent recovery of CAF, HC and IBU in the donor chamber, viable skin, tape strips and receptor fluid is shown in Figure 4.3.

At 6 hrs after exposure, approximately 90% of the applied dose of all drugs was recovered. The majority of drug remained in the outermost layers of the skin as 83.8 ± 5.5%, 78.2 ± 6.0% and 68.5 ± 5.9% of the applied dose of CAF, HC and IBU was extracted from the 8 sequential tape strips. Though not significantly different, the lower recovery of IBU from the SC surface may be due to the higher amount of drug recovered

in the viable skin (17.0 ± 3.9%) compared to that recovered of CAF (9.6 ± 2.8%) and HC (9.5 ± 2.6%). Approximately 1.6% of the applied dose of IBU was detected in the receptor solution while CAF and HC could not be detected. A small percentage of drug was also detected to have spread beyond section 2 of the tape stripped skin area and onto the surrounding donor chamber. The percentage of CAF and IBU extracted from the donor chamber was significantly higher than that of HC, indicating a higher tendency to diffuse laterally across the SC.



(*) denotes significant difference to CAF and HC.

(**) denotes significant difference to CAF and IBU.

Figure 4.3 *In vitro* mass balance recovery of CAF, HC and IBU at 6 hrs relative to the applied dose (mean ± SEM, n=8).

The lower tendency of HC to spread laterally both on the SC surface and within the SC bilayers is further displayed by its distribution profile in Figure 4.4 whereby the concentration of HC in section 2 of each subsequent tape strip is less than half the concentration detected in section 1 of the corresponding tape strip.

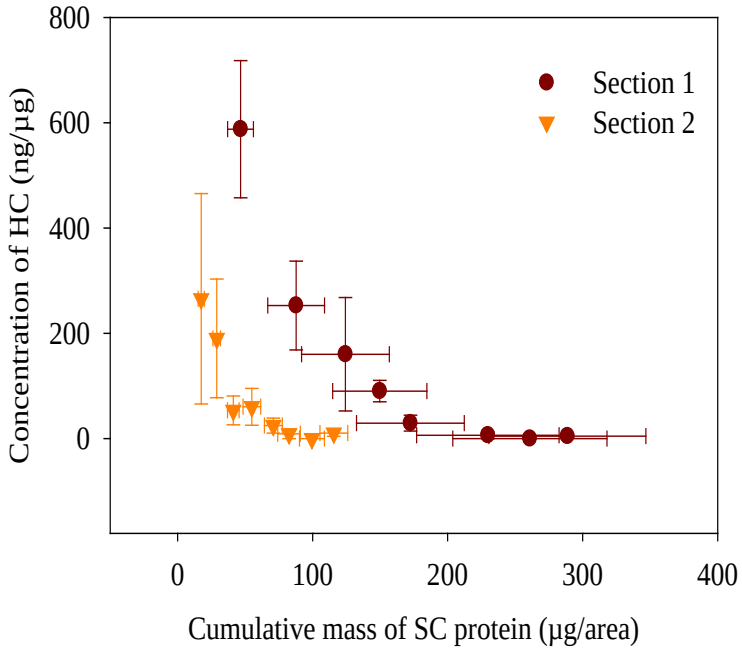


Figure 4.4 *In vitro* distribution profile of HC across dermatomed human skin at 6 hrs after application (mean ± SEM, n=8).

CAF exhibited high lateral diffusion with percentage of the applied dose recovered in the donor chamber being significantly higher than that of HC. The spreadability of CAF on and within the SC is also highlighted by its distribution profile shown in Figure 4.5. As section 2 is 8 mm from the application area and sectiosn 1 is 4 mm from the application area, the similar concentration of CAF found in the outer section compared to that of the application area signifies that the process of lateral diffusion across and within the SC had occurred.

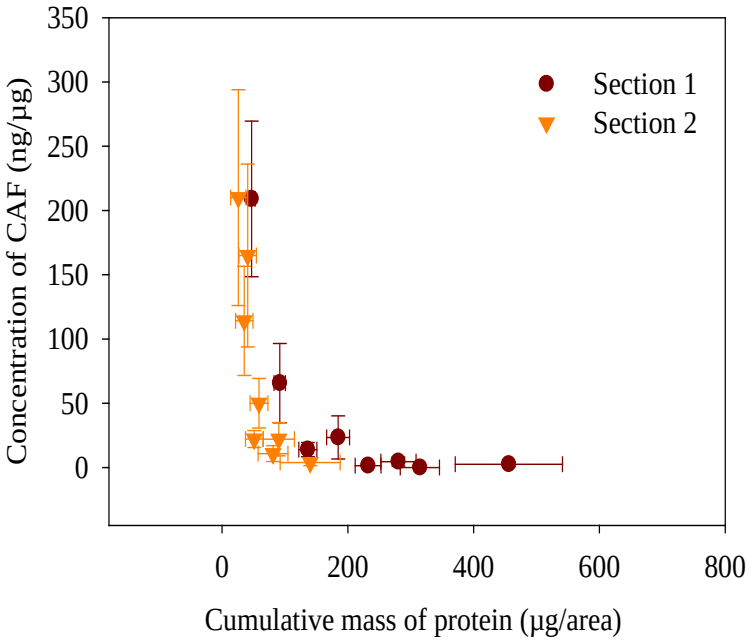


Figure 4.5 *In vitro* distribution profile of CAF across dermatomed human skin at 6 hrs after application (mean ± SEM, n=8).

IBU also demonstrated greater lateral diffusion as the percentage of IBU recovered from the donor chamber relative to the applied dose was significantly higher than that recovered for both CAF and HC. The radial movement of IBU in the uppermost region of the SC is also displayed in Figure 4.6 as the concentration of IBU removed per unit area from section 2 is similar to that removed from section 1 of the corresponding tape strip.

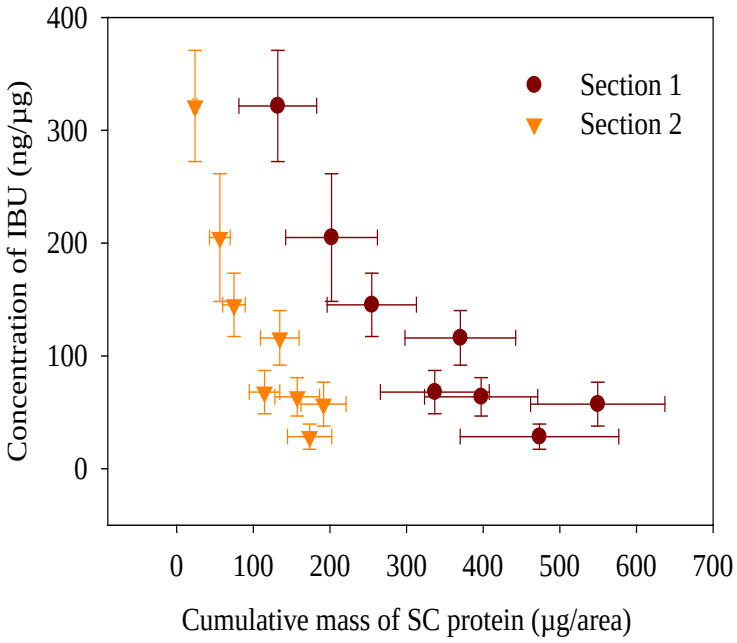


Figure 4.6 *In vitro* distribution profile of IBU across dermatomed human skin at 6 hrs after application (mean ± SEM, n=8).

4.6 DISCUSSION

Studying the *in vitro* permeability of solutes through excised skin using a flow-through diffusion cell set-up is a useful tool for screening the diffusivity of solutes during the initial stages of formulation development. Thus, it was of interest to employ *in vitro* diffusion studies to assess what portion, if any, of the reduced CAF, HC and IBU recovery from the SC surface at 6 hrs *in vivo* was due to penetration into the deeper viable skin. In addition to the permeation recovery of CAF, HC and IBU, a mass balance recovery was executed at 6 hrs whereby the concentration of each drug was determined in the upper SC, the stripped viable epidermal and dermal layers, as well as the amount of drug remaining in contact with the donor chamber.

The total recovery of CAF, HC and IBU from the donor chamber, viable skin, tape strips and receptor fluid at 6 hrs after application was between 88 – 95% as shown in Figure 4.3, demonstrating that the extraction techniques are robust to account for close to 100% of drug applied. The amount of IBU recovered from the donor chambers (5.3 ± 0.5%) was significantly higher than that recovered of CAF (2.0 ± 0.4%) and HC (0.7 ± 0.3%). In addition, the percentage of CAF recovered was also significantly higher than that of HC. As the portion of drug reaching the donor chamber is representative of the drugs' lateral spreading on the SC surface, these results suggest that CAF and IBU both exhibit higher lateral diffusion behaviour than HC. This is supported by the distribution data depicted in Fig 4.3, whereby the amount of CAF extracted from section 2 was comparable to that extracted from section 1 of the corresponding tape strips. Much like the lateral diffusion behaviour observed *in vivo* (refer to Chapter 3), these trends may be attributed to (a) the higher initial drug loading of IBU compared to CAF and HC due to its higher solubility in EtOH, and (b) the relatively small molecular size of CAF and IBU (<300 g/mol) for which lateral spreading is more pronounced[154]. In addition, lateral diffusion of

CAF may occur owing to the less tortuous route of travel on the SC surface than traversing between the alternating lipophilic domains and aqueous corneocytes of the SC[190]. HC, on the other hand, does not display lateral movement but instead, remains concentrated at the site of application as seen in the distribution profile in Figure 4.4. The concentration of HC in section 2 is approximately 50% of that extracted from section 1 of the corresponding tape strips, suggestive of reservoir formation in the uppermost layers of the SC. This further demonstrates less lateral movement of HC, both on the SC surface and within the SC bilayers. The reservoir formation characteristics of HC have been previously reported[184,204-206] and has been credited to slow diffusion and poor solubility within the SC[207].

The compartment containing the highest quantity of each drug was the SC – that removed within the eight tape strips. In Figures 4.4 – 4.6, it was observed, as Potard et al.[208] and Trebilcock et al.[85] did, that the quantity of drug removed decreases according to tape strip layer and thus according to the depth of the SC. After the sixth tape strip, the amount of drug removed is very low and effectively becomes negligible. Nevertheless, Figure 4.3 shows that 83.8 ± 5.5%, 78.2 ± 6.0% and 68.5 ± 5.9% of CAF, HC and IBU was found in the 8 tape stripped layers, respectively. Though not significantly different, the higher recovery of CAF and HC in the SC compared to IBU are supported by their relative physicochemical properties. Among the physicochemical factors influencing the percutaneous absorption of drugs, lipophilicity plays the most important role[209]. In an experiment using 14 steroids, Scheuplein et al.[210] demonstrated that percutaneous absorption may be increased with an increase in the organic/water partition coefficient (logP), that is, an increase in lipophilicity, owing to the lipophilic nature of the SC. CAF and HC are both relatively hydrophilic drugs with a logP of -0.07[115] and 1.43[110], respectively. When topically-applied, CAF and HC would be subjected to a tortuous route

of diffusing around the dead corneocytes of the SC and through the intercellular lipids. The SC is thus not a favourable environment for the penetration of hydrophilic substances and consequently, forms the rate-limiting barrier for the percutaneous penetration of CAF and HC. Hence, the majority of CAF and HC remain as a depot on the outermost layers of the SC while a small amount is able to penetrate through and be extracted from the viable skin.

On the contrary, the overall lower recovery of IBU in the tape stripped samples is accredited to its lipophilicity (logP 3.51[209]). As lipophilic drugs are soluble in the continuous lipid mortar, diffusion through the SC poses less resistance due to the favourable environment[190]. Hence, the ease of movement of IBU through and out of the SC may be responsible for the lower recovery of IBU in the uppermost region of the SC. In turn, this was reflected by a higher recovery of IBU in the viable skin compared to that recovered of CAF and HC as seen in Figure 4.3.

The viable skin section immediately below the SC is hydrophilic in nature and can in turn act as the rate-limiting step in the permeation of highly lipophilic substances. However, it is accepted that for optimal percutaneous absorption, a permeant should be reasonably soluble in both hydrophilic and hydrophobic media. Permeants with a logP between 1 – 3 are deemed ideal for percutaneous penetration[40]. Therefore, though the permeation of IBU across the viable skin will encounter some resistance, permeation beyond this section is still likely. This is verified by the permeation profile of IBU across dermatomed membrane as shown in Figure 4.2. Following a 6 hr period, 1.6 ± 0.3% of the applied dose of IBU was detected in the receptor fluid, whereas no detectable amounts of CAF or HC were present.

Unlike the documented *in vitro* permeation studies whereby the concentration of CAF or HC dosed was at saturated concentrations[168,178,208,211-213] or in the presence of

additives that influence penetration[214,215], the lower drug loading in addition to the sub-saturated concentration at which CAF and HC was dosed in this study is most likely to be responsible for the absence of CAF and HC in the receptor fluid. However, some studies have found that even at saturated concentrations, a longer lag time of >6 hrs is required before any CAF[168,211] or HC[215] is detected in the receptor fluid. Even more interestingly, only approximately 1 % of HC is detected after 5 days[216] and 10 days[217] following application.

It should be noted however, that saturated concentrations were not used in this study as the maximum permitted concentration of IBU to be tested was to match that of usual topical concentrations of 5% w/v. IBU dissolved with ease in EtOH to achieve a concentration of 5% w/v. As the concentration of IBU was not at saturation, the concentration of CAF and HC chosen was below their saturated concentration in an attempt to maintain consistency. Literature on the permeation of CAF and HC at sub-saturated concentrations is scarce. However, as the results in this study show – permeation across dermatomed membrane does not occur or a longer receptor fluid collection time frame is required due to a potentially long lag time.

It should also be acknowledged that the permeation profile of IBU shown in Figure 4.2 does not resemble that of a commonly observed permeation profile due to the absence of a lag phase prior to the accumulative of drug in the receptor fluid. Other literature reports have measured the permeability of IBU over a longer exposure times of 24 hrs[191,218,219] or 48 hrs[167,219]. Therefore, the short experimental time frame employed in this study may not be sufficient to reach steady state and thus may not be reflective of IBU permeation in *in vivo* conditions.

As only IBU was detected in the receptor fluid at 6 hrs after application *in vitro*, it is reasonable to assume that the loss of IBU *in vivo* from the SC surface at the equivalent time point was partially due to absorption into the deeper viable skin tissue. Conversely, the *in vivo* loss of CAF and HC from the SC surface at 6 hrs after exposure is likely to be largely due to loss to the surrounding external environment. However, further studies are required to confirm this.

4.7 CONCLUSION

Following 6 hrs of exposure, most of the drugs remained in the uppermost layers of the SC. For IBU, however, absorption into the deeper viable skin layers was evident by its presence in the receptor fluid. This suggests that the lower recovery of IBU from the SC surface *in vivo* at 6 hrs following application (refer to Chapter 3) is, at least partially, due to absorption of this drug. On the other hand, the reduced recovery of CAF and HC from the SC surface *in vivo* at 6 hrs following application is likely to be almost entirely a result of drug lost to the surrounding environment as no drug could be detected in the receptor solution *in vitro*. For this reason, subsequent studies in this project will only assess the effect of excipients on the lateral diffusion and penetration of only IBU across human skin.

CHAPTER 5

ASSESSING THE EFFECT OF EXCIPIENTS ON THE LATERAL DIFFUSION AND PENETRATION OF IBU *IN VIVO* AND *IN VITRO*

5 ASSESSING THE EFFECT OF EXCIPIENTS ON THE LATERAL DIFFUSION AND PENETRATION OF IBU *IN VIVO* AND *IN VITRO*

5.1 INTRODUCTION

Transdermal drug delivery offers an advantageous mode of drug administration over more conventional methods by avoiding first pass hepatic metabolism, has direct access to diseased topical sites with local treatment[5] and has improved patient compliance due to ease of application and removal[7]. However, the heterogeneous lipid and aqueous domains of the SC limit the range of drug candidates suitable for this route of delivery. Drug molecules currently administered via the transdermal route fall within a narrow range of molecular weight (<500 Da) and lipophilicity (logP between 1 and 3[58]), thereby taking advantage of the natural selectivity of the skin membrane[7]. To overcome this barrier for molecules falling outside these ranges and to improve the systemic availability of compounds delivered via the skin, the incorporation of chemical penetration enhancers into transdermal and topical formulations has been explored.

Penetration enhancers are chemicals that are able to reversibly reduce the barrier function of the SC without damaging viable cells. It is thought that they exert their effect either by extracting lipids from the skin and thereby creating diffusion pathways for the drug to permeate through or by intercalating between the lipid bilayers to create spatial disruption and lipid fluidization[7,40,220]. Various hydrocarbons, terpenes, fatty acids, alcohols and surfactants have been shown to enhance transdermal permeability of compounds[214,221-224]. A sunscreen agent – octisalate (OS) was also demonstrated by Morgan et al.[69] to enhance the skin permeation of hormones across the skin. It has been suggested that the possible lipid fluidising effect of OS increases the free volume within the SC lipid bilayers thereby facilitating partitioning of permeants across the SC[40].

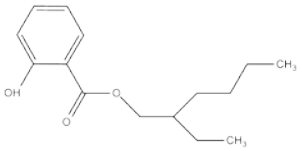
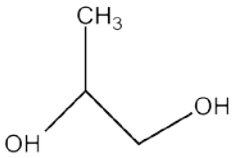
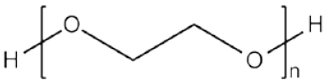
Propylene glycol (PG) has also been shown to improve the transdermal permeability of compounds on its own[225] or when used as a cosolvent to produce a synergistic affect[39,70,226]. A cosolvent system might be referred to as a mixture of two or more solvents used to facilitate drug transport across the skin. The mechanism by which such systems increase transdermal flux may be by increasing the thermodynamic activity of permeant within the SC, by interacting with the SC to increase drug solubility within the SC or by reducing the resistance of the transport pathway[66,74].

Polyethylene glycols (PEGs) are widely used in cosmetics as emollients and solvents. In pharmaceutical products their function can be extended to that of a penetration enhancer. By decreasing the surface tension of the SC, PEGs may enable or enhance the diffusion of other molecules through the skin[123]. De Vos et al.[227] also demonstrated that PEGs affect the structure of the SC by insertion in between the lipid bilayers and thereby improving drug penetration. However, a reduction in skin permeability has also been found following application with PEGs by the formation of complexes and/or increasing viscosity[228]. The contrary effects of PEGs was also verified by Cross et al.[229] when assessing whether the viscosity of formulations affect the skin penetration of sunscreen agents.

While there have been many studies assessing the individual effects of the above mentioned excipients on penetration, the potential synergistic effects of OS, PG and PEGs on the penetration enhancement and lateral spreading of model compounds across human skin *in vivo* and *in vitro* have not yet been explored.

The chemical structure of OS, PG and polyethylene glycol 200 (PEG 200) are shown in Table 5.1.

Table 5.1 Chemical structure of excipients.

	OS	**PG**	**PEG 200**
Chemical structure			

5.2 OBJECTIVES

The purpose of the work presented in this chapter was to:

- Determine whether different combinations of PG, PEG 200 and OS influenced the *in vivo* lateral diffusion of IBU on the SC surface and within the SC bilayers.
- Determine whether the presence of various combinations of PG, PEG 200 and OS modified the penetration behaviour of IBU across the human SC *in vivo.*
- Determine whether PG, PEG 200 and OS enhanced the *in vitro* percutaneous absorption of IBU.
- Determine whether the distribution profile, lateral spreading behaviour and total surface recovery of IBU *in vivo* correlated to that observed *in vitro*.

5.3 MATERIALS

All chemical used in the following studies were obtained from the same manufacturers and distributors as detailed in Section 2.3 and Section 4.3.

5.4 METHODS

5.4.1 Choice of formulation

As discussed in Chapter 2, the application method that this project aimed to replicate is that of a Metered Dose Transdermal System. This evaporative system is sprayed onto the skin to produce a rapid-drying solution containing a volatile component that leads to an increase in surface drug concentration. Meanwhile, the presence of a non-volatile chemical component keeps the drug in solution to prevent drug precipitation or crystallization during evaporation of the volatile solvent component and encourages high drug diffusivity[58].

Therefore, PG, PEG 200 and OS (all which exhibit different functions when incorporated into transdermal products) were added to formulations to not only form the non-volatile component to prevent drug precipitation, but to also assess what effect they would have on the penetration and lateral diffusion of IBU at various exposure times. Hence, the following studies consisted of ethanolic solutions of the following formulations:

- IBU + PG + PEG 200
- IBU + OS
- IBU + PG + PEG 200 + OS

Data relating to the lateral spreading and penetration behaviour of IBU applied alone were taken from Chapter 3 and 4.

OS was incorporated into the formulations at 5% w/v to match that commonly used in sunscreens while PG and PEG 200 were present in formulations at a concentration of 10% w/v as it has been suggested that concentrations above 10% w/v are capable of producing skin sensitization[230-232].

5.4.2 Effect of excipients on *in vivo* IBU lateral spreading and penetration

5.4.2.1 Participants

Eight healthy volunteers (4 males and 4 females) provided written consent to participate in the study that was approved by the SCERH, Monash University, Victoria, Australia (Project #CF08/1125 – 2008000555). The participants were aged between 24 to 37 years of age and had no history of skin disease. Each participant was asked to refrain from applying any topical medicaments to their left and right flexor forearms at least 48 hrs prior to the experiment.

5.4.2.2 In vivo study design

To obtain a distribution profile of IBU applied with and without the presence of PG and PEG 200 and/or OS across human SC *in vivo*, a 1.8 µL aliquot of ethanolic solution was dosed onto individually marked areas on the left and/or right forearm of volunteers as described in Section 3.4.1.2. The forearm remained flat and rested on the bench for at least 30 secs at which all of the solvent had evaporated. At 3 mins, 3 hrs sand 6 hrs after application, 10 concentric adhesive tapes were applied and removed from the sample sites as detailed in Section 3.4.1.3. The tapes were then split into two equal halves. This enabled one half of the tape to be used to quantify the amount of extracted drug while the other half was used to quantify the amount of SC protein removed. To quantify the amount of drug removed, the perforated ring segments from one half of the divided tape was further split into its individual sections and submerged in individual vials containing 10 mL of extraction solvent as detailed in Section 2.4.4. The amount of IBU extracted from each section of each tape strip was analysed using the validated HPLC method as described in Section 2.4.5. The amount of SC removed by the remaining half of the divided tape was also split into its individual sections and immersed into 6-well plates containing 700 µL of NaOH in each well. The Bradford assay was then carried out as outlined in Section 2.4.2.1.

To ensure that residues from each formulation did not impact on the evaluation of other formulations, each forearm was designated one formulation or the formulations were dosed at least one month apart.

5.4.3 Determination of the lateral spreading and penetration behaviour of IBU in the presence of PG, PEG 200 and/or OS across human skin *in vitro*

As will become apparent in Section 5.5.4, a substantial amount of IBU was unaccounted for following tape stripping the uppermost layers of the SC. While it is likely that a portion of the unrecovered drug was lost to the surrounding environment, it is possible that some IBU may have penetrated beyond the depth which was tape stripped and consequently absorbed into the bloodstream. Therefore, in an attempt to get a measure of this, *in vitro* permeation studies were performed to assess the percutaneous absorption of IBU applied with and without excipients.

5.4.3.1 Skin preparation

Surgically excised samples of human female skin were obtained after abdominoplasty with informed consent under a protocol approved by the SCERH, Monash University, Victoria, Australia (Project #2006/565). The preparation of skin for permeation studies was carried out as detailed in Section 4.4.2.1

5.4.3.2 In vitro diffusion studies

The permeation of IBU applied with and without excipients across dermatomed human skin was performed as described in Section 4.4.2.2. The amount of drug permeated over time was quantified using HPLC assays that were previously validated by Acrux Ltd and detailed in Section 4.4.4. The permeation of IBU across human skin was studied across three donors, with four replicates conducted for each donor (n=12 in total for each formulation).

5.4.3.3 Mass balance analysis in vitro

A mass balance recovery of IBU from each formulation was performed *in vitro* immediately following receptor phase sample collection 6 hrs after drug dosing. As drug may have spread laterally across the SC surface and onto the donor chamber following dosing, drug was extracted from the donor chamber as described in Section 4.4.2.3.2. Drug was also extracted from the uppermost layers of the SC (through tape stripping) as detailed in Section 4.4.2.3.1 and in the remaining skin sample (post-tape stripping) as specified in Section 4.4.2.3.3. However, it should be noted that when tape stripping, only section 1 and section 2 of the concentric rings were applied and removed from *in vitro* human skin due to the size constraint of the diffusion cells (3 cm^2). Furthermore, 8 tape strips were removed *in vitro* compared to 10 tape strips removed *in vivo* as it came to light that by the 8^{th} tape strip, the entire epidermis had become detached from the rest of the skin sample.

5.4.4 Validation of IBU extraction from adhesive tape in the presence of PG, PEG 200 and OS

The studies in this chapter involved the extraction of IBU when applied in the presence of PG and PEG 200 and/or OS. Thus, it was necessary to ensure that the presence of these excipients did not interfere with the IBU extraction method as detailed in Section 2.4.4. The extraction of a low, medium and high concentration of IBU in the presence of PG, PEG 200 and OS from adhesive tape was carried out and compared to that of the solvent standards to ensure that adequate extraction of IBU could still be achieved.

Adhesive tape (3M Product #8440, ATA Distributors, Victoria, Australia) measuring 1.5 x 1.5 cm were laid flat with the adhesive side up. Ethanolic solutions of a low, medium and high concentration of IBU (0.02, 1 and 5 mg/mL) with the addition of OS 5% w/v, PG 10% w/v and PEG 200 10% w/v, were applied to individual adhesive tape samples at a dose of 25 µL in order to deposit 0.50, 25 and 125 µg of IBU, respectively. These masses

were chosen as they cover the range of material that was present in the skin in *in vivo* studies described in Chapter 3. The samples were left at ambient conditions on the workbench for 30 mins. This exposure time was arbitrarily chosen to allow the EtOH to evaporate. At the end of the 30 min period, each tape strip was placed separately into 20 mL vials and submerged in 10 mL of extraction solvent which was made up of HPLC grade MeOH:HCl (36% v/v) (90:10). The vials were securely sealed with a Teflon-lined lid and the enclosed tape strip and solvent was vortexed for 10 secs and then placed in a shaking water bath (Ratek, Victoria, Australia) set at 80 strokes per min at 60°C for 30 mins. At the end of the 30 mins, the vials were removed from the water bath and left on the bench under ambient conditions overnight. The samples were then placed in a sonicator (FXP-10M Unisonics, New South Wales, Australia,) for 1 hr, after which time, the amount of IBU was quantified by HPLC as described in Section 2.4.5. The recovery of IBU from tape samples was determined by comparing the peak areas of IBU extracted from blank adhesive tape to that obtained from the solvent standards prepared in the same way as mentioned above, with the exception that the IBU standards were applied directly into 20 mL vials (in the absence of tapes). The accuracy was determined by repeating the extraction IBU at low, medium and high concentrations and comparing the recovered amount to that of the theoretical amount applied.

5.4.5 Measurement of contact angles of IBU applied with and without PG, PEG 200 and/or OS on human skin

As will be shown in Section 5.5.2, IBU applied with and without PG, PEG 200 and/or OS displayed differing degrees of lateral spreading on the skin surface. Therefore, the contact angle that each IBU formulation made with the human skin surface was measured to assess whether the different distances of lateral spreading may be due to

differences in the interfacial tension between the skin surface and IBU applied with and without PG and PEG 200 and/or OS.

Surgically excised samples of human female skin were obtained after abdominoplasty with informed consent under a protocol approved by the SCERH, Monash University, Australia (Project #2006/565). The preparation of skin for contact angle measurements was carried out as detailed in Section 4.4.2.1. As required by the laboratory at which the contact angle measurements were carried out, dermatomed skin was sterilised with gamma irradiation at a dose 25 kGy (Steritech, Victoria, Australia). Contact angle measurements were performed on abdominal skin obtained from three female donors.

To determine the contact angle between ethanolic solutions of IBU with and without the presence of PG, PEG 200 and/or OS against skin, a sessile drop of between 3 – 4 µL of each formulation was placed on sterilised dermatomed skin (as described above) using a manual syringe. The KSV CAM 200 device was used to measure the contact angle of the formulations with skin. The contour of the sessile drop was recorded by an integrated Charge-Coupled Device firewire camera at a rate of 1 picture/sec during the first 30 secs after placement of the formulation drop. The recorded pictures were subjected to contour analysis using the Atension Theta® analysis software which calculates the contact angles of recorded pictures between a virtual baseline (skin surface) and the virtual tangent going through the contact point of the formulation with skin. The contact angle of a droplet of each formulation against skin was measured in triplicate.

5.4.6 Viscosity measurement of OS

As will be discussed in Section 5.6, it is possible that the viscosity of PG, PEG 200 and OS may influence the lateral diffusion behaviour of IBU across human skin. Therefore, it was necessary to obtain the viscosity values of PG, PEG 200 and OS in order

to draw such conclusions. Following extensive literature research, the viscosity value of OS could not be obtained, while that of PG and PEG 200 was available[233,234]. Therefore, only the viscosity of OS was measured in this study.

Viscometric measurement for OS was performed using a Brookfield Cone and Plate Rheometer, Model LVDV-III+CP, (Brookfield Engineering Laboratories Inc, Massachusetts, USA). 1 mL of neat OS was added to a spindle plate and left to equilibrate with room temperature (24°C) for 20 mins. OS viscosity was determined using a CR-42 spindle at a torque of ~28.7%. The stability of the OS viscosity was observed over 30 mins.

5.4.7 Data analysis

The amount of drug removed from each concentric tape section from *in vitro* and *in vivo* studies was normalised to the mass of SC protein removed by the same concentric tape section in order to eliminate the inter-subject variability in the total mass of SC protein removed per layer of tape strip. The normalised concentration of drug was plotted against the cumulative weight of SC protein per unit area. To allow for the easy visualisation of both lateral diffusion and penetration across the SC, the normalised data was schematically shown on contour plots. The percentage recovery of IBU applied with PG, PEG 200 and/or OS at 3 mins, 3 hrs and 6 hrs relative to the applied dose was calculated in order to evaluate whether any difference in recovery across human skin *in vivo* may be due to possible further penetration. In the following sections, the distance of drug spread from the application site will be referenced as ‘section 1’, ‘section 2’, ‘section 3’ and ‘section 4’, which equates to distances of between 0 – 4 mm, 4 – 8 mm, 8 – 12 mm and 12 – 16 mm, respectively.

5.4.8 Statistical analysis

The one-way ANOVA test (using SPSS Statistics 19) was applied to determine whether there was a significant difference between (i) the percentage of IBU recovered at each time point when applied with and without PG, PEG 200 and/or OS *in vivo,* (ii) the compartmental recovery of IBU from the various formulations at 6 hrs *in vitro*, (iii) the amount of IBU recovered per unit area in each concentric ring, (iv) the percentage recovery of IBU relative to the applied dose under *in vivo* and *in vitro* conditions, (v) the total mass of SC protein removed from the uppermost region of the SC under *in vivo* and *in vitro* conditions and (vi) the contact angle that each formulation made with skin over 30 secs. A one-way ANOVA test was also used to determine whether there was a significant difference between IBU uptake when applied in the formulations *in vitro*. A probability of $p<0.05$ was deemed statistically significant. All data are presented as mean ± SEM, unless otherwise stated.

5.5 RESULTS

5.5.1 Validation of IBU extraction from adhesive tape in the presence of PG, PEG 200 and OS

Recovery and accuracy values for the extraction of IBU in the presence of PG, PEG 200 and OS are presented in Table 5.2. The recovery of IBU from adhesive tape was between 94 – 104% for all concentrations. The accuracy of extraction, as determined by %CV, was within accepted limits (<10%) at all concentrations, demonstrating that the presence of excipients did not impact on the assay conditions for IBU.

Table 5.2 Recovery and accuracy of IBU in the presence of PG, PEG 200 and OS (mean ± SD, n=5).

Theoretical amount (µg)	Spiked amount (µg)	Recovery (%)	Accuracy (%)	%CV
0.5	0.51 ± 0.1	103.2 ± 9.5	104.5 ± 9.6	9.2
50	51.9 ± 4.3	94.6 ± 8.4	98.2 ± 8.4	8.8
125	129.8 ± 7.3	101.6 ± 5.6	105.5 ± 5.5	5.5

5.5.2 Lateral spreading and penetration of IBU across human skin *in vivo* in the presence of PG, PEG 200 and/or OS

IBU was dosed in an ethanolic solution with and without the presence of excipients that are reported to enhance penetration and control viscosity. From each treatment area, 10 tape strips were removed using concentric adhesive tapes to allow for the assessment of the extent of lateral diffusion.

As shown by the contour plots in Figure 5.1, a droplet of 1.8 µL (containing 90 µg of IBU) displays various degrees of lateral diffusion on and within the SC depending on the type of excipient present. At 3 mins after application, the application of IBU in the absence of excipients was detected to have spread laterally across the skin surface into section 3 (Figure 5.1a). When IBU was dosed in the presence of PG and PEG 200 or PG, PEG 200 and OS, IBU did not spread further than section 3 (as seen in Figure 5.1d and j, respectively). Instead, IBU formed a drug depot mainly concentrated within the boundaries of section 1. However, when IBU was applied with OS, IBU appeared to exhibit lateral diffusion relatively rapidly after application by forming a flat film on the SC surface that spread into section 4 as seen in Figure 5.1g.

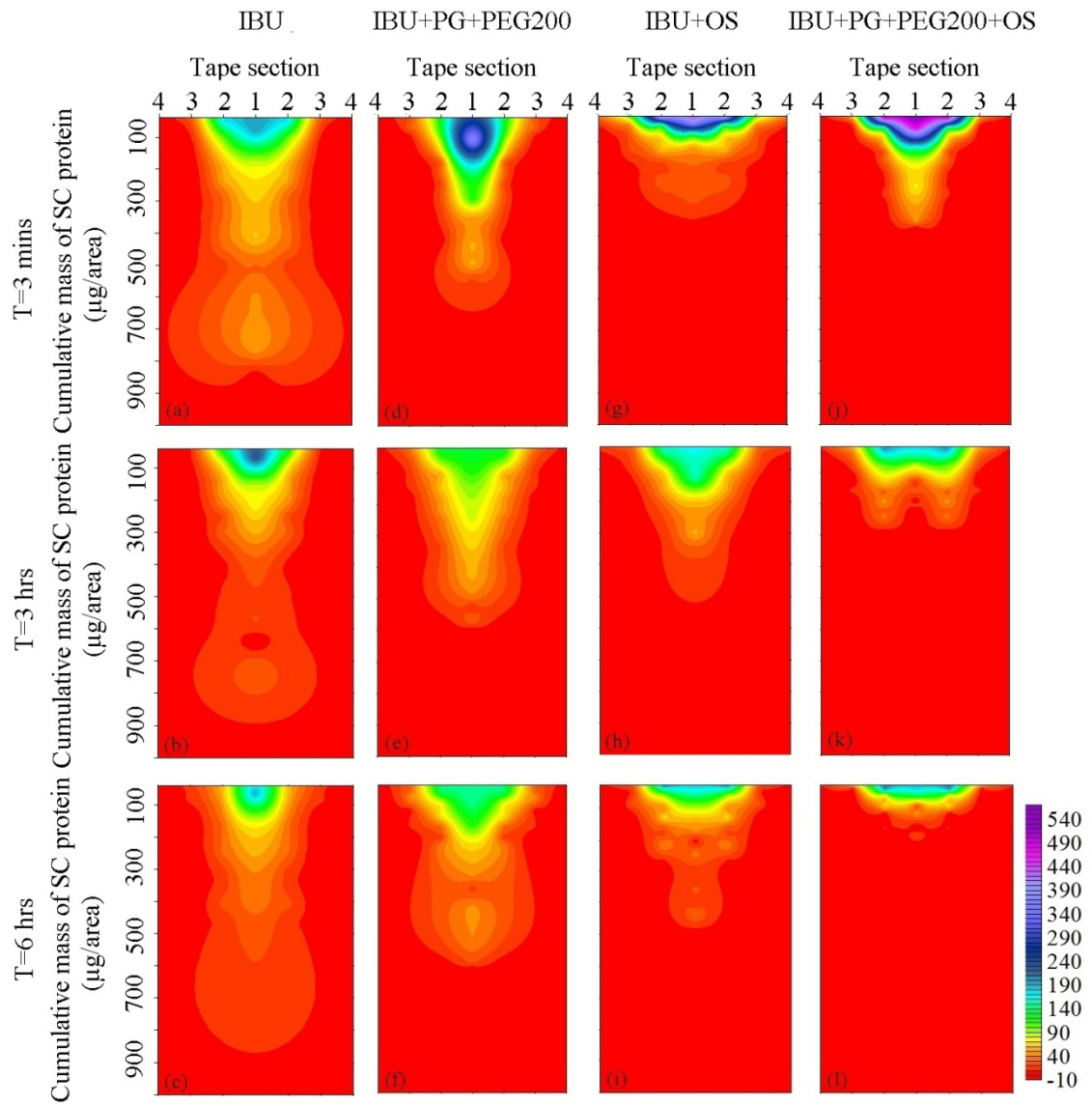


Figure 5.1 Contour plot displaying the extent of lateral spread and penetration IBU applied with and without excipients at 3 mins, 3 hrs and 6 hrs after *in vivo* application to humans. Note the legend values are expressed as concentration of IBU (ng/µg).

Further penetration of IBU through the SC and/or lateral diffusion of IBU across and within the SC bilayers were evident over time when applied with and without excipients. As stated in Chapter 3, when IBU was applied alone, the concentration of IBU removed from section 3 at a SC depth of 400 – 600 µg/area appeared to increase between 3 hrs and 6 hrs as shown in Figure 5.1b – c.

Similarly, as seen in Figure 5.1e – f, the concentration of IBU removed from section 2 between the SC depths of 400 – 500 µg/area also increased between 3 hrs and 6 hrs when IBU was applied with PG and PEG 200. However, it cannot be deduced whether this increase in IBU concentration within the SC was due to lateral diffusion or penetration or both.

Nonetheless, it is evident that when IBU was applied in the presence of PG, PEG 200 and OS, IBU demonstrated further lateral diffusion across the SC surface into section 4 at 3 hrs after application which was not previously detected at 3 mins as shown in Figure 5.1k and j, respectively. At 6 hrs after application, however, IBU (applied in the presence of PG, PEG 200 and OS) was no longer detected in section 4 (as shown in Figure 5.1l), suggesting a possible redistribution back into section 3 or depletion from the SC surface.

No evidence of further penetration and/or lateral spreading of IBU when applied with OS were observed with increased time. However, for all formulations applied, the concentration of IBU decreased with an increase in horizontal and vertical distance from the application area. Furthermore, the recovery of IBU with and without PG, PEG 200 and/or OS from the outermost regions of the SC decreased over 6 hrs as demonstrated by the reduction in colour intensity of the contour plots shown in Figure 5.1. A portion of the unrecovered drug, however, may have absorbed into the deeper layers of the SC.

The lateral spreading behaviour of IBU applied with and without PG, PEG 200 and/or OS is further supported in Figures 5.2a – d. At 3 mins, the normalized concentration of IBU applied alone (Figure 5.2a) and in the presence of OS (Figure 5.2c) in section 2 is similar to that detected in the application area (section 1), while the concentration of IBU applied with PG and PEG 200 (Figure 5.2b) and PG, PEG 200 and OS (Figure 5.2d) in section 2 of each subsequent tape strip is less than half the concentration detected in section 1 of the corresponding tape strip. This further verifies that IBU applied alone or together with OS undergo a relatively rapid process of lateral diffusion and a higher tendency to spread than those formulations containing PG and PEG 200.

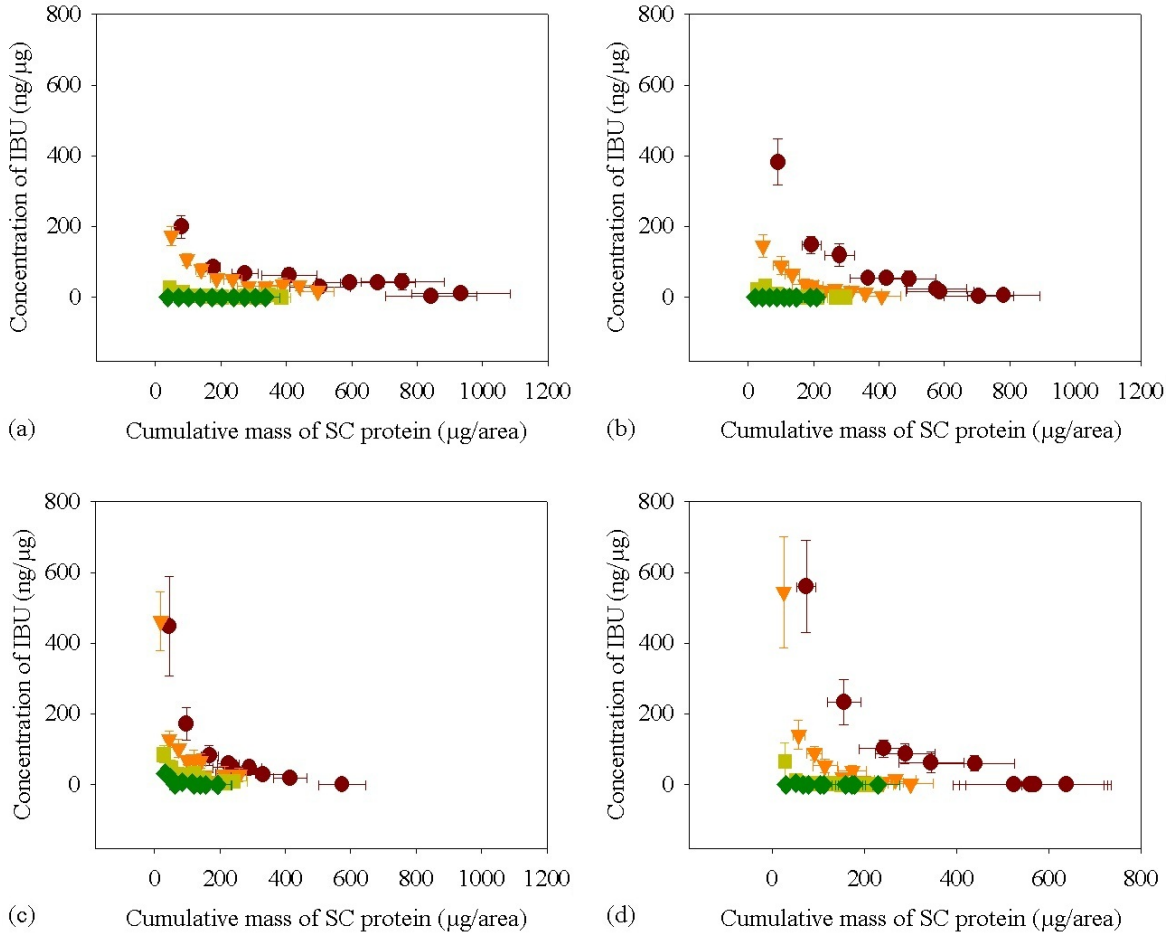
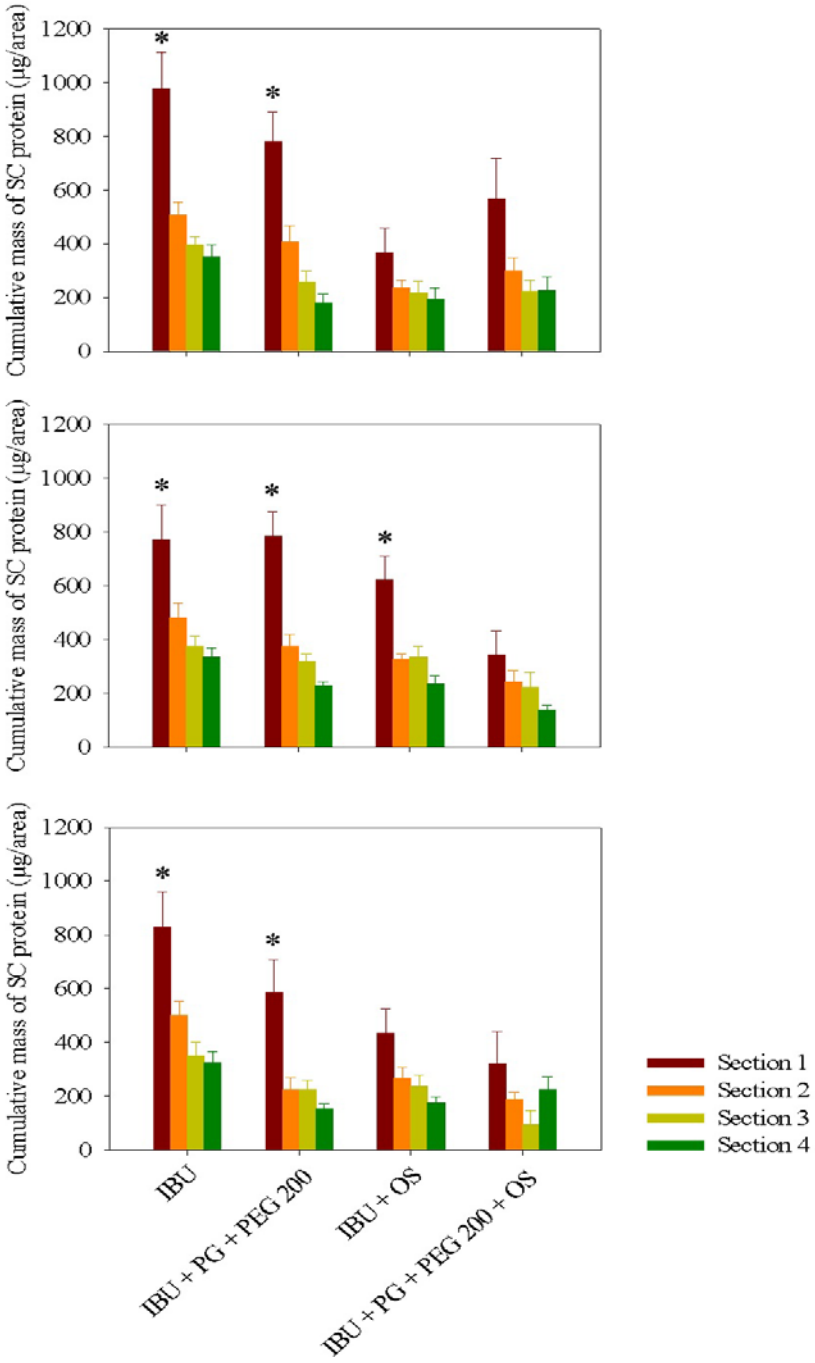


Figure 5.2 Distribution profile of IBU removed from (●) section 1, (▼) section 2, (■) section 3 and (◆) section 4 applied (a) alone, (b) with PG and PEG 200, (c) with OS and (d) with PG, PEG 200 and OS across human SC at 3 mins after application *in vivo* (mean ± SEM, n=8).

5.5.3 Effect of vehicle on SC protein removal using adhesive tape

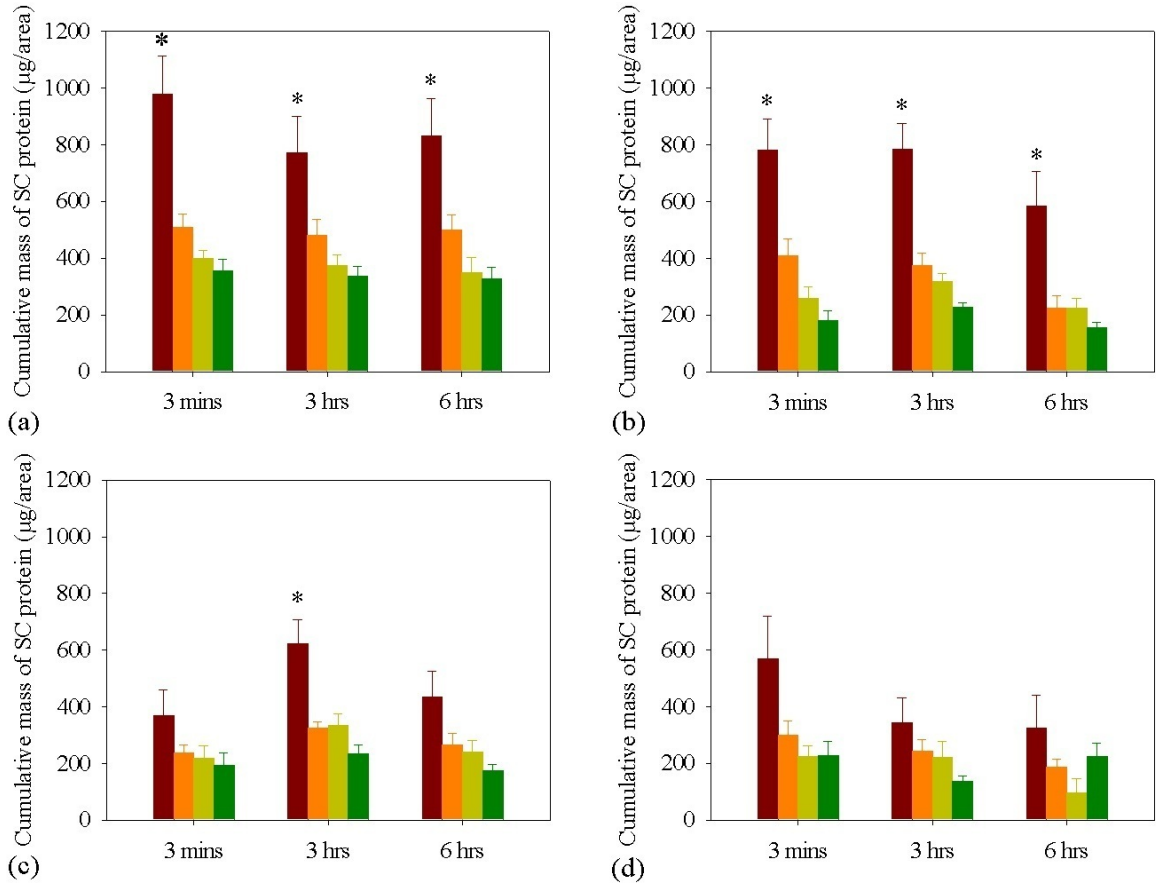
As the concentration of IBU removed per tape strip was normalised against the mass of SC protein removed, it was of interest to assess whether different vehicles affect SC protein removal, as displayed in Figure 5.3. Although not statistically significant for all formulations, a trend appears whereby the cumulative mass of SC protein removed from section 1 is generally higher than that removed from section 2, 3 and 4 at 3 mins, 3 hrs and 6 hrs after application. However, the cumulative mass of protein removed in section 2, 3 and 4 at 3 mins, 3 hrs and 6 hrs after application are not significantly different. Furthermore, the presence of PG, PEG 200 and OS did not affect the mass of SC protein removed compared to that of the IBU applied alone.



(*) denotes significant difference in cumulative mass of SC protein compared to that removed from section 2, 3 and 4.

Figure 5.3 Effect IBU applied with PG, PEG 200 and/or OS on the cumulative mass of SC protein removed at (a) 3 mins, (b) 3 hrs and (c) 6 hrs after application compared to IBU applied alone (mean ± SEM, n=8).

Figure 5.4 presents the cumulative mass of SC protein removed per unit area from skin sites under section 1 – 4 of the concentric adhesive tape over time when ethanolic solutions of IBU with and without PG, PEG 200 and/or OS were applied. It is evident that the length of exposure time does not affect the reproducibility of SC protein removal with adhesive tape. However, the cumulative mass of SC protein removed from skin sites directly under section 1 is significantly higher than the cumulative amount removed at section 2, 3 and 4 when IBU was applied alone and in the presence of PG and PEG 200. The difference in cumulative mass of protein removed in section 2, 3 and 4 is not significant, despite a trend whereby the mass of cumulative protein removed decreases with increased distance from application site.



(*) denotes significant difference of cumulative mass of SC protein removed from section 1, compared to section 2, 3 and 4 of concentric adhesive tape.

Figure 5.4 Cumulative mass of SC protein removed from (■) section 1, (■) section 2, (■) section 3 and (■) section 4 of adhesive tape following application of IBU applied (a) alone, (b) in the presence of PG and PEG 200; (c) in the presence of OS and (d) in the presence of PG, PEG 200 and OS on humans *in vivo* (mean ± SEM, n=8).

5.5.4 Recovery of IBU from adhesive tape strips *in vivo*

The percentage recovery of IBU applied with and without PG, PEG 200 and/or OS from the combined 10 tape strips at 3 mins, 3 hrs and 6 hrs is displayed in Table 5.3. At 3 mins after application, on average approximately 20% of IBU could not be accounted for; suggesting a possible rapid penetration of drug into the deeper viable tissues as the validated IBU extraction method is able to account for 100 ± 10% of drug applied (refer to Chapter 2). The amount of IBU present on the uppermost layers of the SC substantially decreased at 3 hrs for all formulations and remained unaltered between 3 hrs and 6 hrs.

Table 5.3 % Recovery of IBU at 3 mins, 3 hrs and 6 hrs after application to that of the applied dose on humans *in vivo* (mean ± SEM, n=8).

	% Recovery of IBU			
Time	**IBU**	**IBU + PG + PEG 200**	**IBU + OS**	**IBU + PG + PEG 200 + OS**
3 mins	86.6 ± 5.8	82.0 ± 7.7	86.6 ± 7.0	77.0 ± 7.0
3 hrs	61.3 ± 6.7*	61.4 ± 7.3*	59.8 ± 6.3*	52.2 ± 3.9*
6 hrs	47.7 ± 5.7*	60.3 ± 15.5*	46.0 ± 11.4*	46.5 ± 3.9*

(*) denotes a significant difference in IBU recovery to that at 3 mins.

5.5.5 *In vitro* diffusion study

The permeability profile of IBU applied with and without excipients through dermatomed human skin is shown in Figure 5.5. At 6 hrs after application, less than 3.5% of the applied dose of IBU when applied with and without PG, PEG 200 and/or OS was detected in the receptor solution. No enhancement in IBU uptake was observed in the presence of PG, PEG 200 and/or OS over the 6 hr period studied.

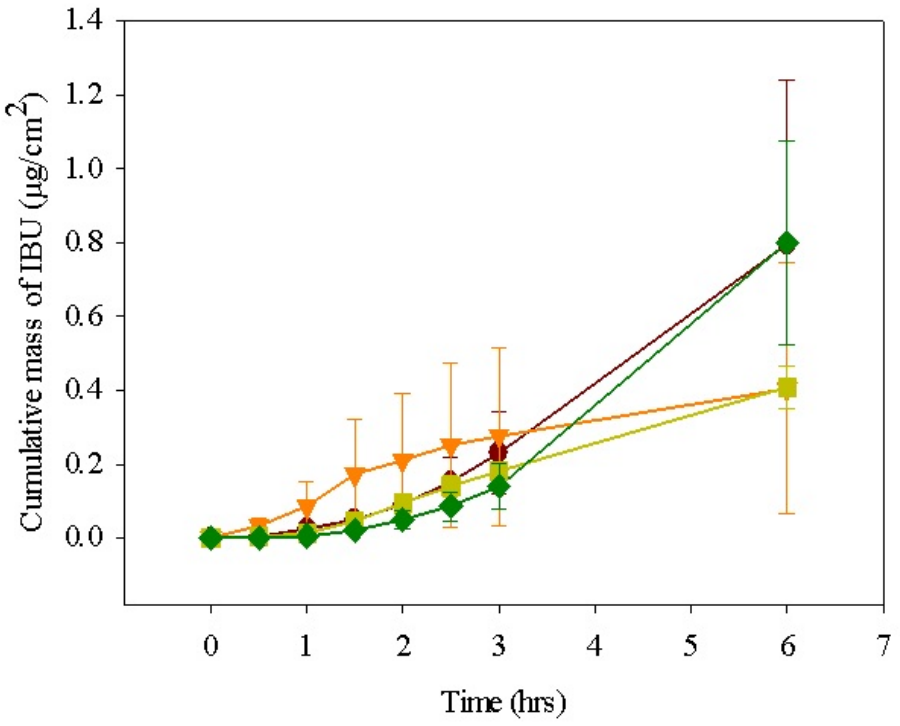


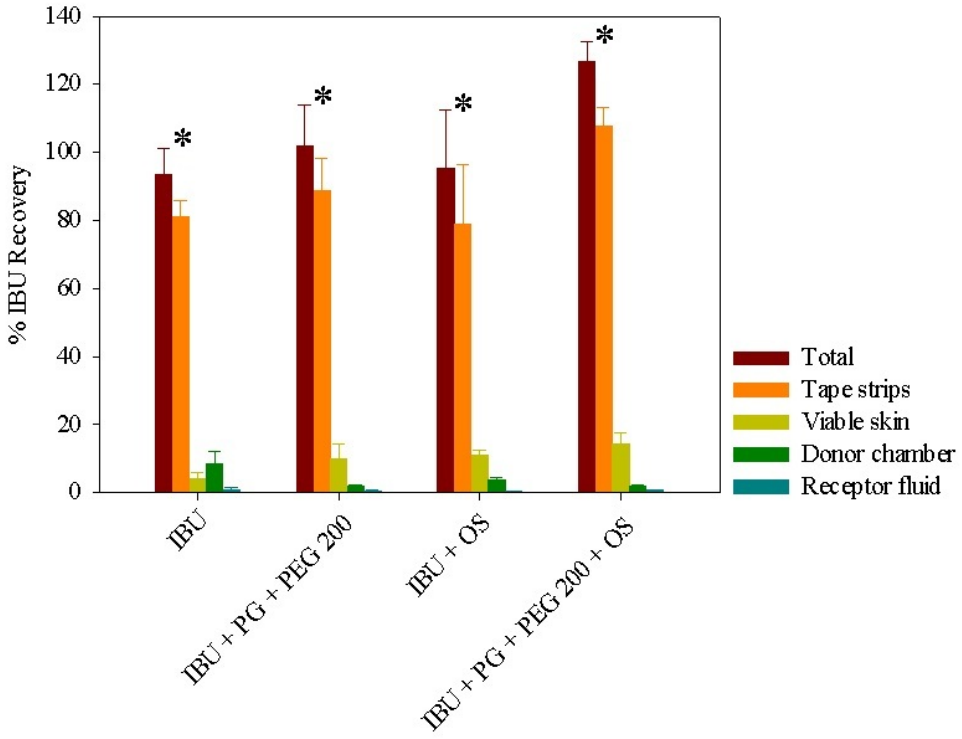
Figure 5.5 Permeation profile of IBU alone (●), with PG and PEG 200 (▼), with OS (■) or with PG, PEG 200 and OS (◆) across excised dermatomed human skin *in vitro* (mean ± SEM, n=12).

5.5.6 Mass balance drug analysis *in vitro*

A mass balance analysis of IBU applied with and without PG, PEG 200 and/or OS was performed whereby the amount of IBU residing in the uppermost layers of the SC, the viable epidermal and dermal skin sections, the donor chamber of the diffusion cell set-up, as well as the amount of drug permeated through the dermatomed membrane into the receptor solution was determined. The percent recovery of IBU in each compartment at 6 hrs after application is shown in Figure 5.6.

At 6 hrs after exposure, recovery of IBU when applied in the presence of PG, PEG 200 and OS was higher than the recovery of IBU when applied with PG and PEG 200 or OS alone, and indeed greater than the theoretical applied dose. Approximately 100% of the

applied dose of IBU when applied alone or in the presence of PG and PEG 200 or OS alone was recovered. The majority of drug remained in the outermost layers of the skin as ~80% of IBU was extracted from the 8 sequential tape strips. Approximately 10% of the applied drug dose was found to penetrate into the viable dermis while less than 3.5% was detected in the receptor solution. The amount of IBU detected to have spread onto the donor chamber was significantly higher for IBU applied alone. No other notable differences in the recovery of IBU between formulations were observed in other compartments.



(*) denotes significant difference in % recovery of IBU compared to that recovered from the viable skin, donor chamber and receptor fluid.

Figure 5.6 *In vitro* mass balance analysis of IBU applied with and without PG, PEG 200 and/or OS at 6 hrs after application (mean ± SEM, n=12).

5.5.7 Comparison of *in vivo* and *in vitro* recovery of IBU and SC protein at 6 hrs

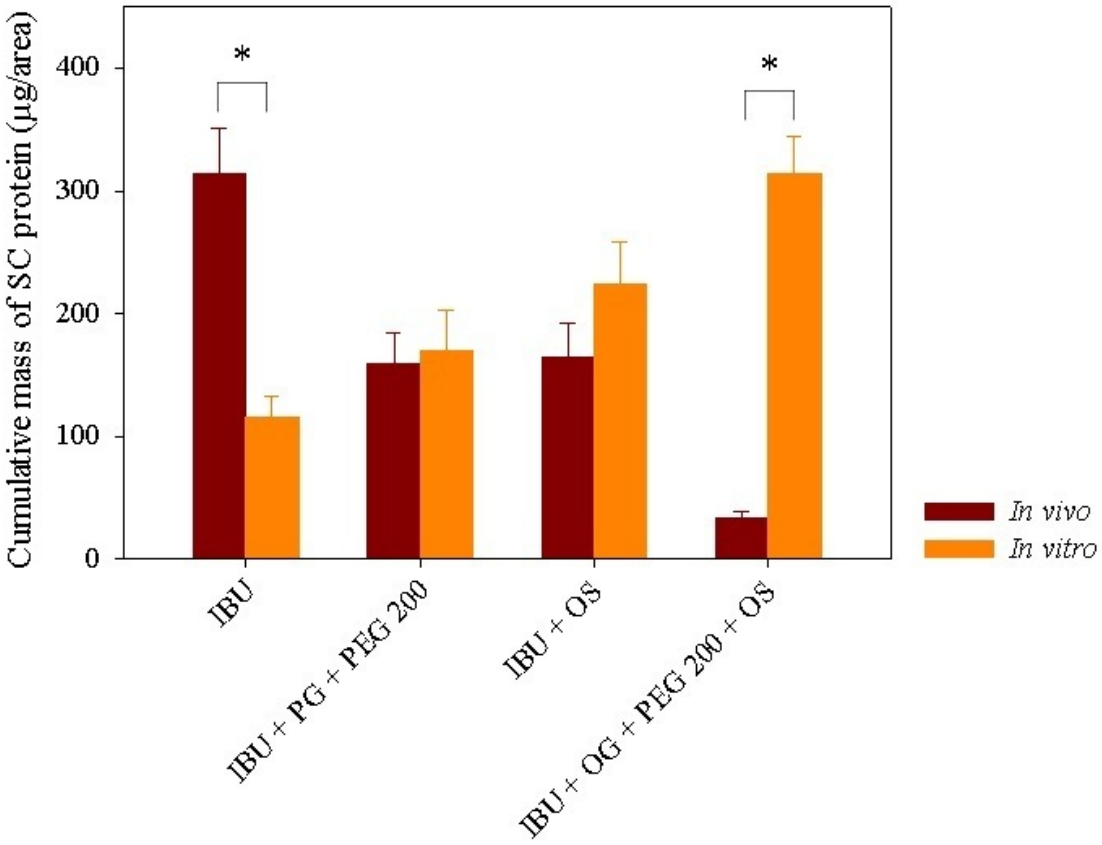
Tape stripping of the SC was performed at 6 hrs after application *in vivo* and *in vitro* to allow for comparisons to be made between data obtained under the two different conditions. The percent recovery of IBU at 6 hrs *in vivo* and *in vitro* is displayed in Table 5.4. It should be noted that due to the size constraint of the diffusion cells (3 cm^2), only section 1 and 2 of the concentric tapes were used for tape stripping *in vitro*.

Generally, *in vitro* recovery of IBU at 6 hrs is 2-fold higher than that recovered *in vivo* at 6 hrs, but is comparable to the amount of IBU recovered *in vivo* at 3 mins after application, as also presented in Table 5.4.

Table 5.4 % Recovery of IBU *in vivo* and *in vitro* (mean ± SEM; *in vivo*, n=8; *in vitro*, n=12).

	% Recovery of IBU from 8 tape strips (section 1 & 2)		
Formulation	***In vitro* 6 hrs**	***In vivo* 6 hrs**	***In vivo* 3 mins**
IBU	81.1 ± 3.8	38.4 ± 4.1	75.7 ± 4.9
IBU + PG + PEG 200	88.8 ± 6.5	49.7 ± 11.0	74.5 ± 5.6
IBU + OS	78.8 8.8	49.8 ± 7.6	66.3 ± 6.4
IBU + PG + PEG 200 + OS	107.9 ± 4.3	48.8 ± 6.9	82.6 ± 7.6

The tape stripping studies performed *in vivo* and *in vitro* also allowed us to assess whether the amount of SC protein removed by tape stripping *in vivo* is reflective of the amount removed *in vitro* and vice versa. Figure 5.7 displays the cumulative mass of protein removed per unit area from 8 repeated tape strips at 6 hrs *in vivo* and *in vitro* following the application of IBU with and without PG, PEG 200 and/or OS. As demonstrated, no pronounced correlation exists between the removal of SC protein *in vivo* and *in vitro.* However, it is interesting to note that while the cumulative mass of SC protein removed from the combined eight tape strips is comparable between *in vivo* and *in vitro* conditions when IBU is applied with PG and PEG 200 or OS alone, the cumulative mass of SC protein removed when IBU was applied alone *in vivo* was considerably higher than that removed *in vitro*. On the other hand, when IBU was applied the presence of PG, PEG 200 and OS, the cumulative mass of SC protein removed *in vivo* was significantly lower compared to that recovered *in vitro*.



(*) denotes significant difference in cumulative mass of SC protein between *in vivo* and *in vitro* conditions.

Figure 5.7 Comparison of cumulative mass of protein removed from human skin *in vivo* and *in vitro* (mean ± SEM; *in vivo*, n=8; *in vitro*, n=12).

5.5.8 Measurement of contact angles of IBU applied with and without PG, PEG 200 and/or OS on human skin

The contact angle formed by ethanolic droplets of IBU with and without the presence of PG, PEG 200 and/or OS on skin was measured over the first 30 secs following application to determine the possible differences in interfacial tension of IBU applied with and without excipients on the skin surface. The change in contact angle of ethanolic solutions of IBU applied with and without PG, PEG 200 and/or OS at 0, 1, 8, 20 and 30 secs after application is shown schematically in Figure 5.8 and numerically in Table 5.5.

Immediately after application (T=0 secs), the droplet of all formulations formed a sphere on the skin surface. The contact angle formed by IBU when applied in the presence of PG and PEG 200 were significantly larger than that formed by IBU applied alone or in the presence of OS over 30 secs. The droplets gradually decreased in angle with increasing residence time on the skin. For IBU applied alone or in the presence of OS, the droplet was no longer detectable on the skin at 20 secs after application while that of IBU applied in the presence of PG and PEG 200 were still measurable. As the contact angle of each droplet of the formulations decreased, an increase in contact area (or spreading) was evident.

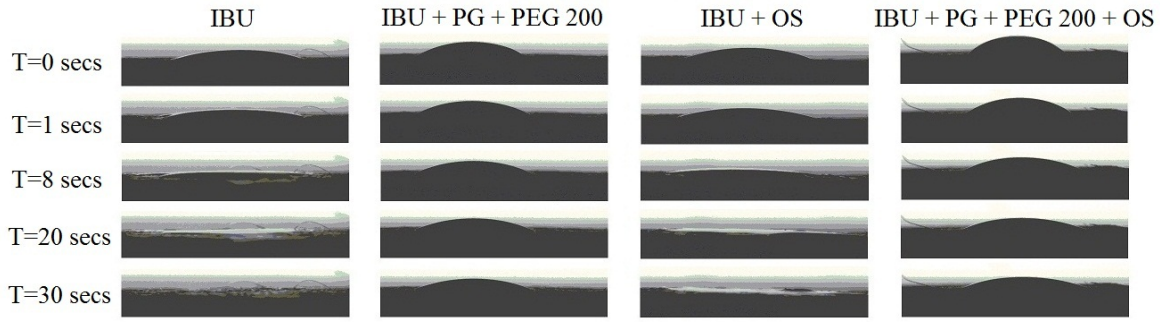


Figure 5.8 Screenshot of change in contact angle of IBU applied (a) alone, (b) with PG and PEG 200, (c) with OS and (d) in the presence of PG, PEG 200 and OS at 0, 1, 8, 20 and 30 secs after application onto human skin.

Table 5.5 Contact angle (°) IBU applied with and without excipients on skin over 30 secs (mean ± SD, n=3).

Time (secs)	IBU	IBU + PG + PEG 200	IBU + OS	IBU + PG + PEG 200 + OS
0	21 ± 2	30 ± 4*	22 ± 3	50 ± 4*$^{\#}$
1	14 ± 1	24 ± 3*	17 ± 2	33 ± 3*$^{\#}$
8	3 ± 1	23 ± 3*	8 ± 2	25 ± 3*
20	n/a	21 ± 2*	n/a	22 ± 2*
30	n/a	20 ± 2*	n/a	19 ± 2*

(*) indicates significant difference to IBU applied alone and in the presence of OS.

($^{\#}$) indicates significant difference to IBU applied in the presence of PG and PEG 200.

5.5.9 Viscosity measurement of OS

As will become evident in Section 5.6, the viscosity of the vehicle remaining on the skin surface following solvent evaporation may influence the lateral spreading behaviour of drug on and within the SC. The viscosity of OS at 24°C was 8.6 mPa.S over 30 mins as shown in Figure 5.9

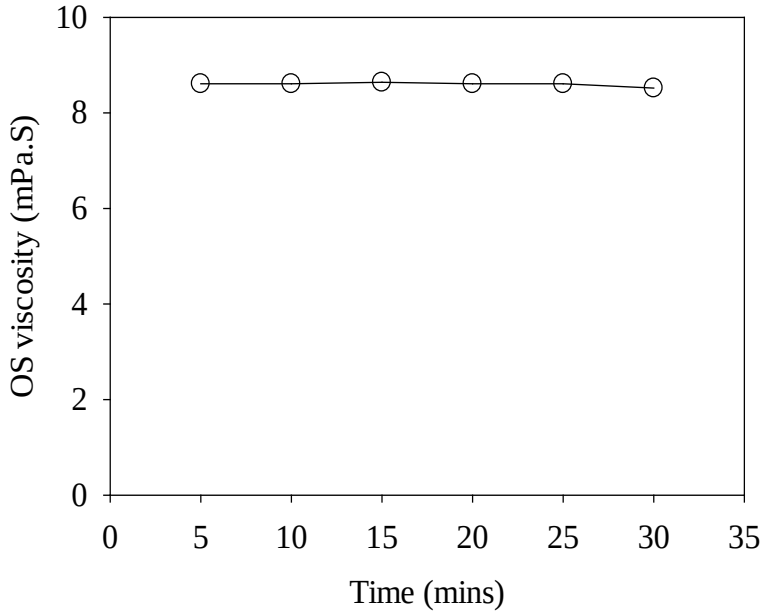


Figure 5.9 Viscosity measurement of OS at 24°C.

5.6 DISCUSSION

Excipients are frequently used in transdermal products to increase drug uptake, improve the appearance of the product and/or enhance its aesthetic appeal. The physical and chemical properties of excipients and vehicles added to a formulation play a major role in determining the rate of uptake and penetration of the medicament through the membrane[235]. Vehicles can modify either the thermodynamic activity of the drug[236] or the barrier properties of the skin[237]. Therefore, it was relevant to assess the effects of PG, PEG 200 and OS on the penetration and lateral diffusion of IBU across the SC at various time points. Furthermore, as the reliability of *in vitro* data to reflect that of *in vivo* conditions is continually discussed in literature, a comparative study on the recovery and distribution profile of IBU (with and without the presence of excipients) across the SC, *in vivo* and *in vitro* was performed.

The lateral diffusion behaviour and penetration of IBU with and without the presence of excipients at various exposure times are displayed in Figure 5.1. At 3 mins after application, IBU applied with OS was already detected in the outermost regions (i.e. 16 mm from site of application), while IBU applied alone or in the presence of PG and PEG 200 could not be quantified beyond section 3. Octisalate is commonly used in sunscreen products as an active UV-absorber[233] and has been reported to act as a successful penetration enhancer[69,70,233]. Though the enhancement mechanism of OS has not yet been completely elucidated, it is thought that penetration enhancers generally exert their activity by spatial disruption of the normally ordered arrangement of the intercellular lipids[66] and thereby creating less diffusional resistance for penetrating molecules. Therefore, upon dosing IBU in the presence of OS, it was anticipated that the penetration enhancing effect of OS would promote a larger amount of IBU to penetrate into the deeper layers of the SC and thereby leaving less drug available for lateral diffusion. However, the contrary findings

observed in this study may be attributed to the initial contact angle formed between OS and skin as shown in Figure 5.8 and Table 5.5.

At 3 mins after application, the amount of IBU recovered in the outer regions of the concentric tapes when applied in the presence PEG 200 and PG is significantly less than the amount of IBU recovered in section 1 as shown in Figure 5.2. PG has been widely used as a cosolvent whilst PEG 200 has been reported to modify viscosity and to enhance drug penetration. However, it appears that the both PG and PEG 200 also play a vital role in the lateral diffusion behaviour of permeants. As Ho et al.[238] pointed out, the pH value of the vehicle, the drug solubility and the viscosity of the vehicle are three important factors to consider in the evaluation of drug penetration across a membrane. Similarly, these factors can also be applied when assessing the lateral diffusion of solutes across the skin. The rate of absorption of a chemical across the skin is believed to be dependent on the concentration of drug in the vehicle. Partitioning of the chemical between the vehicle and the SC results in a concentration gradient developing perpendicularly to the skin surface[239], and most probably laterally across the SC. The partitioning of the drug out of the vehicle is influenced by the chemical-vehicle interactions, which is in turn governed by the solubility of the penetrant and its diffusive mobility within the vehicle[240]. Therefore, a high affinity of IBU for PG and/or PEG 200 may be one explanation for the smaller degree of lateral diffusion of IBU applied in the presence of these excipients.

Taking into account that viscosity may also control drug release[241], it can also be postulated that the relatively high viscosity of PG and PEG 200 retards the radial movement of the formulation from the application area. Following evaporation of the ethanolic solvent, the non-volatile components remaining on and within the SC includes IBU with and without PG (180 µg), PEG 200 (180 µg) and/or OS (90 µg). It is probable that IBU forms a strong complex with PG, PEG 200[242,243] and/or OS, thereby making it

difficult for IBU to diffuse out from the vehicle to the skin. Instead, the lateral diffusion of IBU is dependent on the movement of the vehicle remaining on the skin and is thus faced with the same resistance to spread as PG, PEG 200 and OS. At 24°C, PG, PEG 200 and OS exhibit a viscosity of 46.4 mPa.S[244], 50.7 mPa.S[234] and 8.6 mPa.S, respectively. The viscosity of PG and PEG 200 is significantly higher than the viscosity of OS and indicating a higher resistance of vehicle to flow (as also reflected in results shown in Section 5.5.2). Therefore, the viscosity of a topical formulation will affect its application and delivery of the active agent through and across the skin which could then possibly result in variance in its therapeutic performance[245].

The contact angle formed between a sessile droplet of IBU applied with and without PG, PEG and/or OS may further provide insight into the lateral diffusion behaviour of IBU when applied with and without excipients across the SC. The contact angle is the internal angle in a droplet of liquid in contact with a surface and represents the energetic equilibrium between the solid, liquid and gas phase[246]. As shown in Figure 5.8 and Table 5.5, the initial contact angle formed by IBU applied alone and in the presence of OS is significantly lower than that formed by IBU applied in the presence of PG and PEG 200 with skin and indicating greater adhesion to the skin. The contact angle of all formulations decreased with an increase of residence time on the skin. While the gradual reduction in contact angle may be attributed to the evaporation of the ethanolic solvent, the penetration of formulation into the SC as well as the influence of interfacial tension, close inspection of Figure 5.8 reveals that an increase in droplet radius also occurred. By 8 secs after application, the contact angle of a droplet of IBU applied alone and in the presence of OS approached 0° while the contact angle of IBU applied in the presence of PG and PEG 200 was measured to be approximately 23 – 25°. A droplet is generally accepted to display spreading behaviour if the contact angle is approximately 0° or 180°[246,247]. Thus, in

agreement with the lateral diffusion behaviour of IBU applied with and without excipients as demonstrated in Figure 5.1, IBU applied alone or with OS exhibited a higher tendency to spread across the skin surface, while the presence of PG and PEG 200 retained IBU in closer proximity to its site of application.

It has been documented that vehicles used in topical formulations change the skin's stripping properties in that both stripping profile and total skin harvest are conditioning time sensitive[86]. Tsai et al.[86] demonstrated that an increasing amount of tissue is obtained with increasing treatment time of the skin with formulation. However, as presented in Figure 5.4, our studies showed no significant difference in the cumulative amount of protein removed from each tape strip section from each formulation with increasing exposure time. Nevertheless, the various excipients appear to change the skin's permeability for IBU – probably due to the varying changes in the mechanical strength of the intercellular spaces. In these studies, the mass of SC protein is associated to the depth of IBU penetration into the SC. The depth of IBU penetration from the various vehicles at 3 mins, 3 hrs and 6 hrs after exposure is shown in Figure 5.1 and although not significantly different between all formulations, a trend appears whereby the depth of IBU penetration from section 1 is greatest when applied alone, than in the presence of PG, PEG 200 and OS.

When applied together with excipients, IBU was localised closer to the skin surface across all exposure times. This may be attributed to the inability of IBU to partition out from the encompassing vehicle (as explained above) within the 6 hr time frame studied. In contrast, the application of an ethanolic solution of neat IBU penetrated deeper within the SC. As well as being a common solvent of choice, ethanol is also known to enhance the penetration of topically-applied substances by modifying the barrier function of the skin through lipid fluidization[248], lipid disordering[249], lipid extraction[250] and modulation of the

lipid barrier and keratinized protein conformation at low ethanol concentrations[251]. In addition, as the small dose of ethanol evaporated soon after application, a saturated concentration of IBU was left on the skin surface which resulted in a higher concentration gradient across the SC (compared to other formulations with excipients) and consequently, a greater driving force for IBU penetration into the deeper skin layers.

While the concentric tape stripping method employed in these studies cannot reveal whether the penetrated IBU found at sites immediately adjacent to the application area is a result of lateral diffusion within the lipid bilayers or penetration from the preceding skin layer, the concentric tapes have successfully demonstrated that an applied drug dose is not confined to the area at which it was applied and may be lost from that site via lateral diffusion. In addition, these results verify that the process of percutaneous penetration occurs against the simultaneous process of lateral spread[182].

While it was expected that the recovery of IBU on the outermost layers of the SC would be lower for formulations incorporating excipients that have been reported to enhance transdermal drug delivery, no statistical difference is observed between the percent of IBU recovered with and without the presence of excipients at various exposure times as listed in Table 5.3. For all formulations, the total recovery of the applied dose of IBU from all sections of the combined 10 tape strips at 3 hrs and 6 hrs after application is significantly lower than that recovered at 3 mins. The reduction in colour intensities of the contour plots shown in Figure 5.1 suggests that the unrecovered IBU is mainly lost from the uppermost layers of the SC (i.e. the first few tape strips) to the surrounding environment as participants are free to go about their daily activities between 3 mins and 3 hrs. Any drug that was lost to the surrounding environment was unable to partition out of their corresponding vehicles and/or partition onto the SC to be absorbed and was thus loosely bound. The remaining IBU in the uppermost layers of the SC at 3 hrs after

application did not penetrate further into the SC as the recovery of IBU between 3 hrs and 6 hrs after application was unchanged.

However, as a small percentage of IBU does in fact penetrate into the viable epidermis by 6 hrs after application as shown in Chapter 4, it was also possible that the reduced recovery of IBU when applied in the presence of PG, PEG 200 and/or OS may be partially due to further penetration into the viable skin tissue. Therefore, *in vitro* diffusion studies were carried out on dermatomed human skin to determine what proportion of IBU lost from the skin surface was due to deeper percutaneous absorption and to determine whether the presence of PG, PEG 200 and/or OS enhance the permeation of IBU as reported in literature.

Figure 5.6 represents the total percentage of IBU recovered from the uppermost layers of the SC (through tape stripping), the donor chamber, the viable skin tissue and the receptor fluid at 6 hrs after application with respect to the applied dose. Approximately 100% of the applied dose of IBU was recovered *in vitro* from when applied alone, in the presence of only PG and PEG 200, or OS alone. However, the total recovery of IBU applied in the presence of PG, PEG 200 and OS was significantly higher at 125.07 ± 6.41%. Prior studies were carried out to assess whether the presence of excipients, the adhesive tape, residues of excised skin and/or the donor chamber may interfere with the process of IBU extraction and quantification. All results indicated that these factors did not impact IBU extraction and quantification and thus, the cause of the higher recovery of IBU when applied with PG, PEG 200 and OS is not known.

Due to the smaller area of skin exposure in the *in vitro* studies, the size of the concentric adhesive tapes used to determine lateral spreading were reduced to cover only section 1 and section 2. Therefore, any drug extracted from the donor chamber was

representative of the portion of drug that had spread further than section 2. As seen in Figure 5.6, a statistically higher percentage of IBU was recovered from the donor chamber when applied without excipients compared to that recovered in the presence of excipients. This comes as a surprise as although only IBU applied with OS had spread into section 4 *in vivo*, no significant difference was detected between the total amount of drug reaching section 3 and 4 *in vivo*. It should also be acknowledged that no significant difference in IBU recovery from the donor compartment was observed when applied in the presence of excipients and this appears to be consistent with the *in vivo* data illustrating that although the viscosity of PG and PEG 200 delayed the immediate lateral diffusion of IBU *in vivo*, radial distribution from the application site does occur with extended exposure time[182].

The compartment retaining the highest quantity of IBU at 6 hrs after application *in vitro* is the SC. Following the removal of the SC with eight successive tape strips, over 80% of the applied drug dose of all formulations still remained in the uppermost region of the SC as shown in Figure 5.6. The presence of penetration enhancers and/or cosolvents did not reduce the amount of IBU on the skin surface and therefore indicates they had no impact on drug uptake within the 6 hr time frame studied. Furthermore, as evidenced in the results obtained in Chapter 4 and as Potard et al.[208] put forward, the quantity of the product found in the SC does not always reflect the quantity penetrated into the viable skin and/or receptor solution.

Between 4 – 14% of IBU applied with and without PG, PEG 200 and/or OS was recovered from the remaining viable dermis *in vitro* immediately after tape stripping, indicating that drug had penetrated beyond the SC depth of which was sampled. As Figure 5.6 shows, the amount of IBU detected in the viable skin was significantly higher when applied in the presence of PG, PEG 200 and OS compared to that recovered of IBU applied alone. However, as the total amount of IBU recovered from all compartments *in vitro* was

significantly higher when applied in the presence of PG, PEG 200 and OS than all other formulations due to reasons that cannot be determined; we are unable to conclude that the higher IBU recovery (when applied in the presence of PG, PEG 200 and OS) in the viable skin is symbolic of the enhancement effect of the excipients.

Nevertheless, the permeation profile of IBU from the various formulations as shown in Figure 5.5 demonstrates that the presence of known penetration enhancers did not in fact enhance IBU uptake over the 6 hr time course. Between 1.2 – 3.5% of the applied drug doses were detected in the receptor solution. It is plausible that the duration of the *in vitro* permeation studies need to be extended beyond 6 hrs as a lag time of 6 hrs or more had been reported for IBU permeation across the SC[252,253]. If this is correct, then any enhancement effect of PG, PEG 200 and/or OS would not be detected until steady-state is reached. Therefore, the results in this chapter suggest that it would be beneficial to extend the duration of the *in vitro* permeation of IBU with and without excipients to evaluate if the presence of PG, PEG 200 and/or OS improved drug uptake and how this would affect lateral spreading.

As the SC of human volunteers and excised dermatomed skin was tape stripped at 6 hrs after application for IBU content and the amount of SC protein removed per strip, it was possible to determine whether the recovery of drug within the SC was comparable between *in vivo* and *in vitro* conditions as have been suggested in various literature[106,254].

The main difference between the *in vivo* and *in vitro* experiments is the absence of perfusion within the *in vitro* tests. The blood circulation still present under *in vivo* conditions causes a high tissue clearance by the blood stream and, under some circumstances, may prevent the build up of drug in the SC[255]. This may contribute to the lower recovery of IBU from the combined tape strips collected at 6 hrs *in vivo* as listed in

Table 5.4. For all formulations, the cumulative amount of IBU recovered at 6 hrs *in vivo* is approximately 2-fold lower than that recovered at the equivalent time *in vitro*. A similar observation was also recorded by Scott et al.[106]. More interestingly, the recovery of IBU at 3 mins *in vivo* is similar to that recovered at 6 hrs *in vitro*. Since *in vitro* skin does not undergo cell turnover, a higher concentration of permeating compound will remain on the SC over time[104,105]; or more fittingly, as *in vivo* subjects are able to resume their daily activities in between sample collection times, it is likely that any drug that was loosely bound to the SC may have been lost to the surrounding environment and/or rubbed off onto clothing or nearby objects. Under *in vitro* conditions however, the excised skin remained stationary over 6 hrs with the absence of a fully active micro-circulation, metabolism and the usual process of skin turnover – all which allows for the accumulation of drug on the SC surface.

Furthermore, no correlation could be made between the cumulative amount of SC protein removed at parallel time points under *in vivo* and *in vitro* conditions as seen in Figure 5.7. Under *in vitro* conditions, the excised skin was continually hydrated with receptor solution and as suggested by Weigand et al.[256], prior hydration of the SC facilitates its removal by cellophane tape. Removal of the hydrated SC can be accomplished with about one-third the number of strips normally required[86] and this was apparent in these studies as the complete removal of the SC was marked when the skin samples appeared to 'glisten' after fewer tape strips. However, the complete removal of the SC did not occur with every *in vitro* skin sample stripped. On this basis, it was important not to compare individual tape strips with corresponding strips for different treatment times, vehicles or condition. Furthermore, it was thus necessary to normalise the amount of drug recovered per tape strip to the amount of SC protein removed to determine the depth profile of the drug.

From the data presented, no correlation can be made for IBU recovery and SC protein removal between *in vivo* and *in vitro* conditions. While *in vitro* experiments provide an important tool for screening drug delivery systems and are a valuable method for predicting the behavior of drugs *in vivo*, results from such studies would be considered with caution.

5.7 CONCLUSION

An assessment of the lateral spreading and penetration behaviour of IBU in the presence of PG, PEG 200 and/or OS on human skin *in vivo* and *in vitro* was conducted. IBU applied in the presence of OS *in vivo* displayed a greater tendency to spread radially from the application site while IBU dosed with PG and PEG 200 showed a resistance to lateral diffusion – most probably due to its higher viscosity. IBU applied without the addition of excipients penetrated deeper into the SC while IBU applied with excipients remained in the outermost layers of the SC. PG, PEG 200 and/or OS did not enhance the permeation of IBU over 6 hrs and no correlation could be deduced between *in vivo* and *in vitro* results.

CHAPTER 6

ASSESSING THE EFFECT OF TIME ON THE LATERAL DIFFUSION AND PENETRATION OF IBU *IN VIVO* AND *IN VITRO*

6 ASSESSING THE EFFECT OF TIME ON THE LATERAL DIFFUSION AND PENETRATION OF IBU *IN VIVO* AND *IN VITRO*

6.1 INTRODUCTION

The diffusional resistance of the skin is primarily due to the unique physico-chemical composition of the SC[257] – often described by the 'brick and mortar' model whereby the corneocytes are the 'bricks' embedded in an intercellular lipid matrix[258]. When a permeant is applied onto the skin surface, its diffusion through the skin is governed by Fick's First Law of Diffusion, whereby the rate of transfer of a diffusing substance through a unit area of a membrane is proportional to the concentration gradient through that membrane:

$$\frac{\quad}{\quad}$$

Equation 6.1

The mass of drug penetration through the membrane per unit area, per unit time (steady-state flux (J)) is governed by the partition coefficient of the drug between the delivery vehicle and the membrane (K), the diffusivity of the permeant in the membrane (D), the diffusional pathlength within the membrane (h) and the concentration difference across the membrane (C)[259]. Hence, there is often a characteristic lag time prior to the accumulation of drug in the plasma to initiate a therapeutic effect or to attain steady-state flux. Similarly, a lag time is also often observed in *in vitro* permeation studies as time is required for the applied drug to traverse through the membrane prior to being detected in the receptor fluid. A lack of understanding of diffusional lag time may bring about inaccurate measurements of actual drug flux and absorption. Thus, it is necessary to characterise the full permeation profile of drug – including the lag time.

Penetration enhancers are often incorporated into formulations to increase the permeation rate of drugs across the SC barrier by reversibly perturbing the SC barrier properties[110]. However, when assessing the influence of penetration enhancers on absorption, any enhancement in drug uptake may not become obvious until steady-state is reached. Therefore, it is recommended that *in vitro* diffusion studies be carried out over 24 hrs[260] or 48 hrs[261] (or longer for permeants with longer lag times).

The 6 hr time frame of which diffusion studies were previously carried out in this research was selected to match the duration of *in vivo* studies (Chapter 5). However, no enhancement in IBU permeation across human skin in the presence of known penetration enhancers and excipients was observed within 6 hrs (Chapter 5). It is possible that steady-state flux was not reached within this short exposure period and consequently the effects of excipients may have been masked. Thus, it was decided to measure a full permeation profile by extending the duration of the measurements.

6.2 OBJECTIVES

The objectives of the studies in this chapter were to evaluate whether an extended duration of drug permeation with and without the presence of PG, PEG 200 and/or OS influenced the *in vitro* permeation of IBU across human skin.

As will become clear in the following sections, PG, PEG 200 and OS enhanced the uptake of IBU with prolonged exposure *in vitro*. Hence, the second objective of the studies described in this chapter was to assess whether the presence of excipients influenced the lateral diffusion and penetration behaviour of IBU after prolonged exposure *in vivo*. Therefore, the aims of this chapter were to:

- Determine whether the permeation of IBU is enhanced by PG, PEG 200 and OS after 48 hrs of exposure *in vitro*.
- Assess any changes in the lateral diffusion, penetration and distribution profile of IBU with and without the presence of PG, PEG 200 and OS *in vivo* after 24 hrs of exposure.
- Determine the effect of PG, PEG 200 and OS on the penetration and lateral diffusion of IBU across human skin *in vitro*.
- Determine whether there is a better relationship between the distribution profiles of IBU obtained *in vivo* and *in vitro* with these prolonged exposure times.

6.3 MATERIALS

All chemicals used in the following studies were obtained from the same manufacturers and distributors as detailed in Section 2.3 and Section 4.3.

6.4 METHODS

6.4.1 *In vitro* permeation of IBU through human skin with and without PG, PEG 200 and/or OS

As no enhancement of IBU uptake was observed over 6 hrs after application to human skin *in vitro* in the presence of excipients (refer to Chapter 5), permeation studies were repeated and the duration of receptor fluid collection extended to 48 hrs after application. Extending the permeation studies to 48 hrs allowed a clearer determination of whether steady-state was achieved within 6 hrs (as this was the longest duration used in studies in Chapter 3, 4 and 5). In addition, it was of interest to evaluate whether enhancement in IBU uptake *in vitro* could be observed after a prolonged exposure of 48 hrs.

6.4.1.1 Skin preparation

Surgically excised samples of human female skin were obtained after abdominoplasty with informed consent under a protocol approved by the SCERH, Monash University, Victoria, Australia (Project #CF08/1125 – 2008000555). The preparation of skin for permeation studies was carried out as detailed in Section 4.4.2.1

6.4.1.2 In vitro diffusion studies

The permeation of ethanolic solutions of IBU applied with and without the presence of PG, PEG 200 and/or OS across dermatomed human skin was performed as described in Section 4.4.2.2. However, as was mentioned in Section 6.2, two sets of *in vitro* diffusion studies were carried out. The first set of studies was carried out over 48 hrs to screen whether adequate time had been given to attain steady-state delivery of IBU. Thus, receptor fluid was collected into vials every 30 mins for the first 3 hrs then at 6 hr intervals (from 6 hrs) until 48 hrs using an automated fraction collector (ISCO Retriever II,

Nebraska, USA). Once the enhancement of IBU uptake in the presence of excipients was observed following a 48 hr exposure, *in vitro* permeation studies were repeated with IBU applied with and without PG, PEG 200 and OS. Receptor fluid was collected into vials every 6 hrs over a 24 hr period. The amount of IBU permeated over time was quantified using HPLC assays that were previously validated by Acrux Ltd and detailed in Section 4.4.4. The permeation of IBU from each formulation was studied across 3 donors, utilizing 4 cells from each donor.

6.4.1.3 Mass balance analysis in vitro

A mass balance analysis of IBU applied with and without excipients was performed *in vitro* immediately following diffusion sample collection at 24 hrs after drug dosing. Drug was extracted from the uppermost layers of the SC (through tape stripping) as detailed in Section 4.4.2.3, in the remaining skin sample (post-tape stripping) as specified in Section 4.4.2.3.3 and from the donor chamber as described in Section 4.4.2.3.2 as drug may have spread laterally across the SC surface and onto the chamber following dosing.

6.4.2 Lateral spreading and penetration of IBU with and without the presence of PG, PEG 200 and OS across human skin *in vivo*

As will come to light in Section 6.5.1, it became evident after completion of the 48 hr permeation studies that IBU exhibits a lag time of approximately 6 hrs before accumulation of drug in the receptor fluid reaches steady-state. Steady-state flux of IBU was achieved between 6 hrs and 24 hrs after application. Furthermore, the presence of PG, PEG 200 and OS significantly enhanced IBU uptake and flux compared to that of IBU applied alone. Hence, *in vivo* tape stripping studies (as performed in Chapter 3 and 5) were repeated over 24 hrs to assess whether a change in lateral spreading and penetration behaviour of IBU applied with and without PG, PEG 200 and OS across the SC could be observed following attainment of steady-state.

6.4.2.1 Participants

3 healthy volunteers (1 male and 2 female) provided written consent to participate in the study which was approved by the SCERH, Monash University, Victoria, Australia (Project #CF08/1125 – 2008000555). The participants were aged between 24 to 29 years of age and had no history of skin disease. Each participant was asked to refrain from applying any topical medicaments to their left and right flexor forearms at least 48 hrs prior to an experiment.

6.4.2.2 In vivo study design

To obtain a distribution profile of IBU with and without the presence of PG, PEG 200 and OS across human SC *in vivo*, the study design as detailed in Section 3.4.1.2 and tape stripping procedure described in Section 3.4.1.3 were followed. However, repeated tape strips of the SC were collected at 3 mins, 6 hrs and 24 hrs, rather than 3 min, 3 hrs and 6 hrs. Between 3 mins and 6 hrs, the solutions were left unoccluded and the participant was required to wear a short-sleeved shirt or have their sleeves rolled up to avoid loss of drug via rubbing off onto clothing. The SC was tape stripped at 6 hrs after the initial dose application. A metal tea leaf strainer made of wire mesh and with a cross-sectional area of 10 cm^2 was then placed over the 24 hr sample site and secured onto the skin with Tegaderm® as shown in Figure 6.1.

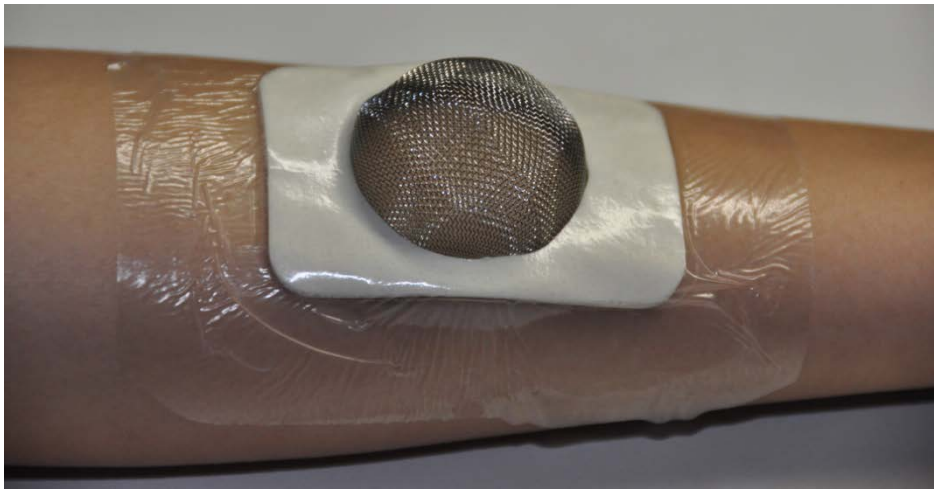


Figure 6.1 A photograph illustrating a tea strainer wire mesh adhered onto the forearm to protect the sampling site between 6 hrs and 24 hrs after application of drug onto the SC of participants.

The strainer was applied to prevent volunteers from rubbing any drug still adhered to the SC while they sleep. Participants were allowed to resume their daily activities, though they were advised to avoid activities that would cause perspiration as it may impact the spreading and penetration of the IBU applied. In addition, the participants agreed not to bath or shower for 24 hrs after the formulations were dosed onto their forearm to prevent any drug from being washed off. The wire mesh was chosen as it was strong enough to resist any deformation in shape if gentle pressure was applied and did not alter the non-occlusive nature of the application. The size of the strainer was chosen as its cross-sectional area is larger than the area of skin which was sampled. The strainer was removed from the volar forearm prior to tape stripping at 24 hrs. To ensure that residues from each formulation did not impact the evaluation of other formulations, each forearm was designated one formulation.

6.4.3 Data analysis

The amount of drug removed from each concentric tape section was normalised to the mass of SC protein removed by the same concentric tape section in order to eliminate the inter-subject variability in the total mass of SC protein removed per layer of tape strip. The normalised concentration of drug was plotted against the cumulative weight of SC protein per unit area. To allow for the easy visualisation of both lateral diffusion and penetration across the SC, the normalised data was schematically shown on contour plots. The percentage recovery of IBU applied with and without PG, PEG 200 and OS at 3 mins, 6 hrs and 24 hrs relative to the applied dose was calculated in order to evaluate whether any difference in recovery across human skin *in vivo* may be due to possible further penetration. In the following sections, the distance of drug spread from the application site will be referenced as 'section 1', 'section 2', 'section 3' and 'section 4', which equates to distances of between 0 – 4 mm, 4 – 8 mm, 8 – 12 mm and 12 – 16 mm, respectively.

6.4.4 Statistical analysis

A one-way repeated measures ANOVA test (using SPSS Statistics 19) was applied to determine whether there was a significant difference in (i) the percentage of IBU recovered at each time point from the different formulations *in vivo*, (ii) the recovery of IBU from the various formulations at 24 hrs *in vitro*, (iii) the amount of IBU recovered per unit area in each concentric ring and (iv) the cumulative mass of SC protein removed under *in vivo* and *in vitro* conditions. A one-way ANOVA test was used to determine whether there was a significant enhancement in IBU *in vitro* uptake in the presence of PG, PEG 200 and/or OS with prolonged exposure. A probability of $p<0.05$ was deemed significant. All data are presented as mean ± SEM (*in vivo*, n=3; *in vitro*, n=12).

6.5 RESULTS

6.5.1 Permeation of IBU through human skin over 48 hrs *in vitro*

The absence of enhanced IBU uptake over 6 hrs of exposure when applied in the presence of penetration enhancers (Chapter 5) gave grounds for a follow up permeation study over 48 hrs to determine whether IBU enhancement would occur with prolonged exposure. Figure 6.2 display the 48 hr permeation profile of IBU with and without PG, PEG 200 and/or OS across dermatomed human skin. The permeation profiles reveal that IBU did experience a lag time of ~6 hrs before steady-state was established and consequently provides insight into why it was not possible to measure an excipient-induced enhancement of IBU penetration (Chapter 5). IBU applied with PG, PEG 200 and/or OS shows steady-state flux between 6 hrs and 24 hrs after which IBU permeation plateaus. IBU applied alone, however displays constant flux up to 48 hrs.

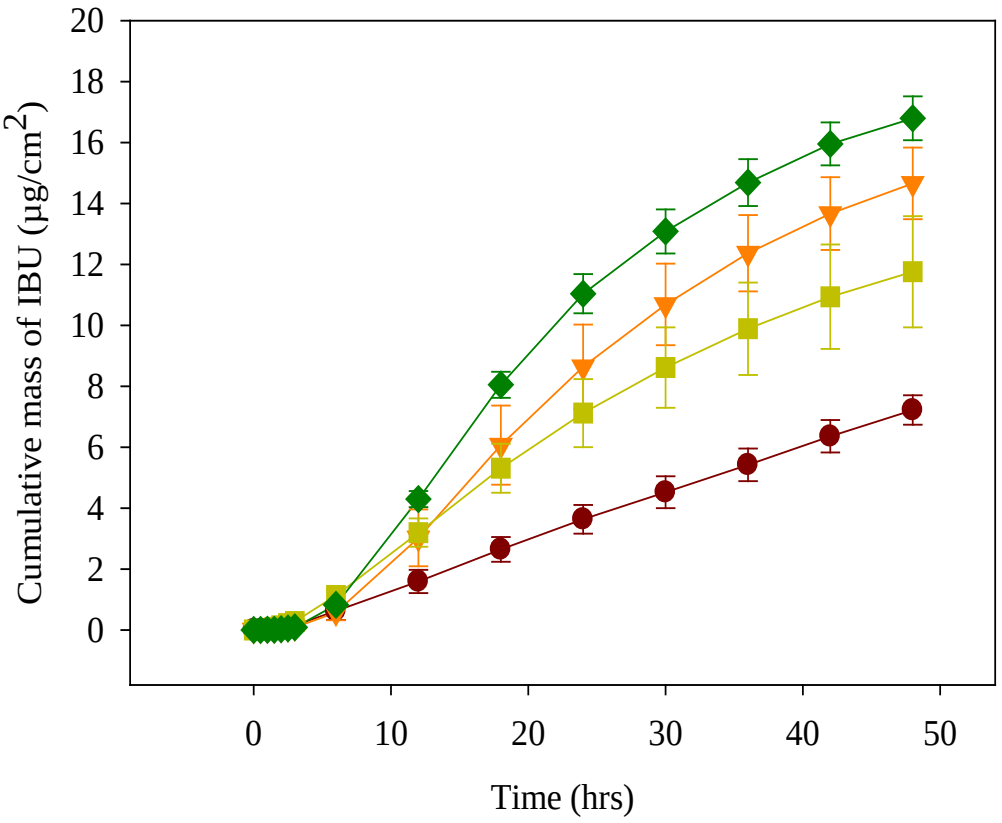


Figure 6.2 Permeation profile of IBU alone (●), with PG and PEG 200 (▼), with OS (■) or with PG, PEG 200 and OS (◆) across excised dermatomed human skin *in vitro* (mean ± SEM, n=12).

The flux values of IBU applied with and without excipients were calculated based on the steady state permeation between 6 hrs and 24 hrs. However, the cumulative mass of IBU permeated across the membrane was measured following 48 hrs of exposure to illustrate the total permeated mass and is listed in Table 6.1. IBU applied with PG and PEG 200, or PG, PEG 200 and OS showed a 2 and 3-fold increase in flux compared to the control, respectively. However, the presence of OS alone did not significantly enhance IBU permeation when using the one-way ANOVA test for significance.

Table 6.1 Cumulative mass of IBU permeated across excised dermatomed human skin *in vitro* at 48 hrs (mean ± SD, n=12).

Formulation	Steady-state flux ($\mu g/cm^2/hr$)	Cumulative mass of IBU at 48 hrs ($\mu g/cm^2$)
IBU	0.2 ± 0.0	7.2 ± 0.8
IBU + PG + PEG200	0.4 ± 0.1*	14.7 ± 2.0*
IBU + OS	0.3 ± 0.1	11.8 ± 3.2
IBU + PG + PEG200 + OS	0.6 ± 0.1*	16.8 ± 1.2*

(*) denotes significant difference to IBU applied alone.

6.5.2 Lateral spreading and penetration of IBU with and without the presence of PG, PEG 200 and OS across human skin *in vivo*

The results from the permeation of IBU applied with and without excipients over 48 hrs prompted the conduct of a second set of *in vivo* studies to evaluate any changes in *in vivo* lateral spreading and distribution of IBU applied with and without PG, PEG 200 and OS at 3 mins, 6 hrs and 24 hrs after application. These two formulations were selected as the combination of PG, PEG 200 and OS showed the most significant enhancement in IBU flux (relative to control). It was not necessary to measure the lateral spreading and penetration of IBU applied with and without excipients at 48 hrs as steady-state delivery was no longer apparent. The lateral diffusion and penetration behaviour of IBU applied alone and in the presence of excipients at 3 mins, 6 hrs and 24 hrs *in vivo* is displayed in the form of contour plots in Figure 6.3.

Unlike the observations in Chapter 5 where IBU did not spread beyond section 3 at 3 mins after application, IBU applied with and without excipients was detected to have spread into section 4 of the sampling site (Figure 6.4a and d). This difference may be attributed to intra and inter-skin variability. However, it should be noted that the majority

of IBU (when applied alone) was retained within section 1 – 3 of the sampling sites while the majority of IBU (when applied in the presence of PG, PEG 200 and OS) was retained within section 1 – 2.

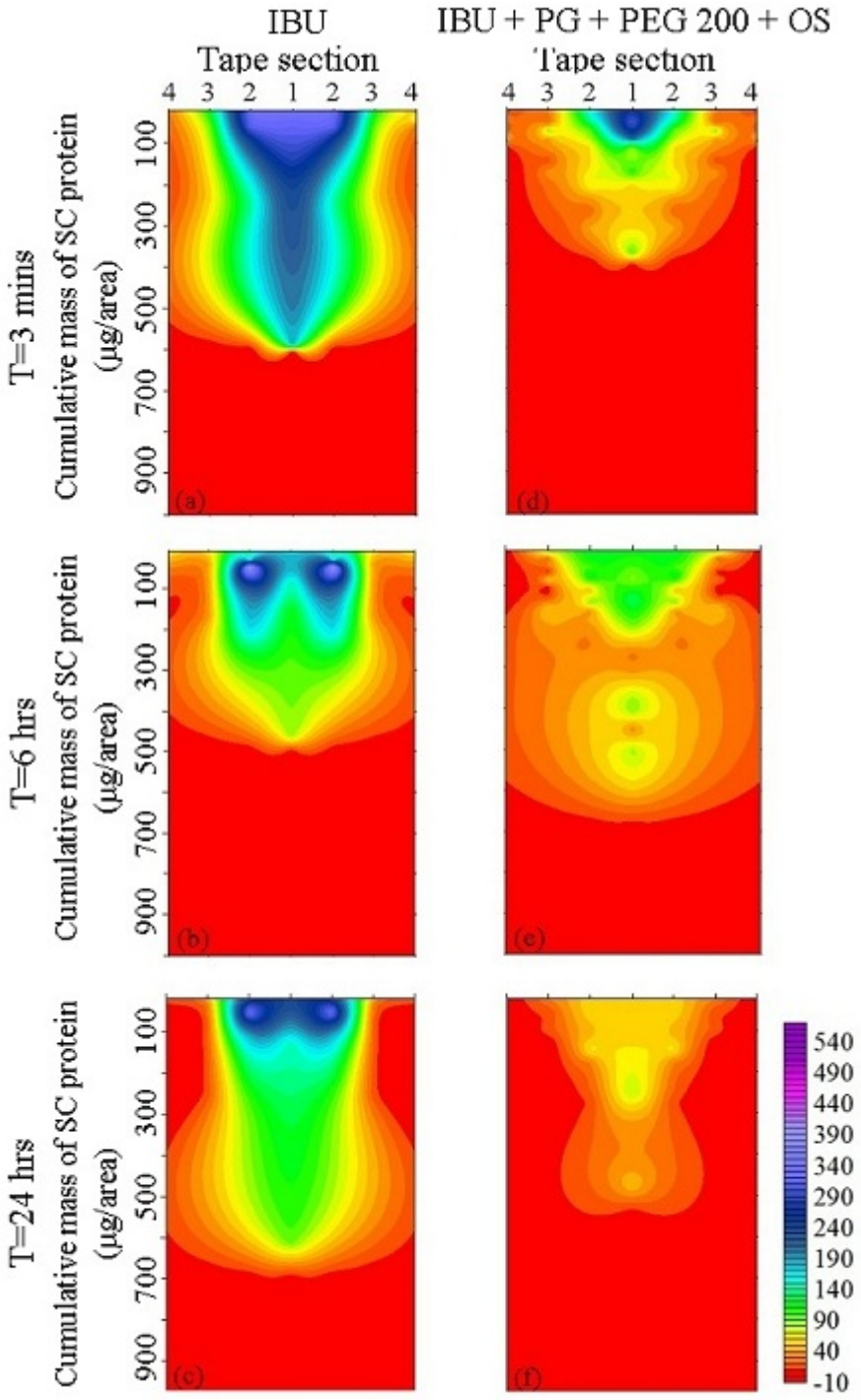


Figure 6.3 Contour plot displaying the extent of lateral spread and penetration of IBU applied with and without PG, PEG 200 and OS at 3 mins, 6 hrs and 24 hrs after application to humans. Note the legend values are expressed as IBU concentration (ng/µg).

The higher tendency of IBU to spread laterally across the SC surface at 3 mins after application when applied alone, compared to that when applied in the presence of PG, PEG 200 and OS is supported by the distribution profile shown in Figure 6.4. In the absence of excipients, it is evident that the concentration of IBU quantified in section 2 is comparable to that measured in section 1 and a substantial amount of IBU was also present in section 3.

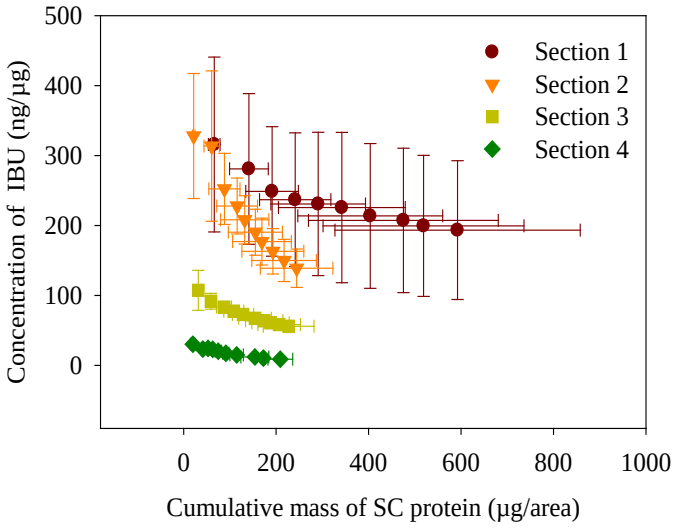


Figure 6.4 Distribution profile of neat IBU across human SC at 3 mins after application *in vivo* (mean ± SEM, n=8).

While the concentration of IBU removed from section 1 and 2 become similar with increasing SC depth, Figure 6.5 shows that in the presence of PG, PEG 200 and OS, the concentration of IBU measured in section 2 of the initial tape strips at 3 mins after application is approximately 2-fold lower than that measured in section 1 – particularly at the uppermost surface of the SC. The higher resistance to lateral diffusion on and within the SC soon after application of IBU when applied with PG, PEG 200 and OS is consistent with findings in Chapter 5.

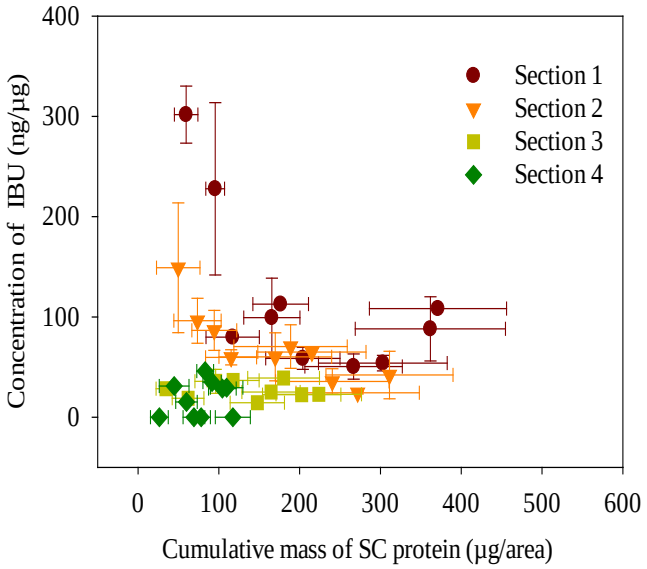


Figure 6.5 Distribution profile of IBU applied in the presence of PG, PEG 200 and OS across human SC at 3 mins after application *in vivo* (mean ± SEM, n=8).

The lateral spreading and penetration behaviour of IBU applied alone does not appreciably change over 24 hrs of exposure suggesting it may have formed a drug reservoir on the SC (Figure 6.3a – c). In contrast, when applied with PG, PEG 200 and OS, IBU is noticeably depleting from the uppermost regions of the SC over 24 hrs as displayed in Figure 6.3d – f, whilst concurrently penetrating deeper into the SC at 6 hrs to reach a depth of approximately 600 – 700 µg/area. Consistent with findings observed in Chapter 5, IBU displayed signs of continued lateral spreading within the SC layers over time when applied with PG, PEG 200 and OS. This is shown by the significantly higher concentration of IBU detected in the deeper SC stripped region at 6 hrs compared to 3 mins under section 3 as displayed in Figure 6.3d – e and further supported by Figure 6.6.

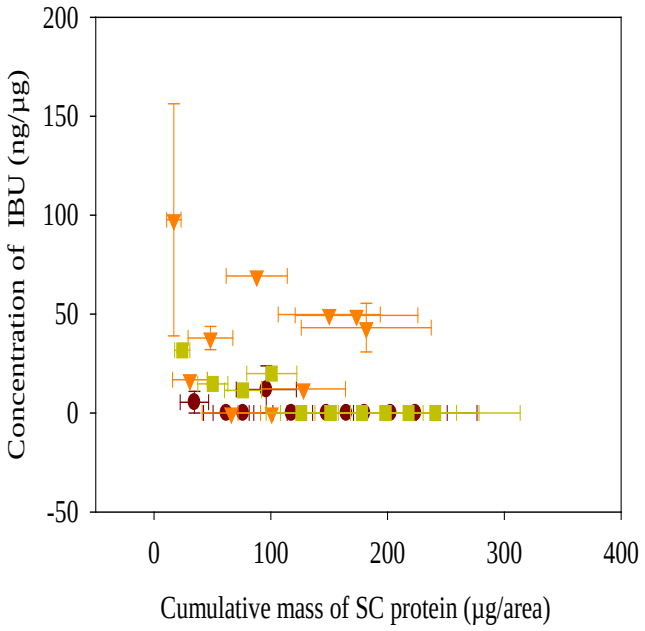


Figure 6.6 Distribution profile of IBU applied in the presence of PG, PEG 200 and OS measured at section 3 across human SC at (●) 3 mins, (▼) 6 hrs and (■) 24 hrs after application *in vivo* (mean ± SEM, n=8).

6.5.3 Recovery of IBU from adhesive tape *in vivo*

The percentage recovery of IBU applied with and without PG, PEG 200 and OS from the combined 10 tape strips at 3 mins, 6 hrs and 24 hrs after application is displayed in Table 6.2. The recovery of IBU (when applied alone) progressively decreased over 24 hrs (though not statistically significant). The recovery of IBU when applied in the presence of PG, PEG 200 and OS at 24 hrs was significantly lower than that recovered at 3 mins and 6 hrs after application. The disappearance of IBU (when applied with excipients) may be due to higher permeation across the skin membrane. Hence, *in vitro* permeation studies were performed as shown in Section 6.5.4.

Table 6.2 % Recovery of IBU applied with and without PG, PEG 200 and OS at 3 mins, 6 hrs and 24 hrs after application to that of the applied dose *in vivo* (mean ± SEM, n=3).

	% IBU Recovery	
Time	**IBU**	**IBU + PG + PEG 200 + OS**
3 mins	94.2 ± 13.7	68.5 ± 5.5
6 hrs	69.2 ± 8.8	55.9 ± 2.5
24 hrs	55.5 ± 18.6	25.3 ± 8.0*

(*) denotes significant difference to that recovered at 3 mins and 6 hrs.

6.5.4 *In vitro* diffusion study

In addition to the repeated *in vivo* studies, parallel *in vitro* studies were carried out to allow a comparison to be made between *in vivo* and *in vitro* data and to determine what portion of the recovered amount of IBU at 24 hrs *in vivo* was potentially made available for systemic absorption. Hence, an *in vitro* mass balance analysis of IBU applied with and without PG, PEG 200 and OS was performed whereby the amount of IBU residing in the uppermost layers of the SC, the viable epidermal and dermal skin section, the donor chamber of the diffusion cell set-up, as well as the amount of drug permeated through the dermatomed membrane into the receptor solution was determined.

The permeation profile of IBU applied with and without PG, PEG 200 and OS over 24 hrs is shown in Figure 6.7. Consistent with the permeation of IBU over 48 hrs, the steady-state flux of IBU applied in the presence of PG, PEG 200 and OS is significantly higher than that of the control.

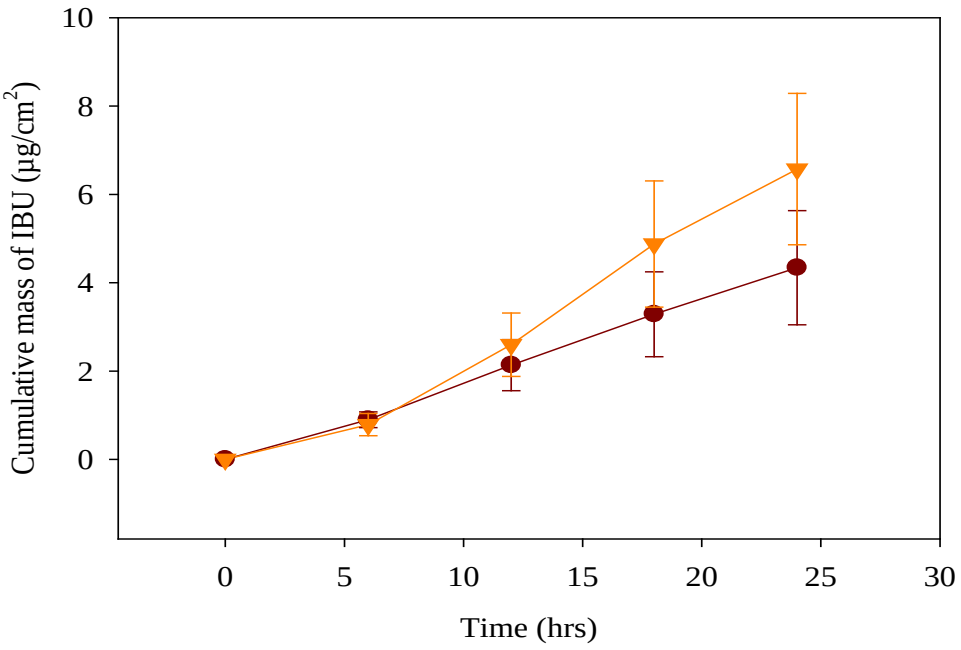


Figure 6.7 Permeation profile of IBU applied alone (●) and with PG, PEG 200 and OS (▼) across excised dermatomed human skin *in vitro* (mean ± SEM, n=12).

The measured steady-state flux and cumulative mass of IBU applied with and without PG, PEG 200 and OS at 24 hrs after application is listed in Table 6.3. The presence of PG, PEG 200 and OS resulted in approximately a 2-fold increase in IBU flux compared to that of IBU applied alone, which was deemed significant. The difference in the degree of IBU uptake shown in Table 6.3 compared to the results listed in Table 6.1 may be due to inter-subject variability.

Table 6.3 Summary of IBU flux and permeated mass when applied with and without PG, PEG 200 and OS over 24 hrs *in vitro* (mean ± SEM, n=12).

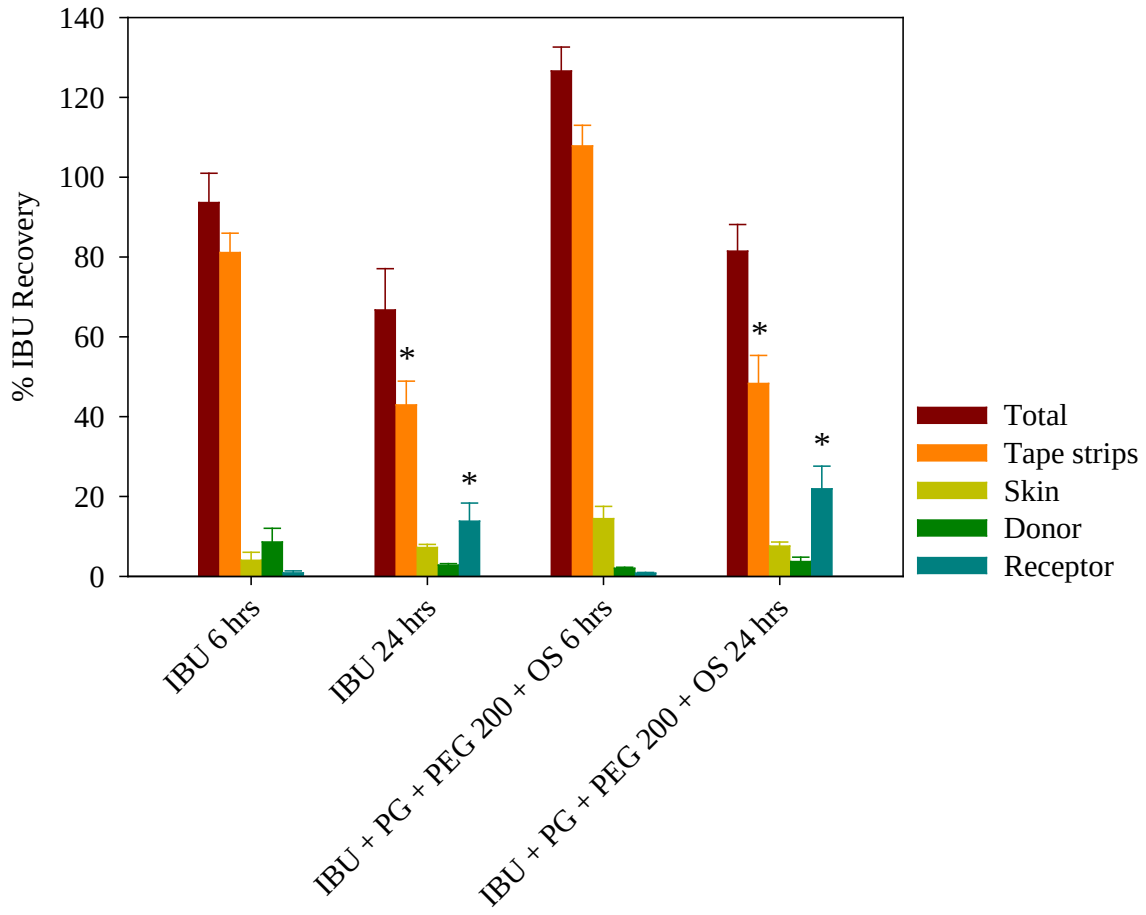
	IBU	**IBU + PG + PEG 200 + OS**
Steady-state flux (µg/cm^2/hr)	0.18 ± 0.03	0.32 ± 0.04*
Cumulative mass of IBU at 24 hrs (µg/cm^2)	4.14 ± 0.80	6.57 ± 0.91

(*) denotes significant difference to that of IBU applied alone.

6.5.5 Mass balance analysis *in vitro*

The percent recovery of IBU when applied with and without PG, PEG 200 and OS in each compartment of the *in vitro* study set-up at 24 hrs after application is shown in Figure 6.8. The recovery of IBU from the *in vitro* study set-up at 6 hrs after application as measured in Chapter 5 are also shown in Figure 6.8 to allow for an assessment of any changes in compartmental recovery over time.

The total recovery of IBU applied with and without PG, PEG 200 and OS from all compartments of the *in vitro* study set-up decreased over time. The uppermost layers of the SC displayed the most significant loss in IBU with time. However, a portion of the loss from the SC may be due to the increased amount of IBU reaching the receptor fluid at 24 hrs. The amount of IBU detected in the skin and donor chamber did not change with time.



(*) denotes significant difference in % recovery of IBU relative to 6 hrs.

Figure 6.8 Mass balance analysis of IBU applied with and without PG, PEG 200 and OS *in vitro* at 6 hrs and 24 hrs after application (mean ± SEM, n=12).

6.5.6 *In vivo* and *in vitro* correlation

Performing tape stripping studies at 24 hrs *in vivo* and *in vitro* allowed for comparisons to be made between the cumulative mass of SC protein removed per unit area in the two experimental conditions. In addition, the effect of the presence of PG, PEG 200 and OS on the mass of SC protein removal was also assessed. Contrary to the results obtained in Chapter 5, there was no significant difference in the amount of SC protein removed with or without the addition of excipients or experimental conditions as shown in Figure 6.9.

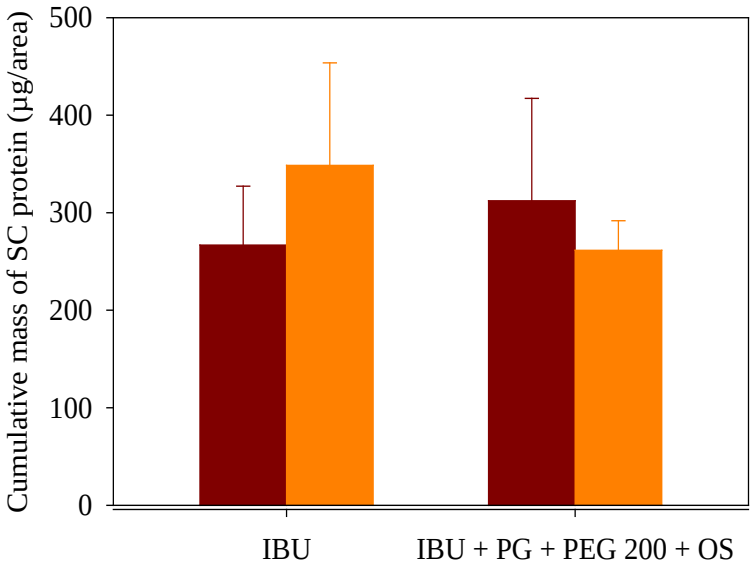


Figure 6.9 Comparison of cumulative mass of SC protein removed (■) *in vivo* and (■) *in vitro* following application of IBU with and without PG, PEG 200 and OS at 24 hrs (mean ± SEM, *in vivo*, n=3; *in vitro*, n=12).

Similarly, the normalized distribution profile of IBU applied alone is comparable between *in vivo* and *in vitro* conditions as shown in Figure 6.10a. However, in the presence of PG, PEG 200 and OS, the normalized *in vitro* distribution profile of IBU over-estimates that of *in vivo* conditions as shown in Figure 6.10b below.

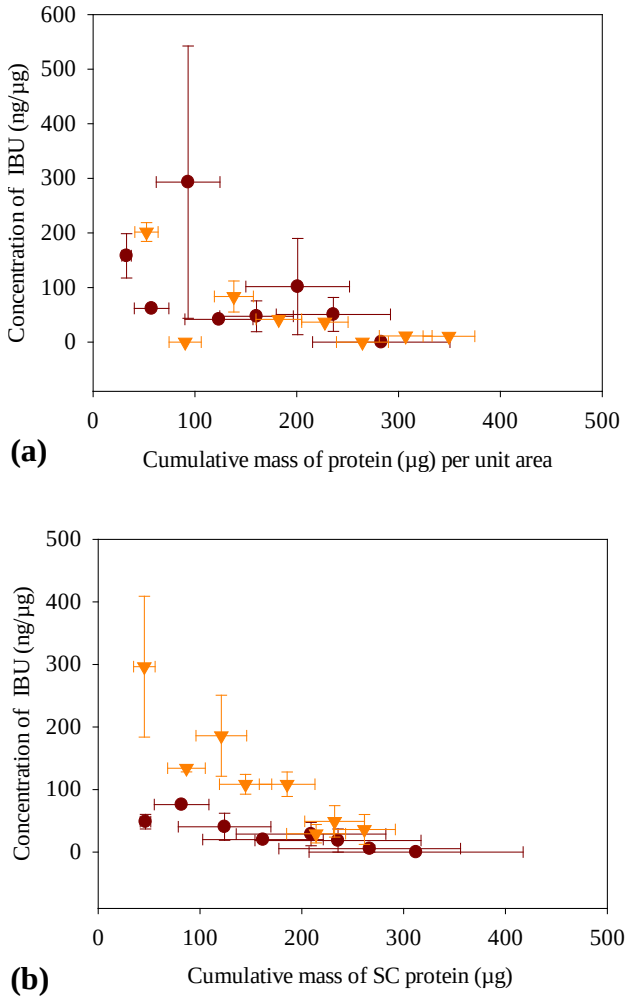


Figure 6.10 Comparison of the (●) *in vivo* and (▼) *in vitro* normalized distribution profile of IBU applied (a) alone and (b) with PG, PEG 200 and OS at 24 hrs (mean ± SEM, *in vivo*, n=3; *in vitro*, n=12).

6.6 DISCUSSION

Topically applied drugs will rarely exhibit instantaneous therapeutic effect due to the efficient barrier properties of the SC. The time elapsed between the period of skin exposure to a permeating substance and the time of therapeutic onset reflects a complex sequence of events including the release of the drug from its vehicle, the reorganization of the skin barriers and the diffusion of permeant through skin[226]. The physicochemical properties of drugs influence the rate at which a drug will traverse the SC structure and diffuse into the systemic circulation and therefore, different topically-applied drugs show varying times of onset[262,263].

In many *in vitro* permeation studies on human skin, IBU has been reported to display a lag time of either less than 1 hr, 4 hrs or 12 hrs[167,264,265] when applied with or without the addition of penetration enhancers. The IBU permeation studies carried out in Chapter 5 suggest that the IBU formulations tested display a lag time of 1 – 3 hrs. However, as no enhancement of IBU uptake was observed in the presence of the known penetration enhancers, it was considered possible that a longer lag time of IBU permeation had occurred, which had been ignored. This would potentially not only lead to an underestimation of IBU permeation, but may have masked the influence of penetration enhancers on IBU uptake.

The lack of understanding of the relatively long lag time of IBU as shown in Chapter 5 came to light following extension of the duration of IBU diffusion studies to 48 hrs as displayed in Figure 6.2. It appears that although IBU is detected in the receptor solution between 1 and 3 hrs after application, a lag time of ~6 hrs is evident before a steady-state flux is attained. In addition, the enhancing effects of PG, PEG 200 and OS are clearly manifest upon the establishment of steady-state. The steady-state flux and cumulative permeated mass of IBU applied with and without PG, PEG 200 and/or OS are listed in

Table 6.1 and show that the presence of PG and PEG 200 significantly enhance the uptake of IBU compared to the control. However, the presence of OS alone did not appear to significantly enhance the flux of IBU compared to IBU applied alone when using the one-way ANOVA statistical test.

PG and PEG 200 were both present in formulations that showed the highest increase in IBU flux. PG is a commonly used cosolvent and penetration enhancer and various mechanisms of action have been proposed. Takeuchi et al.[266] examined the spectral behaviour of rat SC and found that PG altered the conformation of SC keratinized proteins. Furthermore, PG may partition into the SC and facilitate drug uptake by the process of 'solvent-drag'[267] or by disturbing the intercellular lipid packing of the SC bilayers[74]. Similarly, PEGs have also been reported to have the potential to act as penetration enhancers. Though the use of PEG 200 specifically, as a penetration enhancer has not been studied, it has been reported that other PEGs and their derivatives exert their action by decreasing surface tension and conditioning the SC to enhance diffusion of other drugs through skin[123] or by inserting themselves in between the SC bilayers and thereby disrupting the structure of the SC[227]. Hence, the mode of action of PG and the possible effect of PEG 200 may explain the 2 to 3-fold increase of IBU flux over an extended period of exposure.

It is also interesting to note here that while the addition of PG and PEG 200 showed resistance to lateral spread and no enhancement in drug uptake within the first 6 hrs after application, the viscosity modifying effect of PEG 200 may have facilitated penetration with extended exposure. This is consistent with findings by Cross et al.[229] whereby a finite dose of a thickening agent increased drug penetration over time compared to an infinite dose. This may be due to slower water evaporation which may result in a higher water

content in the residual film and an increase in skin penetration due to a higher diffusivity through a more hydrated membrane[268].

IBU applied with OS showed approximately a 2-fold increase in steady-state flux compared to the control. Despite this, the flux of IBU did not appear significantly different to that of the control when compared with the flux of IBU applied in the presence of PG and PEG 200 using the one-way ANOVA test of significance.

It is also noteworthy that the cumulative mass of IBU applied in the presence of PG, PEG 200 and/or OS permeated across the skin membrane plateaus after 24 hrs of application, while IBU applied alone displayed constant flux up to 48 hrs as shown in Figure 6.2. It is possible that PG, PEG 200 and/or OS deplete from the SC surface after 24 hrs and may consequently result in the simultaneous reduction of IBU as suggested by Trottet et al.[121].

The 48 hr permeation studies clearly showed that the flux and uptake of IBU applied in the presence of excipients was enhanced compared to the control. However, the enhancement is only evident following a lag time of ~6 hrs, after which steady-state flux is maintained until 24 hrs. Additionally, the presence of PG, PEG 200 and OS displayed the most pronounced enhancement of IBU uptake compared to that of the control. For this reason, additional *in vivo* and *in vitro* studies that were carried out to evaluate the changes (if any) on the lateral diffusion and penetration behaviour of IBU applied with and without PG, PEG 200 and OS over 24 hrs.

In the presence of PG, PEG 200 and OS, the majority of IBU resided as a depot on the outermost surface of the SC at 3 mins after application. IBU applied alone however, penetrated to a depth almost double that reached by IBU applied with PG, PEG 200 and OS and also demonstrated significant lateral diffusion. Nevertheless, it should be

recognized that further penetration and lateral diffusion of IBU into the deeper SC region was evidenced with prolonged exposure when applied with PG, PEG 200 and OS as shown in Figure 6.3.

When applied with PG, PEG 200 and OS, the recovery of IBU from the SC at 24 hrs was significantly reduced compared to that recovered at 3 mins and 6 hrs after application. This reduced recovery of IBU is reflective of the establishment of the constant flux between 6 hrs and 24 hrs (as discussed above) coupled with the enhanced IBU uptake compared to that of IBU applied alone. In the absence of PG, PEG 200 and OS, the *in vivo* recovery of IBU at 24 hrs, however, is comparable to the amount recovered at 6 hrs after application and further demonstrated by the sustained reservoir on the outermost layers of the SC as shown in Figure 6.3a – b. The reservoir function of the SC has long been established for solutes such as steroids[184,206], where continued penetration is observed for significant amounts of time after removal of donor vehicles from the skin's surface[269]. Santi et al.[270] also observed the formation of an IBU reservoir in rat skin post-iontophoresis after which IBU was concomitantly released for passive diffusion through skin. Hence, it is likely that the reservoir formation of IBU (when applied alone) is the driving force for the constant flux of IBU up to 48 hrs due to the high concentration gradient across the SC as observed *in vitro* and displayed in Figure 6.2. It is also noteworthy that the comparable recovery of IBU (applied alone) between 6 hrs and 24 hrs attests to a robust methodology in implementing the use of the non-occlusive mesh tea strainer to prevent volunteers from rubbing or coming in contact with the sampling site overnight.

The *in vitro* permeation profile of IBU with and without PG, PEG 200 and OS across dermatomed human skin over 24 hrs is displayed in Figure 6.7. Though the extent of enhancement of IBU uptake when applied in the presence of PG, PEG 200 and OS compared to the control is not as pronounced as was seen in the permeation profile over 48

hrs, the steady-state flux at the end of the 24 hr period is still significantly higher than that of the control as listed in Table 6.3. In the addition of PG, PEG 200 and OS, the somewhat lower degree of IBU flux enhancement obtained from the 24 hr *in vitro* study compared to that obtained from the 48 hr *in vitro* study, may be a result of inter-subject variability which has been reported to be up to 40 – 67%[2].

When conducting a mass balance study, the Scientific Committee on Consumer Products (SCCP)[158] states that the recovery of the applied dose must be in the range of 85 – 115%. A total *in vitro* recovery of approximately 100% of IBU applied with and without PG, PEG 200 and OS was achieved at 6 hrs but was considerably reduced to 66.7 ± 10.3% and 81.5 ± 6.7%, respectively at 24 hrs as shown in Figure 6.8. This suggests that IBU is possibly metabolized within the SC between 6 hrs and 24 hrs after application. In addition, the percentage of IBU recovered from the combined 8 tape strips was significantly reduced by 24 hrs. However, a portion of the IBU lost from the SC surface can be accounted for by the significantly higher amount of IBU reaching the receptor solution at 24 hrs compared to that at 6 hrs. The amount of IBU detected in the donor compartment and viable skin remained unchanged between 6 hrs and 24 hrs. This implies that the amount of IBU penetrating the viable skin and into the receptor fluid remained constant between 6 hrs and 24 hrs such that no accumulation of IBU in the viable skin was measured over time.

As *in vitro* models are often used to assess percutaneous penetration, it was useful to evaluate whether the *in vitro* data obtained in this chapter was consistent with the *in vivo* data also obtained. Figure 6.10 compares the *in vivo* and *in vitro* normalized distribution profile of IBU applied with and without PG, PEG 200 and OS at 24 hrs after application. It is evident that while the normalized distribution profile of IBU applied alone is comparable between *in vivo* and *in vitro* conditions (but contrary to that obtained in Chapter 5), the normalized *in vitro* profile of IBU applied with PG, PEG 200 and OS is substantially

higher than seen *in vivo* and consistent with findings in Chapter 5. Various studies have also observed the over-estimation of *in vitro* results when compared to that obtained under *in vivo* conditions[213,255]. Therefore, the comparable normalized penetration distribution behaviour of IBU applied alone between *in vivo* and *in vitro* conditions shown in Figure 6.10a should be interpreted with caution as the smaller *in vivo* sample size (n=3) compared to the *in vitro* sample size (n=12) of studies in this chapter may not be reflective of a true population and therefore poses a limitation of this comparison study.

Figure 6.9 shows the cumulative mass of SC protein removed from *in vivo* and *in vitro* human skin at sites directly under section 1 and 2 of 8 successive concentric tape strips. The cumulative mass of SC protein removed is comparable between *in vivo* and *in vitro* conditions and independent of the type of formulation applied onto the skin. This finding is contrary to that obtained in Chapter 5 and may be attributed to inter-subject variability[2] as well as a change in hydration state of *in vitro* skin[86] between 6 hrs and 24 hrs after exposure.

The inconsistency of *in vivo* and *in vitro* correlations shown in this chapter illustrates the limitations of making direct comparisons between data obtained in *in vivo* and *in vitro* conditions. Under *in vivo* conditions, the contribution of local blood supply on the tissue targeting potential of drug is not well understood. However, it is suggested that the use of local blood circulation facilitates the distribution of transdermal drugs by increasing the circulatory levels of drug[271]. *In vitro* however, the drug has to permeate through the whole skin before an effect comparable with the transport with the blood stream takes place[255]. Mirejovsky et al.[213] also suggested that the variability between *in vivo* and *in vitro* data is due to the temporary modification of the penetration barrier of the living SC, as compared with the permanent modification of dead skin. In addition, an application *in vitro* brings

about a lasting loosening of the SC cells. This highlights not only the intra and inter-subject variability in SC protein removal, but also the effect of skin condition.

To obtain the best estimate of a drug concentration across the SC, the amount of drug measured in each tape strip should be normalized with respect to the amount of tissue removed in each individual strip. Moreover, to make the best comparisons of formulations, the amounts of tissue removed from the skin per strip should be near the same and formulation independent[86]. Although the amount of SC protein removed from the studies in this chapter was similar between formulations applied and experimental conditions, disparity is evident when compared to those in reported in Chapter 5. The amount of SC protein removed by tape stripping depends on a variety of parameters, e.g. experimental conditions and inter-individual as well as inter-seasonal differences in the SC structure[272-274]. Under *in vitro* conditions, continual hydration of the SC (such as that in permeation studies) facilitates the removal of SC protein due to the loosening of cells[189]. This can explain the occasional higher removal of SC protein *in vitro* over *in vivo* conditions. However, due to the discrepancy between the cumulative mass of SC protein removed in Chapter 5 and 6, coupled with the reported 60% inter-subject and inter-conditional variability[2], it is difficult to ascertain a direct correlation of the effect of tape stripping on SC protein removal between *in vivo* and *in vitro* conditions. Evidently, as each tape strip may remove a different mass of SC protein, it is important not to compare individual strips with corresponding strips since there is no comparable physical meaning to each strip[86]. Therefore, in order to obtain reliable concentration-versus-depth profiles of drugs penetrating into the SC, it is essential to normalize the amount of drug measured in each tape strip to the amount of SC protein removed in the corresponding strip.

6.7 CONCLUSION

The permeation profile of IBU applied with and without PG, PEG 200 and/or OS over 48 hrs clearly demonstrated that a relatively long lag time of ~6 hrs is required before steady-state penetration of IBU is established. The addition of PG and PEG 200 with or without OS enhances the uptake of IBU following the establishment of steady-state. The reported viscosity modifying effects of PEG 200 may have contributed to the increased penetration of IBU in addition to the increased lateral spreading within the SC over time. In the absence of PG, PEG 200 and OS, IBU forms a reservoir on the outermost regions of the SC that may be responsible for its sustained permeation after prolonged exposure. However, the amount of IBU residing in the SC is considerably decreased in the presence of PG, PEG 200 and OS after prolonged exposure and this may partially be due to increased permeation. Furthermore, the parallel *in vivo* and *in vitro* mass balance analysis allowed for the successful assessment of the fate of applied drugs. However, caution is required when comparing results obtained from the two experimental conditions.

CHAPTER 7

SUMMARY, IMPLICATIONS AND FUTURE DIRECTIONS

7 SUMMARY, IMPLICATIONS AND FUTURE DIRECTIONS

Transdermal drug delivery is limited to a small range of compounds due to the formidable barrier of the human SC. It is generally accepted that the bioavailability of non-occlusive, topically-applied drugs is in the order of 5 – 10% and is often lower. However, the fate of the 90 – 95% of unabsorbed topically applied drug is not well documented and there is increasing regulatory interest in this phenomenon. Therefore, to better understand the fate of transdermal drug applications, this research investigated the lateral diffusion and penetration behaviour of drugs applied onto the SC surface. The role of excipients on the lateral diffusion and penetration of topically applied drugs was also assessed.

To determine the amount of drug subject to lateral diffusion on the SC surface and within the SC bilayers at various exposure times following topical application, the approach taken in this study was to tape strip the uppermost region of the SC of humans. Tape stripping was performed using specially designed concentrically perforated adhesive tape in four sections of known diameters. The drug removed by each tape strip region was extracted via solvent extraction and the amount extracted was normalized to SC protein. In order to obtain a concentration-depth profile of the drug, tape stripping of the site was repeated 10 times. Tape stripping in this way allowed for a three-dimensional distribution profile of drug across the SC to be constructed.

CAF, HC and IBU were chosen to assess the impact of physicochemical properties on the lateral spreading and/or penetration behaviour of drugs across the SC *in vivo*. CAF, a hydrophilic drug, formed a flat reservoir on the surface of the SC and resided in the uppermost region of the SC. Conversely, HC, a relatively more lipophilic drug than CAF exhibited a lower tendency to spread and instead, formed a narrow drug depot in the uppermost regions of the SC. IBU, a lipophilic drug, demonstrated greater drug penetration

(reaching a greater depth of the SC) and lateral spreading across the SC in comparison to CAF and HC.

The lateral spreading and penetration behaviour of CAF, HC and IBU may be attributed to the lipophilicity of each drug. The SC is a heterogeneous system comprised of both lipophilic and aqueous domains, with paths through which hydrophilic and lipophilic substances can traverse. As lipophilic drugs are soluble in the continuous lipid medium, the SC poses less resistance to their diffusion. Thus, IBU is able to diffuse across and through the SC more rapidly than CAF or HC.

The distribution of CAF, HC and IBU across the SC was measured after various exposure times up to 6 hrs. At 6 hrs, the total recovery of CAF, HC and IBU from the combined tape strips was approximately half of the applied dose. However, it is highly unlikely that systemic absorption accounted for all of the unrecovered drug at 6 hrs of exposure. To confirm this, *in vitro* permeation studies were undertaken.

In vitro permeation studies revealed that at 6 hrs after application, neither CAF nor HC could be detected in the receptor fluid, whilst only 1.6% of the applied dose of IBU permeated into the receptor fluid. Furthermore, less than 10% of the applied dose of each permeant penetrated into the viable skin (remaining skin post-tape stripping). This indicates that while approximately half of the applied dose of CAF, HC and IBU was recovered from the uppermost region of the SC through tape stripping *in vivo*, a further 10% of the applied dose may have penetrated beyond the tape stripped region into the viable skin; and for IBU, 1.6% of the applied dose may be systemically available. Hence it is likely that the remaining ~40% of the applied dose of each compound after 6 hrs of exposure is metabolised or lost to the surrounding environment via desquamation or being rubbed/washed off the skin surface or a combination of these.

As only IBU demonstrated the ability to be systemically absorbed after 6 hrs, further studies were performed to assess the influence of a cosolvent, penetration enhancer and viscosity modifier on the lateral diffusion and distribution of IBU across human skin *in vivo*. IBU was applied with and without the presence of PG, PEG 200 and/or OS onto the forearm of humans and tape stripped to obtain a distribution profile. The concentration of IBU applied with and without the presence of PG, PEG 200 and/or OS decreased with an increase in distance from the application area. However, the presence of PG and PEG 200 appeared to retard the lateral movement of IBU and retain the majority of the applied dose within close proximity to the application area. On the other hand, OS promoted lateral diffusion as IBU (applied with OS) was detected furthest from the application site compared to IBU applied with and without PG and PEG 200. Taking into account that viscosity may control drug spread, it can be postulated that the relatively high viscosity of PG and PEG 200, relative to OS, is responsible for the slow radial movement of IBU across the SC. Furthermore, the initial contact area formed between PG, PEG 200 and/or OS may indicate the spreading tendency of the applied formulation.

No difference in IBU recovery (relative to the applied dose) from the uppermost regions of the SC was observed after 6 hrs of exposure *in vivo* when applied with and without the presence of PG, PEG 200 and/or OS despite literature evidence of the penetrating enhancing effects of PG, PEG 200 and OS. Additionally, the presence of PG, PEG 200 and/or OS did not enhance the *in vitro* permeation of IBU over 6 hrs. The lack of difference in IBU permeation and recovery at 6 hrs was likely to be due to the fact that IBU experiences a lag phase of ~6 hrs before steady-state flux is attained from 6 hrs. The enhancing effects of PG, PEG 200 and OS are clearly evident upon establishment of steady-state, as the flux of IBU increased by an average of approximately 2-fold in the presence of PG, PEG 200 and OS.

In vivo tape stripping was performed in parallel over an extended exposure time of 24 hrs to assess any changes in lateral diffusion and distribution of IBU with and without the presence of PG, PEG 200 and OS across the human skin following the onset of steady-state. The recovery of IBU applied without PG, PEG 200 and OS remained relatively unchanged with prolonged exposure, suggesting the possible formation of a drug reservoir. On the other hand, the recovery of IBU applied in the presence of PG, PEG 200 and OS was significantly reduced. This is reflective of the attainment of steady-state flux coupled with the enhanced penetration of IBU relative to IBU applied alone. Though the exact mechanism by which PG, PEG 200 and OS enhances IBU permeation cannot be elicited from these studies, it has been suggested that PG may partition into the SC and facilitate drug uptake by the process of solvent drag; whilst PEG 200 may disrupt the structure of the SC by insertion between the SC bilayers and OS may initiate a lipid fluidising effect which increases the ease of permeant diffusion through the SC.

A small degree of lateral spreading of IBU applied with and without excipients was also evident with prolonged exposure. This highlights that lateral diffusion is a continuous process that occurs simultaneously to drug penetration through the skin.

A review of the current literature suggests that this study was the first to demonstrate the concurrent process of lateral diffusion and penetration of drug on the SC surface and within the SC bilayers of human skin using specially designed perforated concentric adhesive tapes. During the course of this study, the extent of lateral spread and penetration of drugs of various lipophilicities have been demonstrated. In addition, the effect of excipients on the lateral diffusion and penetration of a permeant have also been assessed. Depending on the type of drug and/or vehicle applied, a substantial amount of drug (relative to the applied dose) may diffuse away from the application site through lateral spread. Though drug that undergoes lateral diffusion may still remains on the skin (and

may eventually be made available for systemic absorption), drug spread from the application site increases the safety risk to the immediate environment due to the greater area of exposure. Hence, in order to reduce the amount of drug loss via lateral diffusion, it may be plausible to increase the viscosity of the applied formulation which will contain the drug in closer proximity to the application site and consequently increase the drug concentration gradient across the skin within the area of application.

This study revealed that approximately half of an applied drug dose may not be accounted for within the skin as soon as 3 hrs following topical application. The lack of accountability of the unabsorbed drug may be a result of loss to the surrounding environment via desquamation or being rubbed/washed off. A simple solution to prevent the loss of drug via these routes is the use of occlusive transdermal products. However, occlusive products have been reported to cause skin irritation. It would be beneficial to carry out a mass balance study to quantify the amount of drug loss via all the possible routes mentioned above. In addition, it may also be advantageous to determine the effects of excipients on the lateral diffusion behaviour of CAF and HC with prolonged exposure. This will provide insight into whether the lateral diffusion effects of excipients are compound specific. Furthermore, a better understanding of the impact of excipients will allow for the development of more safe and effective transdermal delivery systems by limiting the degree of lateral diffusion and concurrently decreasing the risks of secondary exposure.

In summary, this study provided an understanding into the effect of drug lipophilicity, as well as the addition of cosolvents, penetration enhancers and viscosity modifying agents on the lateral spreading and distribution of drug across human skin. The specially designed perforated concentric tape proved valuable in providing a three-dimensional assessment of the localization and distribution profile of drugs across human

skin and have the potential to improve dermatopharmacokinetics in both industrial and academic settings.

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