

**The impact of clinical application protocols for
multiple topical products on drug delivery to
the skin**

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Abstract

In the treatment of inflammatory skin conditions patients are often prescribed more than one topical product: a topical corticosteroid (TCS), an emollient and a topical antibiotic in cases of clinically infected skin. Despite widespread prescribing, there exists a remarkable lack of consensus between healthcare bodies on the optimum application protocol for the products, with recommendations made on the basis of clinical 'expert' opinion rather than evidence-based findings. Thus, the aim of this thesis was to evaluate the impact of clinical application protocols on the *in vitro* percutaneous absorption and skin retention of TCSs and topical antibiotics. A two component model was initially employed where the model TCSs (Elocon cream and Dermovate cream) were applied before or after six emollients, with a five or thirty minute interval. The Aron mix, a tailored extemporaneous therapy, was subsequently investigated to confirm whether the trends observed with TCSs and emollients were applicable to further complex mixtures of a topical antibiotic (Fucidin cream) and a TCS (Diprosone cream) substantially diluted in an emollient base (Diprobace cream). The findings demonstrated that applying multiple topical products to the skin can induce rapid formulation changes *in situ* or indeed in the extemporaneously prepared Aron mix, resulting in an altered performance of the medicinal products in a formulation specific manner. Mixing of the TCSs or topical antibiotic with an emollient dissimilar to the product base resulted in a multitude of effects including alterations in drug and solvent thermodynamic activities, rapid drug crystallisation and emollient excipients acting with penetration enhancing effects. Complementary drug stability investigations of the extemporaneous Aron mix did not support the typically recommended shelf life for the product (two weeks to one month), with significant decreases in drug content evident after seven days. In disagreement with clinical recommendations for TCSs and emollients, allowing up to thirty minutes between product applications was not sufficient to mitigate emollient effects on TCS drug delivery to the skin. Furthermore, application of the TCS after the emollient largely decreases drug delivery to the skin up to 4.4 fold compared to the TCS alone, findings which counter the clinical opinion that application of a TCS to well moisturised skin can increase drug delivery.

Overall, the work presented in this thesis delivers a body of evidence previously unreported to suggest that applying multiple topical products to the skin at similar times may significantly alter the critical quality attributes of the product(s) to unpredictable extents and upon further investigation, these findings will support the advancement of conclusive clinical guidance.

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Abbreviations

γ	Activity coefficient
$^{\circ}\text{C}$	Degrees Celsius
μg	Microgram
$\mu\text{g mL}^{-1}$	Microgram per millilitre
μl	Microlitre
μm	Micrometre
%	Percent
α	Thermodynamic activity
A	α -hydroxy fatty acid
AMP	Antimicrobial peptides
ANOVA	Analysis of variance
API	Active pharmaceutical ingredient
BAD	British Association of Dermatologists
BDNG	British dermatological nursing group
BDP	Betamethasone dipropionate
BP	British Pharmacopoeia
CER	Ceramides
C_i	Drug concentration in the innermost layers of the membrane
cm	Centimetre
C_o	Drug concentration in the outermost layers of the membrane
CPE	Chemical penetration enhancer
C_v	Concentration in donor solution/vehicle
D	Diffusion coefficient
D/h^2	Pathlength normalised diffusion coefficient
Da	Dalton
dS	dihydrosphingosine
EASI	Eczema Area and Severity Index
ECF	Eosinophil chemotactic factor
ECM	Extracellular matrix
EMA	European Medicines Agency
EO	ω -hydroxy fatty acid
FA	Fusidic acid
FDA	Food and Drug Administration
FFA	Free fatty acid
g	Gram
h	Hour
h	Diffusional pathlength
H	6-hydroxysphingosine
H_2O_2	Hydrogen peroxide
HBD-2	human β -defensin 2
HCl	Hydrochloric acid
HMG CoA reductase	3-hydroxy-3-methylglutaryl-CoA reductase
HPA axis	Hypothalamus pituitary adrenal axis

HPLC	High Performance Liquid Chromatography
ICH	International Conference on Harmonisation
IGA	Investigator Global Assessment
IPM	Isopropyl myristate
J	Drug flux
J_{ss}	Pseudo steady state drug flux
K	Partition coefficient
K_h	Pathlength normalised partition coefficient
K_p	Permeability constant
L	Litre
LL – 37	Cathelicidins
LOD	Limit of detection
Log P (o/w)	Logarithm of partition coefficient octanol/water
LOQ	Limit of quantification
LP	Liquid paraffin
M	Molar
MC	Measured concentration
mg	Milligram
mg mL⁻¹	Milligram per millilitre
MIC	Minimum inhibitory concentration
min	Minute
mL	Millilitre
mL min⁻¹	Millilitre per minute
mm	Millimetre
mΩ	Megaohm-cm
N	non-hydroxy fatty acid
NaOH	Sodium hydroxide
NES	National Eczema Society
NHS	National Health Service
NICE	National Institute for Health and Clinical Excellence
nm	Nanometre
nM	Nanomoles
NMF	Natural moisturising factor
NPC	National Prescribing Centre
O/W	Oil in water
OECD	Organisation for Economic Co-operation and Development
P	Phytosphingosine
p	Probability
PBS	Phosphate buffer solution
PCDS	Primary Care Dermatology Society
Ph Eur	European pharmacopoeia
PTFE	Polytetrafluoroethylene
Q	Cumulative amount of drug permeated
r	radius
R²	Coefficient of determination of a linear regression
RCT	Randomised Controlled Trial
RSD	Relative standard deviation

S	Sphingosine
S	Laplace variable
<i>S. aureus</i>	Staphylococcus aureus
SD	Standard deviation
SEM	Standard error of mean
SP	Seine protease
SPC	Summary of product characteristics
STEYX	Standard error of the predicted y-value for each x
t	Time
TC	Theoretical concentration
TCS	Topical corticosteroid
TEWL	Transepidermal water loss
T_L	Lag time
TPE	Therapeutic Patient Education
UV	Ultraviolet
W/O	Water in oil
W/O/W	Water in oil in water
w/w	Weight in weight

Chapter One:

General introduction

1.1 Introduction

In the treatment of inflammatory dermatoses, such as atopic eczema, patients are often prescribed more than one topical product for application to the skin: a topical corticosteroid (TCS), an emollient and a topical antibiotic in cases of clinically infected skin. These products are often applied independently, however are sometimes mixed together in a 'point of care' setting to produce a single extemporaneously dispensed product.

Application of medicinal products to the skin to achieve topical and transdermal drug delivery has been a strategy exploited for many years, especially in the treatment of inflammatory skin conditions where the ability to deliver therapeutic agents directly to the affected sites is advantageous. The benefits of employing topical agents to achieve a localised effect are numerous; in particular it can provide high localised drug concentrations and minimise systemic side effects in comparison to other routes of administration such as oral drug delivery (Walters & Dekker, 2002). However, whilst topical medicinal products may be formulated to achieve such benefits, care should be taken when initiating treatment to ensure that the treatment plan is acceptable to patients, safe and effective (National Eczema Society, 2014). TCSs are one example where the benefits of use must be weighed against the potential for adverse effects, which can be both local and systemic. TCS therapy was first recognised as a treatment for skin conditions in 1952 when Sulzberger and Witten (1952) documented the clinical benefits of applying a hydrocortisone acetate ointment to the skin of patients diagnosed with dermatoses. Since then, the use of TCSs has become widespread and in 2018 alone £55.5 million was spent on dispensing TCSs in England (NHS Digital, 2019).

TCS therapy remains the 'gold standard' treatment for many inflammatory skin conditions; however, whilst TCS use has proven to be effective at treating flare ups of skin conditions such as eczema and psoriasis, the risks associated with potent preparations or the prolonged use of this drug class has also been well- documented. Earliest reports of the adverse effects of TCSs found that topical application of fludrocortisone acetate in certain individuals resulted in weight gain and oedema (Fitzpatrick et al.,

1955). Furthermore, Munro (1976) highlighted the risks of retarded growth in children when exposed to potent topical corticosteroids for a prolonged period.

In order to circumvent the potential for adverse effects associated with TCS use, research has been directed at optimising treatment whilst minimising their side effect profile. One such strategy employed to overcome the challenges associated with TCS therapy involves optimising the physico-chemical properties of TCSs to increase lipophilicity and size, such that skin residence time is increased and drug permeation into the systemic circulation is reduced (Schoepe et al., 2006). However, there is often a balance to be sought between TCS potency and safety, as a significant increase in the intrinsic potency of a TCS is also closely correlated with increased pharmacological action of the drug (Luger, 2011).

In addition to molecular potency, different formulation vehicles have demonstrated an important role in the management of these conditions influencing both delivery of the active ingredient and patient adherence to the prescribed treatments. Sulzberger and Witten (1952) acknowledged the formulation effects on treatment outcomes, specifically that the use of ointments can contribute favourably towards treatment. The improvement of dermatoses was judged when hydrocortisone acetate 2.5 % w/w ointment was applied in comparison to an emollient ointment base of lanolin, liquid petroleum and white petroleum. In 6 out of 19 cases, the use of an emollient ointment alone appeared to improve the treatment area compared to the controlled, untreated site; however, such an improvement was further enhanced on addition of a steroid to the ointment base (Sulzberger & Witten, 1952). Indeed, emollients are now the mainstay of treatment, recommended for use even when flare ups have cleared and selected with consideration to patient preference for different vehicles to improve adherence (NICE, 2007). Considering the complex heterogenous nature of the skin, an organ with the primary aim to prevent the ingress of foreign material into the body, the pharmaceutical industry expends considerable efforts to optimise the drug delivery profile of topical products. Whitefield and McKenzie (1975) elegantly exemplified how tailoring formulation excipients and compositions can optimise the clinical effectiveness of a product. Formulating a 0.1 % w/w hydrocortisone cream with propylene glycol enabled a clinical response (measured by vasoconstriction) equivalent to that of 1 % w/w

Hydrocortisone cream BP, but with one tenth of the drug strength in the formulation (Whitefield & McKenzie, 1975).

Inflammatory dermatoses, such as atopic eczema, are particularly susceptible to infection and in cases of clinically infected eczema patients may also be prescribed a topical antibiotic, alongside the TCS and emollient, for application to the affected sites only (British National Formulary, 2020b). Whilst these products are developed independently by the pharmaceutical industry, clinical guidance recommend that the products are used together. However, these recommendations are made without knowledge of the extent to which the formulation performance may be altered by use with other products and without contributions from those with formulation development expertise. Furthermore, there is disparity in the clinical guidance issued with respect to the optimum order of application and time interval between product applications. The application of multiple products to the skin at similar times may alter the formulation *in situ*, or in premixed extemporaneous systems resulting in an unpredictable effect on critical quality attributes of a marketed formulation (such as the drug delivery profile). These unexpected formulation changes are of particular concern when using potent or very potent TCSs, such as mometasone furoate or clobetasol propionate, which are commonly prescribed in general practice. In such cases an increase in drug delivery may result in an increase in side effects for medicines with an already high side effect profile, distress for patients and resultant non-adherence to therapy. In the case of topical antibiotics, a change in the drug delivery profile of the formulation may result in clinically ineffective doses of the antibiotic being delivered to the skin, potentially resulting in treatment failure and an increase in the risk of antimicrobial resistance. Despite the widespread prescribing of TCSs, topical antibiotics and emollients, the impact of mixing these products *in situ* (on the skin surface) or extemporaneously has only been investigated in three preliminary clinical efficacy investigations: a retrospective uncontrolled case series (Lakhani et al., 2017), a randomised controlled investigator-blinded trial for one TCS and emollient combination, the recommended use for which is a soap substitute rather than a leave on emollient (Ng et al., 2016) and an *in vivo* mouse model for one TCS and emollient product combination (Conner & Tietje, 2018). In all cases, the authors concluded that further studies are required for conclusive guidance to be formed. An understanding of the mechanistic

effects occurring when TCSs, topical antibiotics and emollients are mixed and the consequential impact on drug delivery to the skin has yet to be elucidated. As such there is a need to investigate the effects on the percutaneous absorption and skin retention of TCSs and topical antibiotics when applied with emollients, the associated impact on clinical efficacy and the scope for optimising treatment.

1.2 Structure and function of healthy skin

Healthy human skin is composed of several complex layers, from the outer stratum corneum through to the dermis, each contributing to the sensory, homeostatic and highly effective barrier functions of this organ (Benson & Watkinson, 2012). The homeostatic function of the skin supports thermoregulation of the internal environment whilst allowing for adaptation to varying climatic conditions. Protection from the external environment is also offered through the physical protective features of the skin which guards against chemical and bacterial toxins (Madison, 2003). It has been established that a change in the physical and chemical properties of the skin can promote the ingress of foreign bodies, such as allergens, and may contribute to the development of inflammatory skin conditions (Cork et al., 2009). An understanding of the structure and function of healthy skin must first be established to appreciate the nature of the skin barrier to the absorption of drugs, the altered defences of diseased skin and the action of topical treatments when applied to skin. Thus, a review of the structure and function of the three main layers of healthy human skin ensues (epidermis, dermis and subcutaneous tissue; depicted in Figure 1-1).

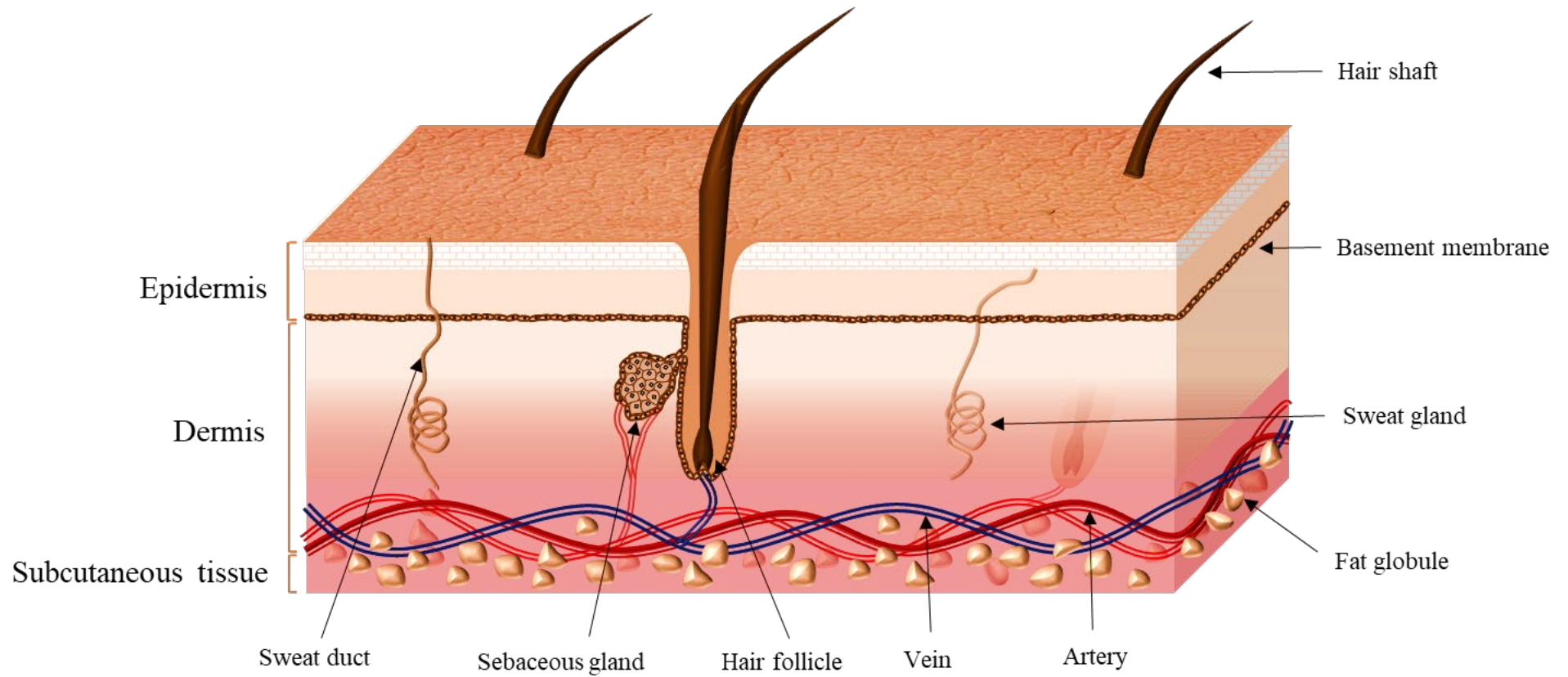


Figure 1-1: : Illustration of the three main layers of human skin (the epidermis, dermis and subcutaneous tissue) and skin appendages (hair follicles and sweat ducts).

1.2.1 The epidermal barrier

The principle barrier properties of the skin have been shown to lie predominantly in the outer epidermal layer. This theory was initially postulated by Homolle (1853) and Duriiau (1856) who found that the skin was not entirely impermeable. The epidermal layer can be divided into several histologically distinct layers as depicted in Figure 1-2: the stratum basale, stratum spinosum, stratum granulosum and the outermost stratum corneum (Wickett & Visscher, 2006).

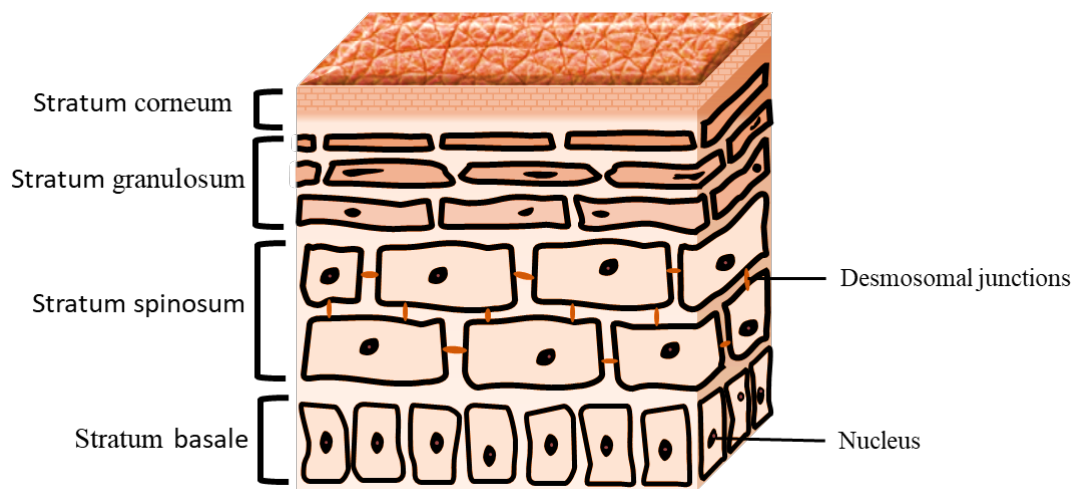


Figure 1-2: Illustration of the distinct layers of the epidermis: stratum corneum, stratum granulosum, stratum spinosum and stratum basale.

Following Homolle and Duriiau's initial findings, extensive research was conducted in an attempt to further isolate the barrier function to a sub-layer of the epidermis. In 1944, the stratum corneum finally emerged as the principle contributor to epidermal barrier function; this was achieved following partial and full removal of the outermost layer by sandpapering and correlating this change with an increase in transepidermal water loss (TEWL) (Winsor & Burch, 1944).

The stratum corneum, the end-product of epidermal differentiation, is a 10-15 μm thick heterogeneous layer with a highly complex composition (Bouwstra & Gooris, 2010). The exact structure and composition of the stratum corneum has been widely discussed with many studies aiming to elucidate the stratum corneum structure, characterise the lipid composition and correlate these features with a healthy barrier performance. The 'bricks and mortar' model presented by Michaels et al. (1975) succinctly characterised the structure of the stratum corneum as an arrangement of annucleated, keratin

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rich corneocytes, analogous to the bricks separated by the mortar, a heterogeneous lipid enriched extracellular domain. Serre et al. (1991) later added to this model, suggesting the presence of specialised desmosomes (corneodesmosomes) which are incorporated into the corneocyte envelope, promoting cohesion of the stratum corneum by binding together adjacent corneocytes.

The stratum corneum corneocytes arise from differentiation of keratinocytes at the stratum granulosum level and TEWL can, in part, be influenced by the constituents of these cells, which help to control water flux and maintain adequate hydration of the stratum corneum (Rawlings & Harding, 2004). Filaggrin, a filament aggregating protein, plays a vital role in corneocyte production. This protein encourages the aggregation of keratin intermediate filaments, promoting the collapse of corneocytes into flat cells for organised packing (Wickett & Visscher, 2006). The resultant organised 'brick' like packing of corneocytes presents a tortuous pathway for molecules, increasing the diffusional path length and promoting the stratum corneum defensive features (Rawlings & Harding, 2004). Following the production of corneocytes, filaggrin is degraded into amino acids which are later involved in the formation of natural moisturising factors (NMFs; Dale et al. (1997)). NMFs, a mixture of amino acids, amino acid derivatives and salts, along with the intercellular lipid matrix help to fulfil the water retaining functions of the stratum corneum, a function vital to maintaining stratum corneum elasticity and flexibility (Jokura et al., 1995). Filaggrin is particularly important when considering the altered barrier function in disease state skin as a clear association between the loss of function filaggrin mutation and atopic dermatitis has been reported in the literature (Harding & Rawlings, 2005; Kezic et al., 2008; Seguchi et al., 1996); particularly, the loss of function filaggrin mutation has been associated with a reduction in NMFs and corneocyte size and elevated TEWL (Cork et al., 2009). Furthermore, Nemoto-Hasebe et al. (2009) reported a correlation between filaggrin related atopic dermatitis and the severity of the condition, postulating that these mutations may play a crucial role in the pathogenesis of atopic dermatitis.

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The intercellular lipid matrix of the stratum corneum is comprised predominantly of three main lipid classes: cholesterol, ceramides and free fatty acids (FFAs; Jungersted et al. (2010)). Support and stabilisation of the stratum corneum lipids is achieved by cholesterol, synthesis of which involves the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase). It has been shown that up regulation of HMG-CoA reductase occurs in the skin following an acute barrier disruption consequently affecting the cholesterol levels in the stratum corneum and stabilisation of this layer (Harris et al., 1997).

Formation of lipid bilayers within the intercellular matrix is thought to be partially attributed to the presence of FFAs within the stratum corneum. This lipid class is the primary provider of ionisable head groups and has a role to play in stratum corneum structure and surface pH (Jungersted et al., 2008; Wickett & Visscher, 2006). The role of FFAs in healthy skin and their effects on barrier function when absent were assessed by Mao-Qiang et al. (1993) who found that upon inhibition of fatty acid synthesis following barrier disruption, recovery was significantly impaired but on topical application of FFAs recovery improved. These findings were mirrored by Fluhr et al. (2001) in a study which also attributed structural changes in the stratum corneum to low FFA content.

The role of ceramides within the stratum corneum has been of particular interest when determining stratum corneum barrier properties; this is due to the great variability in their chemical structure which is important for stratum corneum structure and function. Bleton et al. (2001) suggested the presence of over a thousand different ceramides structures within the stratum corneum. Until recently, ceramides have been categorised according to nine structural classes following a classification system proposed by Motta et al. (1993). However Masukawa et al. (2008) extended this system on discovery of a new sphingoid base, dihydrosphingosine (dS), resulting in two new structural classes and hypothesised the presence of another ceramide class which was later confirmed by van Smeden et al. (2011).

Following the research conducted by Masukawa et al. (2008) and van Smeden et al. (2011), ceramides can now be grouped according to 12 subclasses. However, with improvements in the techniques for ceramides characterisation it is expected that the present structural classification system will expand even further. At present, all structures present a sphingoid base coupled to a fatty acid chain. Sphingoid bases can be classed as one of four structures: sphingosine (S), phytosphingosine (P), 6-

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hydroxysphingosine (H) or dS. These bases are coupled to one of three fatty acids; namely, non-hydroxy (N), α -hydroxy (A) or ω -hydroxy (EO), which vary in chain length. EO fatty acid chains have been found to link to linoleic acid, increasing the chain length and further supporting stratum corneum lipid packing and are of particular significance when attempting to understand the development of atopic dermatitis. Macheleidt et al. (2002) compared the percentage weight of EO ceramides in the epidermis of healthy and atopic dermatitis subjects, finding a change from 52 % in healthy individuals to 10 % in atopic dermatitis sufferers, highlighting a link between ceramides EO presence and disease state skin.

The physical properties of ceramides, such as the long hydrophobic carbon chains, are also thought to aid in the formation of lamellae structures within the stratum corneum by binding adjacent corneocytes, helping to prevent TEWL and increase the mechanical strength of the stratum corneum (Masukawa et al. (2008); Wertz et al. (1985); Figure 1-3). It is thought that as well as performing this role within the lipid matrix, ceramides such as EOS attach, through the ω hydroxy fatty acid group, to proteins of the corneal envelope providing support by anchoring corneocytes into the extracellular lipid matrix (Raith et al., 2004).

The formation of a healthy stratum corneum layer relies primarily on the delicate balance of lipids of the intercellular matrix and successful differentiation of cells to produce a defensive layer analogous to the aforementioned 'bricks and mortar' model. It is the complex nature of this heterogeneous, selectively permeable, differentiated layer that allows the stratum corneum to perform so remarkably as a multifaceted barrier.

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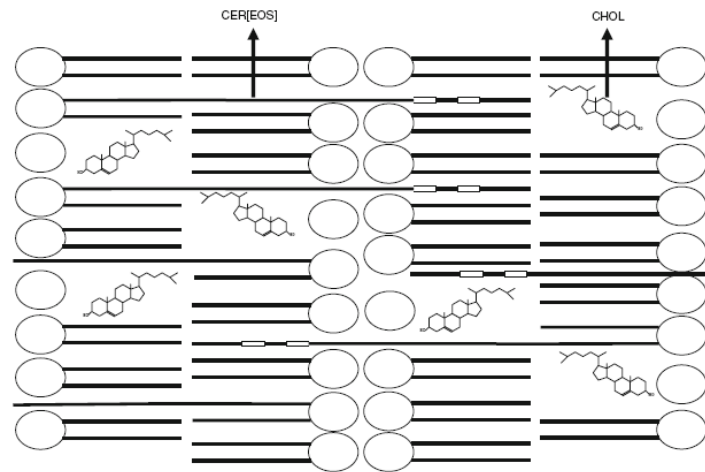


Figure 1-3: Schematic representation of ceramide packing within stratum corneum lipid matrix (Kessner et al., 2008). CER(EOS) extends into adjacent layers providing increased mechanical strength. The variation in chain length of ceramides promotes intercellular lipid space allowing for the incorporation of cholesterol into the matrix.

Below the stratum corneum, lies the stratum granulosum, stratum spinosum and stratum basale, often collectively referred to as the 'viable epidermis'. This multi-layered structure reflects the various stages of cell differentiation, initiated in the stratum basale and terminating in the stratum corneum. The stratum basale comprises a single layer of specialised cells, including keratinocytes, melanocytes, Langerhans and Merkel cells anchored to the basement membrane by hemidesmosomes. In the stratum basale, keratinocytes undergo cell division and upward migration into the stratum spinosum. Within the stratum spinosum, a 2-6 cell layer above the stratum basale, keratinocytes begin the process of differentiation, synthesis of keratins and the formation of keratin filaments (tonofilaments). Condensation of tonofilaments ultimately leads to the formation of desmosomes, responsible for anchoring adjacent keratinocytes in the stratum spinosum. Keratinocyte differentiation continues into the stratum granulosum, 1-3 cell layers in thickness and characterised by the presence of keratin containing granules (keratohyalin). Within these granules are profilaggrin, essential in the formation of keratin bundles and loricin and cystin-A which are major constituents of the cornified envelope. Additionally, at this stage of differentiation membrane coating granules, containing the precursors for intercellular lipid lamellar of the stratum corneum, are synthesised within keratinocytes. As keratinocytes migrate towards the upper granulosum, the membrane coating granules are released into the intercellular space as the cells begin to flatten and compact to form non- viable corneocytes (Brown & Williams, 2019; Eckert & Rorke, 1989).

1.2.2 The dermal layer

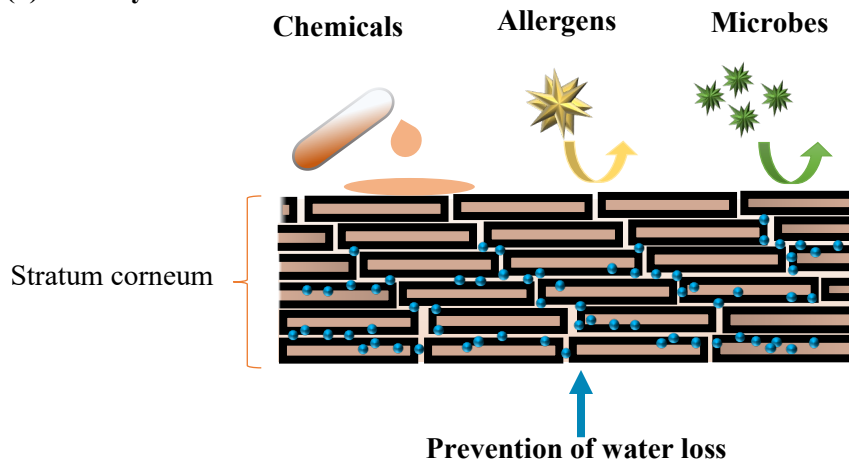
The dermis is a 3 – 5 mm thick heterogenous layer that lies below the stratum basale (Brown & Williams, 2019). Embedded within this layer are blood and lymphatic vessels, nerve endings, pilosebaceous units (hair follicles and sebaceous glands) and sweat glands (eccrine and apocrine). The extensive vasculature in the dermal layer contributes to thermoregulation, delivery of oxygen and nutrients to the skin and clearance of drugs, toxins and waste from the skin (Brown & Williams, 2019). Additionally, the dermis offers an element of mechanical protection afforded by densely packed collagen fibres and flexibility provided by elastic fibres. Dermal fibroblasts, the major cellular component of the dermis, play a pivotal role in the production and regulation of the extracellular matrix (ECM) of which the connective tissue is largely comprised, inflammation and immune cell recruitment to the site of tissue injury. Researchers have sought to elucidate the role of fibroblasts in the pathogenesis of atopic dermatitis and this cell type has proven capable of producing eosinophil chemotactic factors (ECFs) to stimulate the migration of eosinophils to the site of inflammation (Bartels et al., 1996; Mochizuki et al., 1998; Schröder et al., 1996). In the dermis, TCSs have been found to act on fibroblasts, inhibiting interleukin 1 alpha (IL-1 α) and resulting in antiproliferative and atrophogenic effects (Ponec et al., 1980; Wiedersberg et al., 2008).

1.3 Hypotheses of action of inflammatory dermatoses

The primary functions of the stratum corneum are to limit TEWL and safeguard the body from the external environment (Madison, 2003). Physical protection to the external environment is, in part, supported by the arrangement of stratum corneum cells which aid structural integrity and provide tensile strength. Proksch et al. (2008) succinctly categorised the general functions of the stratum corneum barrier as either ‘inside-outside’, pertaining to prevention of TEWL, or ‘outside-inside’ to protect the body from chemical, physical and microbial assaults (Figure 1-4a). However, in cases of diseased skin, quite often the stratum corneum barrier is perturbed, leading to a reduction in the protective features of the skin (Figure 1-4b). Indeed, atopic dermatitis and psoriasis may arise following stratum corneum barrier abnormalities and these changes may be attributed to genetic or environmental

factors (Williams, 2005). In particular, the role of NMFs in maintaining adequate skin hydration and the consequential increase in TEWL when NMF levels are reduced in atopic dermatitis patients has been widely reported (Angelova-Fischer et al., 2014; Horii et al., 1989; Nakagawa et al., 2004; Watanabe et al., 1991). In addition, exposure to an environment of reduced humidity may accelerate TEWL, reduce the hydration and flexibility of the stratum corneum and consequently this layer's ability to perform effectively as a barrier.

(a) Healthy skin



(b) Disease state skin

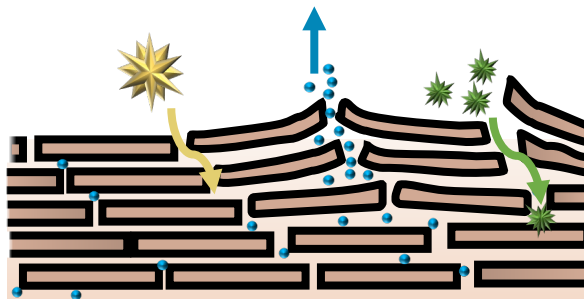


Figure 1-4: Illustration of (a) the defensive features of healthy skin, offering chemical, physical and microbial protection from the environment in addition to preventing transepidermal water loss and (b) a compromised barrier allowing the ingress of chemical and microbial contaminants and egress of water from the stratum corneum.

Some reports suggest that alterations in internal immunological processes, possibly arising from inherited abnormalities, drive a change in the stratum corneum barrier properties and compromise the defensive features of this layer. For example, Elias et al. (2008) detailed the effect of an increased level of serine protease (SP), as a result of inherited abnormalities, on stratum corneum structure and function (Figure 1-5). SP is partially responsible for the degradation of lipid-processing enzymes and

corneodesmosome-constituent proteins. As such, an increase in SP will indirectly hinder the formation of lipids, such as ceramides, a lipid class vital for maintaining mechanical strength of the stratum corneum. Furthermore, degradation of corneodesmosome-constituent proteins may encourage desquamation of the outer layer by weakening the anchorage between corneocytes. It has been suggested that the consequential effect of these internal alterations may alter the barrier properties of the stratum corneum give rise to atopic dermatitis and other inflammatory skin conditions (Elias et al., 2008).

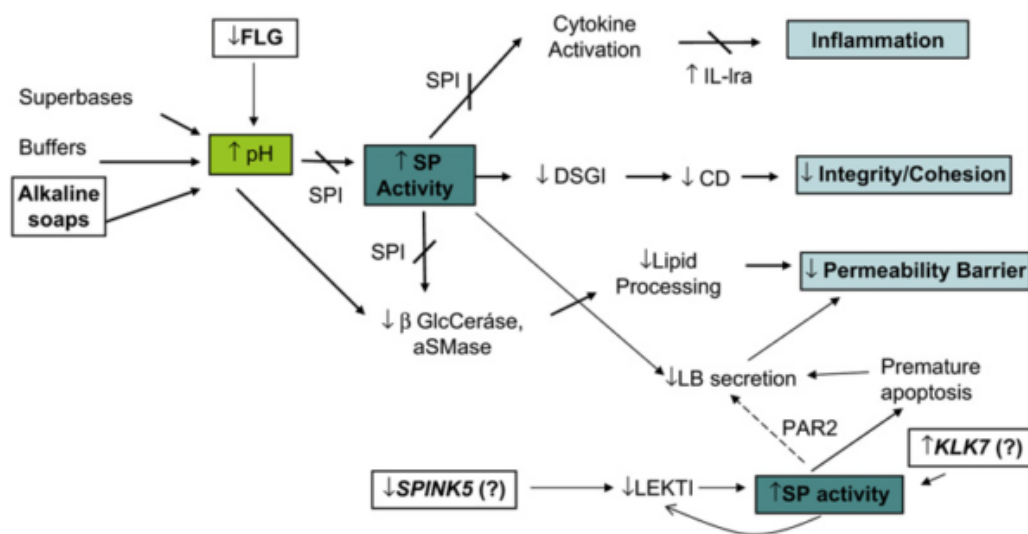


Figure 1-5: Illustration of the effect of an increase in activity of serine proteases on stratum corneum function from Elias (2008). Abbreviations are as follows: SPI (Serine protease inhibitor); DSG1 (desmoglein 1); CD (corneodesmosome); LEKTI (lymphoepithelial Kazal-type trypsin inhibitor); PAR2 (plasminogenactivator type 2 receptor); KLK7 (kallikrein7); FLG (filaggrin); SPINK5 (Serine protease inhibitor Kazal-type 5).

Wickett and Visscher (2006) have also highlighted the role of the skin microbiome, in particular antimicrobial peptides (AMPs), cathelicidins (LL-37) and human β -defensin 2 (HBD-2) found in the stratum corneum lipid matrix, in preventing microbial infections. Interestingly, deficiencies of LL-37 and HBD-2 have been reported in skin lesions of atopic dermatitis patients but not in psoriatic lesions, suggesting a reduced innate ability to resist bacterial infection in cases of atopic dermatitis (Ong et al., 2002). Indeed, this factor may contribute to the increased prevalence of *Staphylococcus aureus* (*S. aureus*) infections in atopic dermatitis lesional and non lesional skin compared to healthy, or indeed psoriatic, skin (Wollenberg et al., 2018b).

Atopic dermatitis and psoriasis are chronic relapsing inflammatory skin conditions with complex aetiologies, yet to be fully understood. However, an interplay between genetics, the environment, skin barrier disruption and immune dysfunction is thought to contribute to development of the conditions (Cork et al., 2009; Raychaudhuri et al., 2014). A clearer understanding of the mechanisms involved may help to then reduce the frequency of relapses, optimise the use of current topical therapies and inform the development of new products for targeted repair of the skin barrier.

1.4 Common topical therapies in the treatment of inflammatory dermatoses

It is estimated that 15-20 % of children worldwide are affected by atopic dermatitis (Nutten, 2015). The prevalence of this condition is increasing worldwide and the impact on the quality of life of sufferers is evidenced through the physical, social and psychological problems experienced in children with atopic dermatitis (Hoare et al., 2000; Lewis-Jones, 2006). It has been found, for example, that over 60 % of children experiencing the associated symptoms of eczema have markedly disturbed sleep patterns as a result, impacting on the quality of life of individuals (Stores et al., 1998). In order to treat such conditions, it is common for patients to be prescribed more than one topical product. Quite often, this treatment package comprises an emollient for skin hydration which forms the basis of continuous management, a TCS to manage inflammatory flare ups and a topical antibiotic in cases of clinically infected skin (Hoare et al., 2000).

1.4.1 Emollients

Emollients are the cornerstone of maintenance therapy for inflammatory skin conditions, predominantly prescribed to treat the symptomatic manifestations of the conditions by increasing hydration of the stratum corneum. Emollients achieve this by occlusion and delivering humectants into the stratum corneum (Loden & Maibach, 1999). The occlusive actions are best achieved through the use of large molecular weight hydrophobic ingredients such as paraffins which remain on the skin surface trapping moisture in the stratum corneum and increasing hydration in the tissue (Rawlings & Harding, 2004). Hygroscopic excipients, such as urea and glycerol, may be incorporated into emollients as they possess water-binding capabilities to varying extents. Thus, once within the stratum corneum these excipients

act to hold water in the skin to hydrate the stratum corneum (Loden, 2003). These actions aid in the symptomatic relief of the condition, such as dryness, tightness and itching, whilst also supporting the repair of the defective skin barrier and improving skin flexibility.

Guidance for the application of emollients suggests that these products should be applied frequently, at times of the day which suit the patient and in generous quantities (250-600 g per week) (Penzer, 2012). The guidance also addresses the role of patients in product selection, as poor patient acceptability of the product can reduce adherence to therapy and result in avoidable treatment failure.

1.4.2 Topical corticosteroids

The use of TCSs in the treatment of skin conditions is well established; TCSs have been employed to treat various inflammatory skin conditions from atopic dermatitis to psoriasis. Since the introduction of hydrocortisone in the early 1950s, there have been many advances in optimisation of the formulation and synthesis of new drug compounds, widening the scope of treatment even further by providing TCSs of varying potencies (Charman et al., 2000).

A majority of the TCSs currently available are synthetic derivatives of hydrocortisone, with modifications made to the basic glucocorticoid structure in an attempt to increase potency, specificity and duration of action. Two widely used topical glucocorticoids arising from the structural modification of hydrocortisone are mometasone furoate (potent UK classification) and clobetasol propionate (very potent UK classification), presented in Figure 1-6. For these compounds, specificity was optimised through the introduction of a double bond at carbon 1 and substitution at carbon 16 (Wiedersberg et al., 2008). Furthermore, it was found that halogenation at carbon 9 would increase the lipophilicity of the steroid, aiding delivery of the drug through the skin, and extending the duration of action (Ponec et al., 1981).

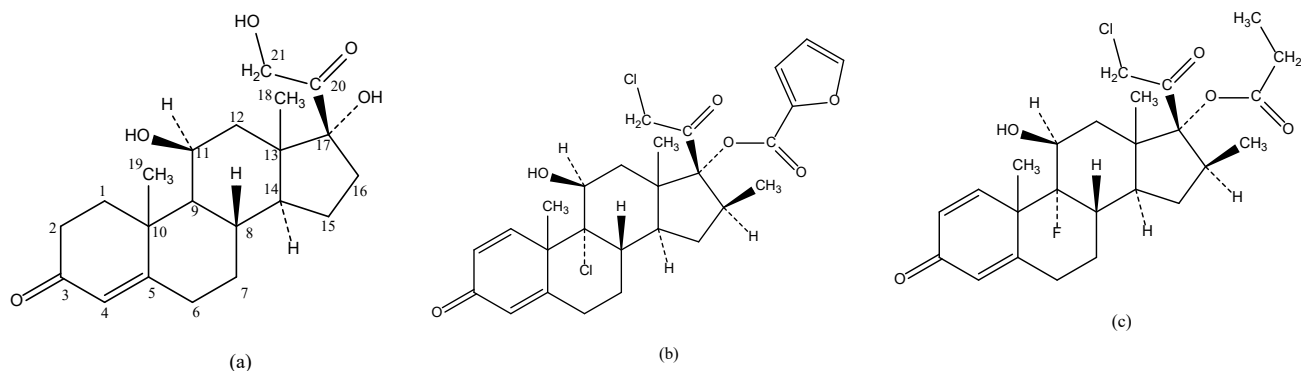


Figure 1-6: The structures of (a) hydrocortisone, (b) mometasone furoate and (c) clobetasol propionate. The respective potency classifications are mild, potent and very potent.

With an increase in molecular potency, the potential for side effects, both local and systemic, such as skin atrophy and adrenal suppression also increases, especially if the TCS is applied for prolonged periods of time and to large areas of skin (Del Rosso & Friedlander, 2005). Knowledge of this fact has influenced the development of clinical guidance; therefore, when prescribing and counselling on the use of TCSs, healthcare professionals should do so with full appreciation for the potency and associated side effect profiles.

Clinical guidance for the use of TCSs in practice is often in support of limiting their use, with the British National Formulary recommending that TCSs should be ‘spread thinly on the skin but in sufficient quantities to cover the affected areas’ (British National Formulary, 2020a). The widely adopted recommendation for the frequency of TCS application is twice daily, stemming from the typical use of well-established TCSs such as hydrocortisone and betamethasone valerate (Williams, 2007). However, whilst such suggestions are typically made, clinical recommendations issued by the NHS Health Technology Assessment programme following a systematic review of the available body of clinical efficacy data, are in support of a reduced frequency of application of TCS to once daily (Green et al., 2004).

In the UK, TCSs are ranked according to a 4-point potency scale: mild, moderate, potent or very potent (British National Formulary, 2020a) based on molecular potency. In contrast, the United States classification system, based on the vasoconstriction assay, accounts for the effect of the vehicle on the

potency of TCSs; for example mometasone furoate 0.1 % w/w cream is classified as a mildly potent TCS, whereas the equivalent strength ointment is ranked as a potent TCS. The latter classification system, accounting for the nature of the vehicle, highlights the importance of considering formulation effects as well as the molecular potency of a drug when determining the potency class (National Psoriasis Foundation, 2019). Use of the vasoconstrictor assay to determine the extent of skin blanching and hence potency as a measure of pharmacodynamic effect was first described by McKenzie and Stoughton (1962). The formulation effects on the extent of skin blanching induced by TCSs have since been well documented; on comparison of commercial TCS formulations, Stoughton (1972) found ointments to exhibit superior *in vivo* blanching responses compared to the equivalent cream or lotion formulations. This was further confirmed in a study documented by Poulsen and Rorsman (1980) where steroids in an ointment formulation were found to be of a higher potency classification, as determined by the skin blanching assay, than the equivalent cream formulation. Stoughton (1987) later used the skin blanching assay to determine the bioequivalence of generic and trade TCSs, finding certain TCS formulations to produce inequivalent vasoconstrictive effects, despite containing equal concentrations of the active drug. The vasoconstrictor assay has since been adopted as a means of determining the potency and bioequivalence of TCSs on the market in the USA (FDA, 1995).

There are currently 15 different proprietary TCS drugs available for UK prescribing, effective in the treatment of atopic dermatitis, seborrheic dermatitis and psoriasis (British National Formulary, 2020a); these can be sub divided by formulation type and drug concentration. Of these 15 drugs mometasone furoate, betamethasone dipropionate and clobetasol propionate are classified as potent, potent and very potent TCSs respectively. As such, the risk of adverse effects, both local and systemic, should be considered before prescribing (National Institute for Health and Care Excellence, 2018a).

1.4.3 Topical antibiotics

Patients with atopic dermatitis exhibit an increased susceptibility to skin infections and it is thought that *S. aureus* is extensively present in clinically affected and unaffected skin of up to 90 % of atopic dermatitis patients (Wollenberg et al., 2018b). This phenomenon has been partially attributed to a reduction in AMPs which are active against a broad spectrum of Gram positive and Gram negative bacteria (Nomura et al., 2003). In cases of clinically infected localised eczema, patients are likely to be prescribed a topical antibiotic with recommendations that the product is applied to the affected area only, for no longer than two weeks to reduce the risk of sensitisation and bacterial resistance (NICE, 2007). Currently available topical antibiotics are mupirocin and fusidic acid, available as cream and ointment formulations. Whilst combined topical antibiotic and corticosteroid preparations are also available such as Fucidin H (hydrocortisone and fusidic acid), the regular use of these preparations are also restricted to reduce the risk of antibiotic resistance (Primary Care Dermatology Society, 2019).

1.5 Application protocols for Emollients, TCSs and Topical Antibiotics

1.5.1 Current clinical guidance

Despite the widespread prescribing of emollients, TCSs and topical antibiotics, there exists a remarkable lack of consensus between healthcare bodies on the optimum application protocol for the products, with recommendations made on the basis of clinical ‘expert’ opinion rather than evidence-based findings (Voegeli, 2017). Furthermore, little consideration is paid to the practicalities of applying emollients, TCSs and topical antibiotics on a daily basis, an important factor as the time required for disease management is burdensome (Drucker et al., 2017; Loden, 2005; Ring et al., 2012). A high variability has been reported within and across studies when investigating the time that patients typically spend on treatment with reports ranging from 17 minutes per day (Jemec et al., 2006) to 63 minutes per day (Holm & Jemec, 2004), despite these studies being conducted in similar settings. Thus, without an understanding of the benefits or drawbacks of particular regimes, adherence to seemingly complex and time-consuming application regimes is likely to be low (Smoker & Voegeli, 2014). Understandably, the lack of an evidence base has resulted in uncertainties amongst healthcare professionals and patients about the safest way in which topical products can be used together to ensure clinical efficacy, adherence to treatment and patient safety (Batchelor et al., 2013). Such uncertainties have been highlighted by a relatively recent eczema Priority Setting Partnership between patients, healthcare professionals and researchers (Batchelor et al., 2013). In this partnership, a key prioritised treatment uncertainty that patients and healthcare professionals have drawn particular attention to is ‘what is the best and safest way of using topical steroids for eczema, for example alternating with other topical treatments?’ emphasising the clinical need for future research to address this issue.

The currently limited understanding and widespread uncertainties surrounding the application of multiple topical products to the skin surface at similar times has a consequential effect on the extent of patient education healthcare professionals can offer, the level of support through medicines management and potentially the quality of care delivered. Smoker and Voegeli (2014) succinctly reviewed the evidence base for combined topical therapy, concluding that ‘there is no substantive

evidence to demonstrate the complex interplay between emollients and TCS and thereby underpin practice’.

A relatively recent randomised controlled trial investigated the effect of the order of application of a TCS and emollient on the severity of atopic eczema in children, finding no difference in the severity of atopic eczema when the TCS was applied 15 minutes before or after aqueous cream (Ng et al., 2016). Whilst this contributes to the limited evidence base, it is noteworthy that the study tested only one emollient, the recommended use for which is as a soap substitute rather than a leave on emollient. Given the variety of formulation strategies employed for the range of emollients currently available, it is expected that the co-application of a particular emollient with a TCSs is likely to result in an emollient specific effect, thus the application of these findings to wider clinical practice is limited.

In addition, whilst it is recommended that topical corticosteroid therapy should continue alongside topical antibiotic treatment (National Institute for Health and Care Excellence, 2018b), to date there have been no reported studies evaluating the impact of one formulation on the performance of the other, or indeed whether the physicochemical properties of one drug may be altered in the presence of a second drug.

Recommendations for the application of a TCS with an emollient are available (Table 1-1), however differ on the time interval between product applications, with suggestions ranging from ‘as soon as absorbed’ (Penzer, 2012) to 60 minutes (Flohr & Williams, 2004). Further disparity is evident when considering the order in which products should be applied, with propositions that the order of application is in fact unimportant (Moncrieff et al., 2013), patient preference should determine which product is applied first (NICE, 2007), TCSs should be applied before the emollient (National Eczema Association, 2019) or that the formulation of the emollient should be considered (cream based emollients 15 minutes before, but ointment based emollients 15 minutes after, the TCS; Ring et al. (2012)). Indeed, there appears to be conflicting guidance within organisations with the clinical knowledge summary for atopic eczema, produced by NICE for primary care practitioners, suggesting that emollients should be applied prior to topical corticosteroids with a several minute interval (National Institute for Health and Clinical Excellence, 2015).

Table 1-1: Summary of the range of clinical recommendations for the order of product applications and time interval in between TCS and emollient applications, adapted from Smoker and Voegeli (2014).

Source of recommendation	Published Date	Topical steroid before emollient	Topical steroid after emollient	Time interval between topical treatments (minutes)
NPC	1998	Yes		Not stated
NPC	1999		Yes	10-20
Flohr, Williams	2004	Yes		60
NES	2004		Not stated	15
NICE	2004		Not stated	Not stated
Prodigy	2004		Not stated	30
Prodigy	2005	Yes		30
BNF	2004		Not stated	30
BNF	2005	Yes		Not stated
Gradwell, McGarvey	2006	Yes		30
Hicklin	2006		Yes	30
PCDS & BAD	2006	Yes		30
NICE	2007	According to patient preference		"several"
NES	2008		Not stated	15
PCDS & BAD	2010	Yes		30
NICE	2010		Yes	30
SIGN	2011	Not stated		Not stated
Penzer	2012		Yes	Emollient has to be fully absorbed
Knott	2012		Yes	10-15
Ring et al.	2012	Yes – if emollient is an ointment	Yes – if emollient is a cream	15
Lawton	2013		Yes	30
NES	2013	Not stated		30-60

Table 1-1 (continued): Summary of the range of clinical recommendations for the order of product applications and time interval in between TCS and emollient applications, adapted from Smoker and Voegeli (2014).

Source of recommendation	Published Date	Topical steroid before emollient	Topical steroid after emollient	Time interval between topical treatments (minutes)
NICE	2013		Yes	"several", emollient has to be fully absorbed
Moncrieff et al.	2013	Not stated		30
American Academy of Dermatology (Eichenfield et al., 2014)	2014	Not stated		Not stated
European Academy of Dermatology and Venereology (Wollenberg, 2018)	2018	Not stated		Not stated
Primary Care Dermatology Society	2019		Yes	20
National Eczema Association	2019	Yes		Not stated
BDNG: How to apply.. Emollients factsheet	Undated a.		Yes	30
BDNG: How to apply.. Topical steroids	Undated b.	Yes		20-30

Note: British Association of Dermatologists (BAD); British Dermatological Nursing Group (BDNG); British National Formulary (BNF); National Eczema Society (NES); National Institute for Health and Care Excellence (NICE); National Prescribing Centre (NPC); Primary Care Dermatology Society (PCDS); Scottish Intercollegiate Guidelines Network (SIGN).

The British Dermatological Nursing Group (BDNG) has issued a statement recommending that moisturisers should be allowed to absorb into the skin before the application of a therapeutic product, however the guidance recognised the need for an evidence base to be developed (Penzer, 2012). Additionally, relatively recent international guidance on the treatment of atopic dermatitis fails to address the issue (Eichenfield et al., 2014; Wollenberg et al., 2018a). These expert opinion statements have arisen in response to concerns that applying topical products at similar times may alter the formulations on the skin surface in an unpredictable manner. However, without an evidence base on which these opinions are formed, there exists a division of opinion, or lack thereof, which highlights the scope for improvement of clinical recommendations and patient education. Indeed, current guidance does advise against the mixing or dilution of TCS with other topical products with concerns that critical quality attributes of the TCS formulation, such as drug stability, formulation stability, the drug delivery profile or consistency of the dose applied may be altered to an unknown extent (Paediatric Formulary Committee, 2019). Thus, a clear understanding of the formulation effects on drug delivery to the skin when different application protocols and product combinations are employed should be gained to better inform current recommendations.

1.5.2 Adherence to therapy

Poor adherence to topical therapy has been widely reported (Aubert-Wastiaux et al., 2011; Richards et al., 1999; Storm et al., 2008; Tan et al., 2012) and it has been found that only 32 % of children prescribed topical therapy for the treatment of atopic dermatitis adhere to treatment (Krejci-Manwaring et al., 2007). In these cases, poor adherence may result in treatment failure, an increase in disease severity and unnecessary ‘stepping up’ to higher risk treatment.

The World Health Organisation defines adherence as “*the extent to which a person’s behaviour – taking medication, following a diet, and/or executing lifestyle changes, corresponds with agreed recommendations from a health-care provider*” (Sabaté & Sabaté, 2003), suggesting that a dialogue between patients/ carers and healthcare professionals is vital for treatment success. Indeed, prescribers typically select topical products in discussion with patients based on a number of factors such as formulation type, patient preference, ease of product use and convenience (Del Rosso & Friedlander,

2005; Feldman et al., 2008; Rapp et al., 1997). Patient preference, in particular, has been found to be an important factor for adherence to topical treatment. For example, a survey of product use amongst patients with psoriasis revealed that 38 % of patients desired less frequent applications of TCSs, including those on once-daily regimes, and non-compliance in 11 % of patients was attributed to the greasy feel of formulations (van de Kerkhof et al., 1998). In agreement with the findings that poor cosmetic characteristics impact negatively on patient adherence, a European wide survey of 1281 psoriasis patients revealed that ‘sticky’ formulations which take ‘too long to rub in’ were two of the prevailing reasons that patients failed to adhere to topical treatment (Fouéré et al., 2005)(Figure 1-7). Moreover, there is a clear correlation between adherence to topical therapy and treatment success and it has been reported that a 10 % decrease in adherence to topical therapy resulted in a 1 point increase in disease severity for psoriasis patients, where severity was measured as the sum score of erythema, scale and thickness (Carroll et al., 2004). Considering the more complex application regimes involved for the use of multiple topical products, it is reasonable to suggest that adherence to these treatment packages may be even lower than the reported findings.

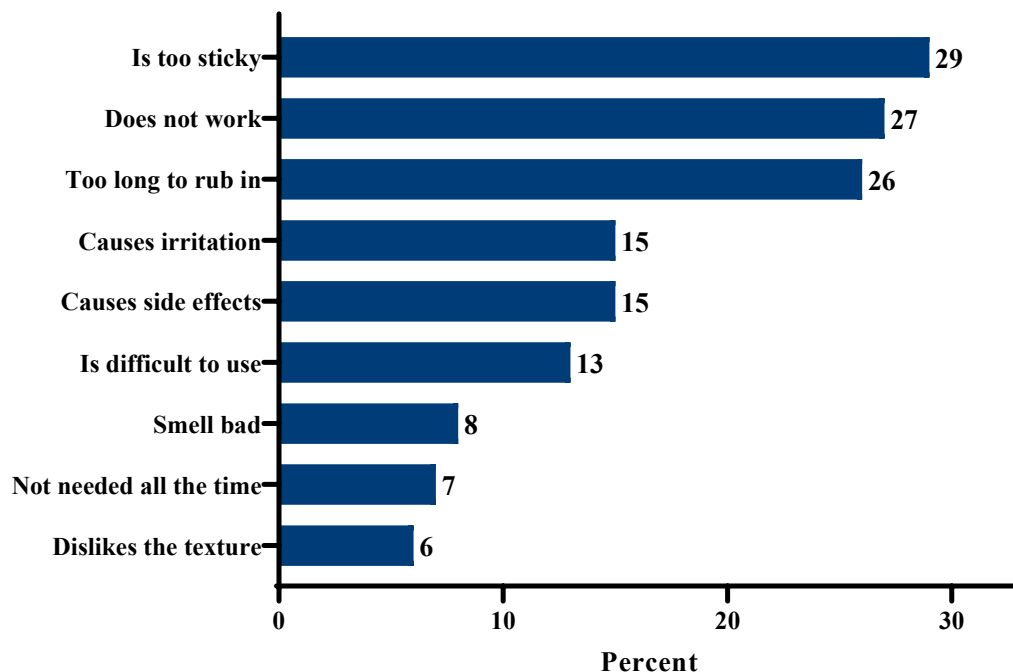


Figure 1-7: The main reason(s) for poor adherence to topical therapy in psoriatic patients, presented as percent of responses, reproduced from Fouéré et al. (2005).

Brown et al. (2006) also reported medication inefficacy to be a predominant reason for poor adherence to topical therapy and this may arise from poor self-management of the condition as a result of inconsistent or insufficient patient education. The application regimes for multiple topical products are complex, time consuming and require patients or carers to modulate product applications in response to an increase or decrease in the severity of the condition. An additional concern reported by patients is fear of the potential side effects of use of topical products, with ‘steroid phobia’ having been reported in 36 – 58 % of patients prescribed TCSs (Aubert-Wastiaux et al., 2011; Mueller et al., 2017; Müller et al., 2016). Thus, clear and consistent patient education on the self-assessment of the condition, the correct applications protocols to employ and how treatment should be adapted for long term therapy should be provided to all patients and carers. Encouraging a positive shift in the educative approaches offered to healthcare professionals, patients and carers can improve self-management of the condition (Williams, 2006) and alleviate concerns surrounding topical corticosteroid phobia. Indeed, a recent approach to empower greater self-management of skin conditions (therapeutic patient education; TPE) has focused on the transfer of skills, in addition to information, centred on evidence based recommendations to support patients and carers in the optimum use of topical products (Barbarot et al., 2013; Stalder et al., 2013).

1.5.3 Unconventional treatments

In severe cases of atopic dermatitis unresponsive to conventional topical treatment, patients may seek alternative, unconventional treatments in an attempt to manage the skin condition. Often, these treatments are centred on the notion that frequent product applications, with strict protocols, can drastically improve the skin condition and negate the need for systemic therapy (Kohn et al., 2016).

One such treatment employs commonly used TCSs but instructs patients to apply the product using a ‘soak and smear’ technique. In essence, this involves a period of soaking in a bath (lukewarm water for 20 minutes exactly), then smearing of a TCS ointment onto wet skin immediately after, without drying (Gutman et al., 2005). It is thought that the period of soaking enables stratum corneum hydration then smearing of the TCS formulation onto wet skin traps in moisture and enhances drug absorption to the skin (Rustad & Henning, 2009). A retrospective study evaluating the effectiveness of the ‘soak and

'smear' technique in achieving disease control reported that 95 % of patients demonstrated an improvement in the severity of atopic dermatitis, measured using the Investigator Global Assessment (IGA), from moderate/ severe to mild (Hajar et al., 2014). However, the study failed to include control groups (the conventional application of the topical product or 'smearing' without soaking) thus did not demonstrate superiority over conventional treatments. Comparatively, Kohn et al. (2016) conducted a randomised controlled trial comparing TCS application to dry skin with TCS application to wet skin (following 10 min of soaking) and reported no significant difference between the control group and treatment group (81.4 -84.8 % reductions in the Eczema Area and Severity Index; EASI). Overall, the current literature base offers conflicting evidence and fails to identify the optimum application regime for the 'soak and smear' technique (Gutman et al., 2005; Hajar et al., 2014; Kohn et al., 2016; Rustad & Henning, 2009). Thus, without evidence of the clinical benefit of the 'soak and smear' therapy, application of potent TCSs in this manner may put patients at an unnecessary increased risk of local and systemic side effects with no clear benefit over traditional application protocols for TCSs.

Another emerging treatment is the Aron Regimen, pioneered by Dr Richard Aron, a therapy with a focus on decolonising the skin of *S. aureus* (Aron, 2019). The causative link between *S. aureus* skin colonisation and atopic dermatitis has been previously reported (Nakamura et al., 2013; Schlievert et al., 2008), and it is thought that *S. aureus* is extensively present in areas of affected and unaffected skin in up to 90 % of atopic dermatitis patients (Wollenberg et al., 2018b). Building on this premise, the Aron regimen employs a three-component system of commonly prescribed topical products in the treatment of skin conditions: an emollient, a topical corticosteroid and a topical antibiotic compounded into one tailored formulation (the Aron mix). However, unlike the conventional application of these products, the Aron regimen entails frequent application of the Aron mix to all affected, and unaffected, areas of the body up to six times a day for one to two weeks, after which the frequency of application is gradually tapered down according to response to treatment. Dr Aron's rationale behind heavily diluting the TCS and topical antibiotic is to allow uninterrupted therapy (and more frequent applications) thereby preventing the risk of 'steroid rebound' or recolonisation of the skin by *S. aureus*. However, it should not be readily assumed that dilution of a TCS reduces the potency of the product,

thus associated side effects, as a poor correlation between the two is largely reported in literature (Gibson et al., 1982; Stoughton & Wullich, 1989).

Evidence supporting the benefits of combined TCS and topical antibiotic therapy in the treatment of infected eczema is lacking (Hoare et al., 2000). One randomised controlled trial (RCT) compared the efficacy of a betamethasone-17-valerate plus fusidic acid to the efficacy of the TCS alone in treating infected atopic eczema, reporting no evidence of improved efficacy for the combined product over the TCS alone (Hjorth et al., 1985). Another RCT compared the efficacy of hydrocortisone and fusidic acid to the efficacy of the TCS alone in the treatment of moderately severe atopic eczema, reporting no superiority of the combined therapy over the TCS alone (Ramsay et al., 1996). It is noteworthy that the latter study investigated the benefits of a topical antibiotic in the treatment of moderately severe atopic eczema with and without bacterial colonisation as also recommended by Dr Aron, rather than the more specific criterion of clinically infected severe atopic eczema, however the workers did not dilute the products in a complex base. Instead, the reported studies employed products which held a marketing authorisation supported by clinical efficacy and stability data and used these products as recommended. The Aron regime fails to use the topical antibiotics and TCSs as intended by the manufactures and in doing so introduces uncertainty about the effects on formulation stability and drug delivery profiles when several marketed products are extemporaneously mixed. As clinical expert opinion and uncontrolled case reports are the only sources of information when evaluating the clinical benefit of employing the Aron regime, further controlled studies should be conducted to evaluate whether applying complex premixed systems (a TCS, emollient and topical antibiotic) to the skin compromises the stability of the drugs in the formulation, the stability of the extemporaneous formulation itself and the expected drug delivery profiles to the skin.

1.6 Drug delivery across the skin

The principal barrier to drug delivery across the skin is the stratum corneum (Williams & Barry, 2012). Successful topical products will be designed to overcome the challenges presented by the stratum corneum, a task made less challenging with an appreciation for the complex structure and function of the stratum corneum, the potential routes of drug delivery across the stratum corneum and the strategies that may be employed to enhance drug delivery to the skin.

1.6.1 Routes of drug delivery

The three main routes by which molecules may traverse the stratum corneum are the intracellular, intercellular and appendageal routes (Hadgraft, 2001) (Figure 1-8).

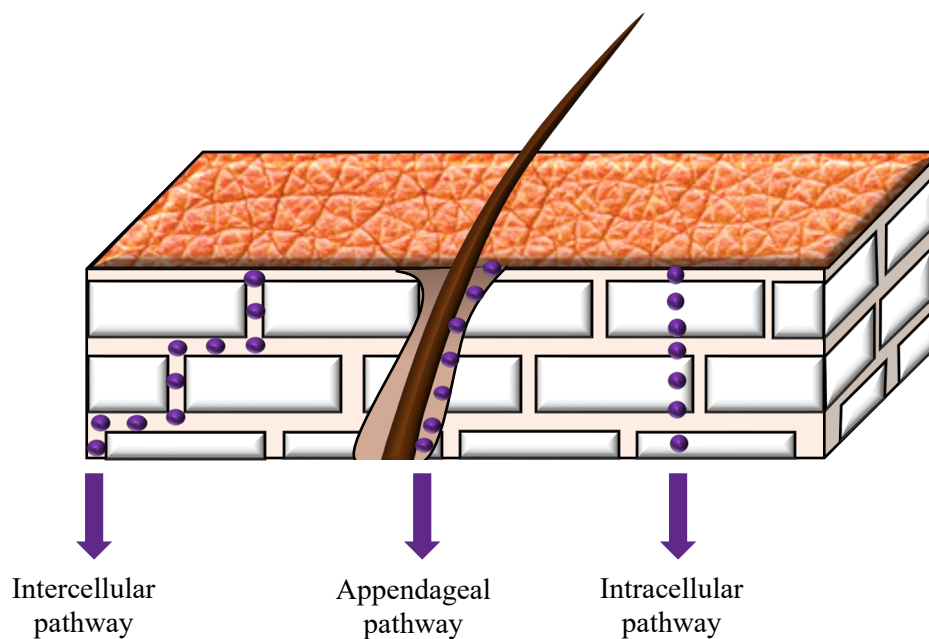


Figure 1-8: Illustration of drug delivery routes through healthy stratum corneum (intercellular, appendageal and intracellular).

The intracellular route requires drug molecules to repeatedly partition into corneocytes of the stratum corneum, diffuse through these cells then partition into the lipid matrix, a route which is thought to be unfavourable for a majority of compounds (Selzer et al., 2013). The intercellular route is comprised of the complex lipid matrix; this route presents a tortuous pathway for drug molecules. However, although the lipid organisation and structure presents a challenge to drug permeation, it is thought to be

comparatively more favourable for drugs to cross the intercellular route as it does not involve the repeated partitioning of drugs through the lipid matrix and corneocytes, as required for intracellular pathways (Scheuplein, 1976).

The appendageal route includes the transport of molecules through sweat glands and hair follicles. Given the small contributory surface area of these routes in comparison to intercellular or intracellular transport, it is thought that this route has a limited contribution to skin penetration (Williams, 2003). However, it has been found that drug concentrations in ducts and follicles are high during early time points, suggesting that this route may contribute more significantly to diffusion during the early stages of drug permeation (Scheuplein, 1967). Furthermore, the appendageal route may be of more importance to drug permeation at particular anatomical sites; Feldmann and Maibach (1967) examined the influence of regional variations on drug absorption across the skin, finding cortisol absorption to increase in areas of larger or more numerous follicles, such as the forehead and scalp.

1.6.2 Influence of the physicochemical properties of the drug on percutaneous absorption

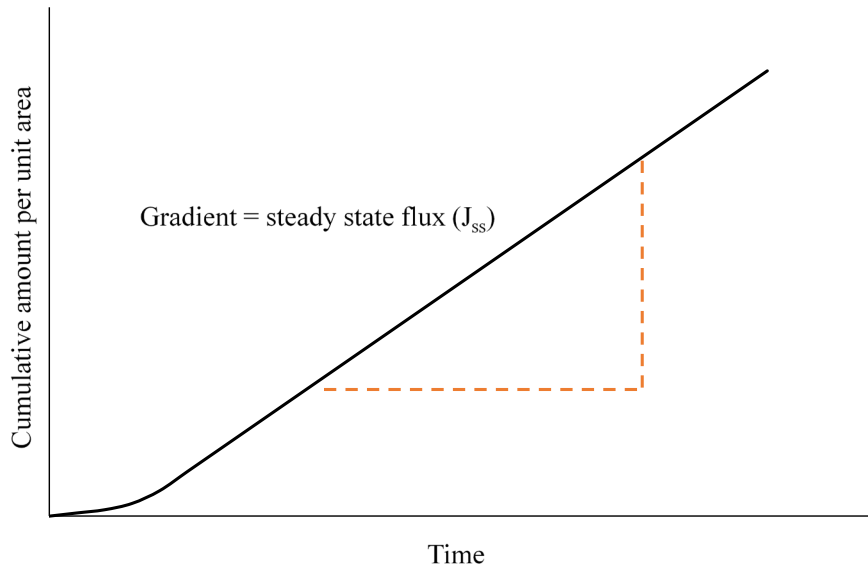
The ability of a compound to cross the stratum corneum barrier has been associated with a number of properties, including: logarithmic octanol: water partition co-efficient (LogP), molecular weight of the drug and the melting point of the drug. The LogP can be used as an indicator of the likelihood of a molecule to partition through the lipophilic stratum corneum and the relatively more hydrophilic environment of the lower dermal layer (Williams, 2003). As the stratum corneum is a heterogeneous layer with both lipophilic and hydrophilic properties, compounds with a LogP between 1 and 3 exhibiting some solubility in both these environments would typically be more likely to traverse the stratum corneum (Finnin & Morgan, 1999). An ideal drug molecule for topical application is also preferred to be less than 500 Daltons (Da), with small molecules having been shown to permeate better than large molecules (Scheuplein et al., 1969). Additionally, molecules with a low melting point have been associated with good permeation (Williams, 2003), as such it is suggested that products with a melting point below 200 °C will be most favourable to formulate into topical products (Finnin & Morgan, 1999). Given the aforementioned ideal properties of a permeant, it is not surprising that

nitroglycerin, a drug with a LogP of 1.6, a mass of 227 Da and a melting point of 13.5 °C, is very effective at penetrating the skin (Hadgraft, 2001). However, few drugs exhibit the ideal properties described above and in such cases several approaches may be employed to enhance skin permeation; in particular chemical enhancement is commonly used by the pharmaceutical industry to improve drug delivery to the skin from topical medicines they develop.

1.6.3 Mathematics of drug delivery to the skin

Drug delivery across the stratum corneum is thought to be a passive process, with no active transport mechanisms having been reported to date (Brown & Williams, 2019). In order to fully understand this a process, an appreciation for the factors governing it must be gained. Delivery of a permeant to the skin occurs in three steps: partitioning of the permeant from the vehicle into the stratum corneum, diffusion of the permeant through the stratum corneum, then partitioning of the permeant from the stratum corneum into underlying tissue. One experimental approach to evaluate drug delivery to the skin from different formulations is through the conduct of *in vitro* drug diffusion experiments. In this two compartment set up, a membrane (e.g. full thickness skin) separates the donor compartment (where the formulation is applied) from the receiver compartment (where drug permeating across the membrane can be monitored). A sound experimental design will tailor the receiver fluid, intervals at which this fluid is sampled and the sampling volume to ensure sink conditions are maintained throughout the experimental period. The data generated following these types of experiments can usually be plotted as the cumulative amount permeated per unit area ($\mu\text{g cm}^{-2}$) against time, and yields a characteristic profile depending on whether steady state drug permeation (infinite dosing; Figure 1-9a) or transient permeation (finite dosing; Figure 1-9b) has been achieved. Additionally, to discern the relative contributions of the drug partitioning and diffusion processes to total drug permeation, the data obtained from these *in vitro* (or *ex vivo*) diffusion studies can be mathematically modelled according to Fick's laws of diffusion, discussed in more detail herein.

A



B

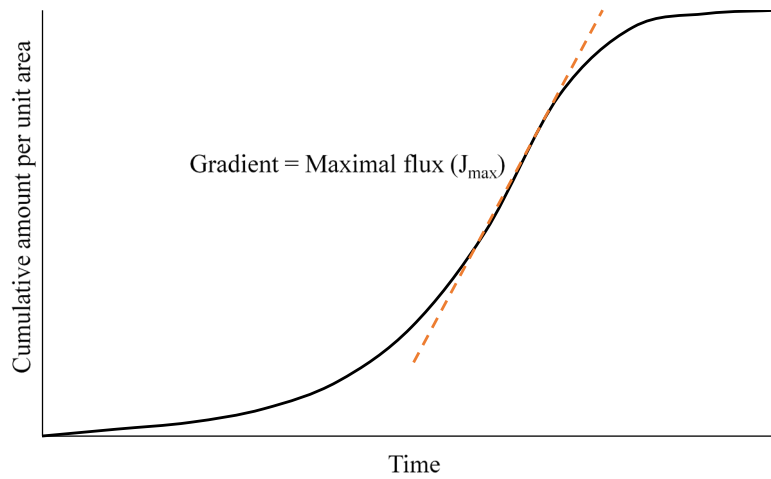


Figure 1-9: The cumulative amount of drug permeation with time following (A) an infinite dose and (B) a finite dose application of a product to human skin. Steady state drug flux (J_{ss}) can be obtained from the gradient of the linear portion of the infinite dose profile. Maximal drug flux (J_{max}) can be obtained from the pseudo steady state (non linear) portion of the finite dose profile.

1.6.3.1 Steady state permeation

The application of an infinite dose of the drug to a membrane maintains a constant drug concentration in the donor phase, despite relatively small amounts of the drug permeating skin tissue over time. Under these conditions, drug permeation reaches a constant rate, often referred to as ‘steady state’ and is easily identifiable as the linear portion of the permeation profile (Figure 1-9a). The time taken to reach ‘steady state’ is the lag time (L_T) and can be calculated by linear extrapolation of the ‘steady state’ region back to the x axis intercept.

Fick’s first law of diffusion states that the rate of transfer of a diffusing substance through the unit area of a section is proportional to the concentration gradient measured normal to the section and can be described by Equation 1-1, where J is mass transfer per unit area, D is the diffusion co-efficient, C is the concentration of the diffusing species and X is the space co-ordinate measured normal to the section (Crank, 1975).

$$J = -D \frac{\partial C}{\partial X}$$

Equation 1-1

The concentration gradient across the membrane (∂C) is calculated as the change in concentration between the outermost layer of the membrane (C_o) and the innermost layer of the membrane (C_i). The distance across which a permeant travels (∂X) is assumed to be the thickness of the membrane (h).

Thus, Equation 1-1 can be simplified to:

$$J = \frac{D(C_o - C_i)}{h}$$

Equation 1-2

It is important to appreciate that when fitting skin permeation data to Fick’s first law, ascribing the distance travelled by a permeant to thickness of the stratum corneum (h) assumes this layer is homogenous and does not account for the tortuous nature of the membrane or the relative contributions of the intercellular, intracellular and transappendageal routes to drug permeation. Thus the values

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obtained provide reasonable approximations, often normalised for pathlength, to allow comparisons across data sets.

A well-executed experimental design will ensure that sink conditions are maintained in the receiver fluid for the duration of the experimental period. Under these conditions, it is expected that the concentration of the permeant in the innermost layer of the membrane (in direct contact with the receiver fluid) is negligible (C_i is essentially zero), thus Equation 1-2 can be further simplified to;

$$J = \frac{DC_0}{h}$$

Equation 1-3

Determining the concentration of the permeant in the outermost layer of the membrane (in direct contact with the formulation) is a challenging task in itself, requiring isolation of the outermost layer of the membrane without contamination from the donor vehicle (Brown & Williams, 2019). It is however known that the concentration of the permeant in the outermost layer (C_0) is related to the concentration of the permeant in the vehicle (C_v) by Equation 1-4, where K is the partition co-efficient of the permeant between the vehicle and the membrane.

$$K = \frac{C_0}{C_v}$$

Equation 1-4

As the concentration of the permeant in the vehicle can be readily obtained, rearranging Equation 1-4 for $[C_0]$ and substituting into Equation 1-3 results in the more conventional form of Fick's first law, widely used to estimate the flux of a permeant under 'steady state' conditions (Equation 1-5).

$$J = \frac{DKC_v}{h}$$

Equation 1-5

1.6.3.2 Drug thermodynamic activity

Fickian diffusion describes a scenario where diffusion is dependent on a concentration gradient existing between the formulation and membrane, where a permeant moves from an area of high concentration to low concentration. Under this assumption, the permeant concentration in the vehicle (C_v) is directly proportional to flux across the membrane (J), as described in Equation 1-5, and it would be expected that the degree of dilution of a product (reduction in C_v) would result in a proportional decrease in J across a membrane. However, this definition does not account for the thermodynamic activity of the permeant in the formulation, the true driver for diffusion across a membrane. Thermodynamic activity describes the ‘escaping tendency’ of a permeant from the formulation with maximum thermodynamic activity (unity) achieved from saturated formulations (Brown & Williams, 2019). Based on this definition, molecules with a high thermodynamic activity in the formulation will favourably partition into the stratum corneum, thus drug thermodynamic activity is related to both drug concentration in the vehicle and the partition co-efficient (term ‘ KC_v ’ in Equation 1-5).

The relationship between thermodynamic activity and drug flux across a membrane was described by Higuchi (1960) where α is the thermodynamic activity of the drug within the formulation and γ is the effective activity coefficient of the drug in the membrane. Thus Equation 1-5 can be rewritten as:

$$J = \frac{\alpha D}{\gamma h}$$

Equation 1-6

The importance of considering the thermodynamic gradient, rather than the concentration gradient, between the formulation and stratum corneum was exemplified by the work of Refai and Müller-Goymann (2002). Dilution of a saturated hydrocortisone cream formulation, by two and four fold in the same base, resulted in no significant change in drug flux across excised human stratum corneum, compared to the application of the undiluted preparation. The authors concluded that though drug concentration was substantially reduced following dilution (C_v reduced up to four fold), hydrocortisone was still suspended in formulations thus at thermodynamic maxima (α unaltered) resulting in a negligible change in drug flux across the membrane.

1.6.3.3 Transient permeation

The use of an infinite dose model enables an evaluation of the fundamental mechanistic processes that impact on drug permeation across a membrane with relative ease. However, to more closely replicate the clinical ‘in use’ application of topical products, the finite dose model is employed. Following a finite dose application to a membrane, drug permeation increases with time then slows to plateau, as depicted in Figure 1-9, and is often indicative of donor depletion from the membrane and formulation interface. From the resultant permeation profiles, maximal drug flux (J_{\max}) and the time to maximum flux (T_{\max}) can be obtained. Generally, for finite dose experiments resulting in non-steady-state diffusion profiles, where the approximation of the apparent partition co-efficient (Kh) and apparent diffusion co-efficient (D/h^2) by Fick’s first law is not possible, further mathematical treatment of the data set is often necessary (Anissimov & Watkinson, 2013; Mitragotri et al., 2011). In such cases, diffusion is governed by Fick’s second law (Equation 1-7), a partial differential equation, which once solved can allow a mechanistic evaluation of drug permeation across the skin.

$$\frac{\partial C}{\partial T} = D \frac{\partial^2 C}{\partial X^2}$$

Equation 1-7

One approach for solving Equation 1-7 is through the Laplace transformation solution (Crank, 1975; Jaeger & Carslaw, 1959), an integral transformation which can be used to calculate drug concentration with respect to position and time (x and t), subject to initial and boundary conditions being set. To solve Fick’s law using this approach, the following assumptions must apply:

- 1) Diffusion is unidirectional (from the formulation towards the skin surface)
- 2) Co-efficients must be independent of time; for example, should D/h^2 changes in response to a change in environment, the Laplace transformation solution would not be suitable
- 3) The diffusion co-efficient must be independent of concentration (constant D/h^2); for example, co-diffusion with a penetration enhancer would result in a nonlinear differential equation rendering the Laplace transformation solution unsuitable.

Alternative approaches to solve Fick's second law include the separation of variables method and the reflection and superposition method (Crank, 1975). The former method has been successfully employed to model a concentration profile in the stratum corneum following short applications of a permeant *in vivo*, using data obtained following tape stripping (Piroet et al., 1997). Fewer studies have reported the use of the latter model, however, the work of Rochowski and Szurkowski (2014) employed both the Laplace transformation solution and reflection and superposition methods to model drug transport into an artificial membrane.

1.7 Formulation considerations for the application of multiple topical medicinal products

1.7.1 Formulation design of topical products

During topical formulation development, excipients are carefully selected and evaluated for their ability to facilitate optimal drug delivery to the skin (within the safe and therapeutic thresholds of the drug). The final formulation should be elegant, easy to use and possess excellent cosmetic properties (e.g. texture, appearance and fragrance) for patient acceptability (Brown & Williams, 2019). When designing topical formulations, the concentration and types of fats, emulsifiers, humectants and preservatives should be considered, in addition to other excipients which play a role in stabilising the formulation such as chelating substances, antioxidants and pH buffers (Loden, 2003). The potential for excipients and the active pharmaceutical ingredient (API) to undergo oxidative degradation may necessitate the inclusion of antioxidants in the formulation to avoid compromising the stability of the topical formulation. Commonly used antioxidants include butylated hydroxytoluene, ascorbic acid and alpha tocopherol (Chang et al., 2013; EMEA, 2003; Sheskey et al., 2019). Adequate control of the pH of a formulation is important for two reasons; first, the pH of the skin surface is thought to lie between 5.5 and 5.8 (Ehlers et al., 2001) and formulations which lie outside this range have the potential to cause skin irritation and exacerbate the skin condition being treated. Second, inadequate pH control of a topical formulation may compromise the chemical stability of the drug or alter the solubility of the drug in the formulation to unpredictable extents.

Topical semisolid formulations can be classed into three general categories: creams, ointments and gels. Creams are ‘semisolid emulsion dosage forms that often contain more than 20 % water and volatiles and less than 50 % hydrocarbons, waxes or polyols as the vehicle for the drug substance’ (USP, 2018). Creams can be water-in-oil emulsions (w/o) where water droplets are dispersed in a continuous phase of oil, oil-in-water emulsions (o/w) where oil droplets are dispersed in a continuous aqueous phase or water-in-oil-in-water emulsions (w/o/w) (Brown & Williams, 2019). Emulsions are inherently unstable systems which often require the addition of emulsifying agents to prevent flocculation or coalescence of dispersed droplets. The benefits of overcoming these formulation challenges to produce an elegant product is clear from the improved patient acceptability of creams over ointment counterparts. Patients report that creams are less greasy and easier to apply compared to ointments, explaining the earlier reported trends that that ‘sticky’ formulations which take ‘too long to rub in’ result in poor adherence to topical therapy (Fouéré et al., 2005).

Ointments are ‘semisolid preparations usually containing less than 20% water and volatiles, and more than 50% hydrocarbons, waxes, or polyols as the vehicle’ (USP, 2018). The higher proportion of hydrocarbons, waxes or polyols in ointment formulations leads to a more occlusive, greasy formulation suitable for application to dry lesional skin and thus may be preferred over cream formulations in cases of severe xerosis. The API may be finely dispersed (suspensions) or dissolved in the ointment base. To improve patient acceptability of this formulation type, excipients which aid spreadability may be incorporated into the formulation, such as cetostearyl alcohol or silicones.

Gels are ‘semisolid systems consisting either of suspensions of small inorganic particles or of organic molecules interpenetrated by a liquid’ (USP, 2018). The gelatinous properties of the continuous liquid phase are achieved by use of thickening agents derived from natural sources, such as carrageenans, or synthetic derivatives, such as hydroxypropyl methylcellulose. Thickening agents vary in swelling strength, pH and temperature response and microbial stability and these factors should be considered when selecting suitable excipients during formulation development.

In addition to essential formulation components, excipients may be incorporated into the formulation to optimise drug delivery to the skin. It can be seen from Equation 1-5 that an increase in the diffusion co-efficient, partition co-efficient or concentration in the vehicle will enhance drug flux across a membrane and various strategies can be employed to achieve this aim. One approach is the inclusion of chemical penetration enhancers (CPEs) which partition into, and temporarily interact with, components of the stratum corneum in a reversible manner (Dragicevic & Maibach, 2016). This strategy is particularly appealing as CPEs are easy to formulate within a topical product, are relatively inexpensive and a wide range of options are available, offering considerable design flexibility with the potential for combined CPEs to exhibit synergistic effects on penetration enhancement (Karande & Mitragotri, 2009). A variety of complex mechanisms have been reported through which CPEs act and can be broadly classified by their ability to directly alter stratum corneum components to promote permeation or indirectly through modification of the formulation itself (Dragicevic & Maibach, 2016).

Membrane perturbation can occur through disruption of the intercellular lipid domain, interaction with corneocytes or by increasing the partitioning of the drug into the stratum corneum, as described by the lipid-protein-partitioning theory (Barry, 1991). In the first case, CPEs may interact with the intercellular lipid domains of the stratum corneum rendering the intercellular pathway more permeable to drug diffusion. Modification of the intercellular lipid domain can result in fluidisation of the lipid bilayer, alteration of the polarity of the intercellular lipids, extraction of lipids from the bilayer or phase separation within the lipid bilayer (Barry, 2004). Alternatively, CPEs may act on intracellular keratin to denature or change the conformation of corneocytes, alter the lipid domains of the cornified envelope to reduce cohesion between corneocytes or weaken the anchorage of corneocytes to the intercellular lipid domain (Barry, 2001).

Finally, direct alteration of the stratum corneum barrier may be achieved by altering the solvent nature of the barrier to promote drug partitioning from the vehicle into the stratum corneum. In this case, ideal solvents will partition from the vehicle into the stratum corneum, increase the solubility of permeants in the tissue and promote partitioning of the permeant into the stratum corneum (Barry, 2001; Benson, 2005).

In addition to direct effects on the stratum corneum, CPEs may also act either directly or indirectly to enhance drug permeation into the stratum corneum through: (i) increasing the solubility of the permeant in the vehicle, a strategy of particular benefit to permeants with low solubility (e.g. steroids in aqueous donor solutions) or (ii) modification of the thermodynamic activity of the drug during formulation development (i.e. ensuring drug saturation in the final product) or *in situ* (e.g. rapid permeation of a solvent from the formulation into the skin or solvent evaporation leaves the drug in a thermodynamically heightened state within the vehicle; see Equation 1-6).

It is, however, worth considering that CPEs may possess the ability to perturb the stratum corneum barrier through more than one mechanism. For example, propylene glycol, an excipient present within Dermovate cream (0.05 % w/w clobetasol propionate), is often employed as a co-solvent in formulations to alter both drug solubility in the vehicle and partitioning into the skin; the latter of which may be achieved by increasing drug solubility within the stratum corneum (Arellano et al., 1999; Schneider et al., 1996). A clinical example where increasing drug thermodynamic activity in the vehicle resulted in enhanced drug delivery to the skin is evidenced by Dioderm, a currently available 0.1 % w/w hydrocortisone cream formulated with propylene glycol to deliver the equivalent clinical efficacy to that of the 1 % w/w Hydrocortisone cream BP formulation (Whitefield & McKenzie, 1975). The formulation design of Dioderm comprised drug dissolved in propylene glycol (to increase the drug solubility in the vehicle) and production of a saturated formulation (to achieve maximum thermodynamic activity). The outcome was a formulation that delivered the equivalent clinical efficacy to that of the pharmacopeial formulation, achieved with one tenth of the drug strength in the formulation (Barry & Woodford, 1976) and classified within the same potency group as the 1% w/w hydrocortisone formulation (mild, UK classification).

Urea, a hygroscopic agent, is commonly used in topical preparations for its capacity to bind with water and increase stratum corneum hydration. However, this agent also exhibits keratolytic properties, potentially interacting with intracellular keratin and enhancing drug permeation into the skin (Trommer & Neubert, 2006). Indeed, formulating a topical 1 % w/w hydrocortisone preparation with 10 % urea resulted in the increased activity and bioavailability of hydrocortisone from Alphaderm cream (Barry

& Woodford, 1977; Woodford & Barry, 1984), resulting in an increase in the UK TCS potency classification to a moderate potency (British National Formulary, 2020b). This further demonstrates how careful selection of the excipients in a topical product may optimise formulation performance.

1.7.2 Considerations for an altered topical formulation

1.7.2.1 Drug thermodynamic activity

The application of multiple topical products to the skin surface may result in formulation changes *in situ*, if applied at similar times, or within the premixed system, if extemporaneously prepared, the implications of which warrant consideration from a formulation and clinical perspective. The extent to which a topical formulation may change and the consequential impact on formulation performance and clinical efficacy is largely governed by the formulation design of the products. It has been established, in Section 1.6.3.2, that the thermodynamic activity of the permeant in the formulation is an important driver for drug delivery to the skin. Mixing of one topical product with another, be this the emollient, TCS or topical antibiotic, may alter drug thermodynamic activities in the mixed formulations, relative to the individual marketed products. If thermodynamic activities are substantially reduced, for example, this may result in clinically ineffective drug concentrations delivered to the skin and, in the case of topical antibiotics, an increased risk of antimicrobial resistance. Indeed, the approach of diluting a topical product with a similar base to reduce the drug concentration (and assumed potency) has been commercially exploited, as exemplified by the potent Synalar cream (0.025 % w/w) which was diluted to produce a TCS product with one tenth of the strength and a lower potency classification (Synalar 1 in 10 dilution; mild potency classification) compared to the original strength formulation (British National Formulary, 2020b). However, it is now recommended that the extemporaneous dilution of topical products to tailor potency should be avoided (British National Formulary, 2020a), considering reports that the extent to which efficacy is altered does not always correlate with the degree of dilution of a product, thus cannot be readily predicted in a clinical setting. Additionally, Gibson et al. (1983) highlighted the impact of using different diluents on potency of the product, finding an extemporaneous 1 in 4 dilution of Betnovate ointment resulted in significantly greater vasoconstriction compared to the proprietary Betnovate 1 in 4 ointment.

1.7.2.2 Solvent thermodynamic activity

During formulation development the degree of saturation of solvents in the formulation is optimised to tailor drug delivery to the skin, thus an equally important consideration is the altered thermodynamic activity of CPEs in the TCS and topical antibiotic formulations when multiple topical products are mixed *in situ* or extemporaneously together. For example, propylene glycol is a commonly employed CPE to increase drug solubility in the vehicle and promote drug partitioning into the stratum corneum (Arellano et al., 1999). The dependence of oxybutynin (Santos et al., 2010) and loperamide (Trottet et al., 2004) flux on propylene glycol activity has been established, with reports that propylene glycol depletion from the skin surface may leave the drug ‘stranded’ in the formulation, reducing drug flux across the skin. Indeed, many of the frequently employed penetration enhancers are most effective within an optimum concentration range; with increasing proportions of propylene glycol in a binary mixture with water found to correlate with an increase in ibuprofen permeation across skin, for example (Watkinson et al., 2009). Typically, the lowest concentration required for penetration enhancing activity is employed given the potential for compounds to cause skin irritation, an effect that would hinder rather than further the treatment goals for patients with an already compromised skin barrier. Thus, it is possible that mixing of multiple topical products together (*in situ* or extemporaneously) may reduce solvent thermodynamic activities in the formulations to an extent that the expected delivery profile of the drug(s) to the skin is also reduced. Equally, mixing of multiple topical products (either extemporaneously or *in situ*) may alter the intricate design of each individual medicinal product and introduce new excipients with penetration enhancing capabilities into the marketed formulations. Depending on the concentrations of these excipients within the original formulations, the thermodynamic activity of the excipients when mixed with other products and the potential to act synergistically with other excipients, the delivery of the one or more drugs to the skin may be altered to an extent which is difficult to predict. With the current understanding of the complex factors that may influence the performance of a TCS or topical antibiotic, there is a clear need to establish the exact formulation changes that may be occurring in complex mixtures of TCSs, topical antibiotics and emollients and the implications for drug delivery to the skin.

1.7.2.3 Formulation stability

During formulation development, critical quality attributes such as the physical and chemical stability of the drug in the formulation, alongside the stability of the formulation itself, are assessed to ensure the desired product quality is achieved (ICH, 2009). In extemporaneous products, substantial dilution of TCSs, topical antibiotics and emollients with diluents dissimilar to the base vehicles of the formulations may alter the intricate balance of preservatives, antioxidants and pH buffers to an extent that the stability of the drug and formulation are compromised, impacting on the anticipated shelf life of the new product.

1.7.2.4 Occlusion and obstruction

The current clinical guidance on the order of application of multiple topical products to the skin is conflicting and it has yet to be established whether applying one topical product before or after another may lead to occlusive or barrier effects on drug delivery to the skin. Barry et al. (1984) reported that occlusion following the application of a TCS to the skin enhanced percutaneous absorption *in vivo*, as measured by the vasoconstrictor assay. This was potentially attributable to an increase in stratum corneum hydration as a result of a decrease in TEWL, swelling of the corneocytes and a resultant increase in the lipid matrix diffusion pathways to enhance permeation of compounds into and across the stratum corneum (Walters & Dekker, 2002). If TCSs or topical antibiotics are applied to the skin surface before a product with occlusive properties, similar enhancements in drug delivery to the skin may be observed compared to the applications of the individual products alone. In the reverse scenario, the application of these products to the skin surface after an emollient, for example, may create an additional barrier to drug delivery to the skin. The formulation characteristics of each product are likely to govern the extent to which a formulation is occlusive or obstructive on the skin surface and it is, to date, unclear whether the short time intervals that are recommended to be left between product applications are sufficient to mitigate these potential effects on drug delivery to the skin.

1.8 Summary

In the treatment of inflammatory skin conditions, it is common for patients to be prescribed more than one topical product. Quite often, this treatment package comprises an emollient which forms the basis of continuous management, a TCS to manage inflammatory flare ups and a topical antibiotic in cases of clinically infected skin (Hoare et al., 2000). Whilst the manufacturers' recommendation for the safe and effective use of TCSs and topical antibiotics is made with a good understanding of the clinical efficacy of the formulation, they are not made with consideration to the application with other topical products. Despite the widespread prescribing of TCSs, emollients and topical antibiotics, there exists a remarkable lack of consensus between healthcare bodies on the optimum application protocol for the products, with recommendations made on the basis of clinical 'expert' opinion rather than evidence-based findings (Voegeli, 2017). When marketed topical medicinal products are mixed together, a range of complex formulation changes may ensue *in situ* or in the extemporaneously prepared mixtures, potentially altering the critical quality attributes of the products to varying extents (drug delivery profile, drug stability within the formulation and stability of the formulation itself). A greater understanding of the complex interplay occurring when multiple topical products are applied at similar times will support the advancement of comprehensive and conclusive guidance on the best way to apply TCSs, topical antibiotics and emollients to maintain the efficacy of treatment and the expected safety profiles.

Considering the wide range of TCS, topical antibiotic and emollient combinations available and the variety of approaches currently recommended for application of these products, ascertaining the impact on drug delivery to the skin exclusively through clinical efficacy studies is unfeasible and offers a limited mechanistic understanding of formulation effects on drug delivery to the skin. Franz et al. (2009) demonstrated a good predictive power of *in vitro* human skin drug absorption experiments and have found such experiments to be beneficial for explaining the findings of clinical efficacy studies (Franz et al., 1999). This experimental method has been widely adopted by the pharmaceutical industry as a relevant model for *in vivo* studies, particularly during formulation development, and thus can be employed to elucidate the effects on skin retention and percutaneous absorption of TCSs and topical antibiotics when the products are applied with emollients according to various application protocols.

1.9 Aim and objectives of the thesis

The overarching aim of this thesis was to develop a body of evidence evaluating the impact of clinical application protocols for multiple topical products on drug delivery to the skin, *in vitro*. To achieve this aim, the following objectives were set:

1. Conduct a mechanistic evaluation of the effect of applying various combinations of TCSs and emollients in a premixed system on the *ex vivo* percutaneous absorption and skin retention of the TCSs.
2. Establish whether *in situ* formulation changes occur when a TCS was applied with an emollient according to various clinical application protocols and discern the effects on TCS formulation performance.
3. Evaluate whether the trends observed with TCS and emollient systems were applicable to further complex mixtures of topical products (a TCS, topical antibiotic and emollient).
4. Assess the chemical stability of a TCS and topical antibiotic in an extemporaneous preparation (Aron mix) over the recommended period of use.

Chapter Two:

A mechanistic evaluation of the impact of mixing Elocon cream and emollients on the percutaneous absorption and skin retention of mometasone furoate

2.1 Introduction

Atopic dermatitis is a chronic or chronic-relapsing inflammatory skin condition characterised by pruritus, erythema and dry skin (NICE, 2007). It is estimated that 15-20 % of children worldwide are affected by atopic dermatitis (Nutten, 2015) The prevalence of this condition is increasing worldwide and the impact on the quality of life of sufferers is evidenced through the physical, social and psychological problems experienced in children with atopic dermatitis (Drucker et al., 2017; Flohr, 2011). It has been found, for example, that over 60 % of children experiencing the associated symptoms of eczema have markedly disturbed sleep patterns as a result, impacting on the quality of life of individuals (Stores et al., 1998). In order to treat such conditions, it is common for patients to be prescribed more than one topical product. Quite often, this treatment package comprises an emollient which forms the basis of continuous management and a topical corticosteroid (TCS) to manage inflammatory flare ups (National Institute for Health and Care Excellence, 2018b).

In a clinical setting, it is likely that TCS and emollient formulations are applied at similar times with some guidance recommending that the time interval between product applications can be ‘as soon as absorbed’ (Penzer, 2012) or ‘several minutes’ (NICE, 2007). The guidance on the order of application and the time interval between applications of TCSs and emollients is unclear, giving rise to uncertainties amongst healthcare professionals and patients about the safest way in which the two products can be used together to ensure clinical efficacy and patient safety (Smoker & Voegeli, 2014). Such uncertainties have been highlighted by a relatively recent eczema Priority Setting Partnership between patients, healthcare professionals and researchers where particular attention was drawn to the question ‘what is the best and safest way of using topical steroids for eczema, for example alternating with other topical treatments?’ (Batchelor et al., 2013).

Whilst the manufacturer recommendations for the safe and effective use of TCSs are made with the support of clinical data and a good understanding of the clinical efficacy of the particular formulation, they are not, however, made with consideration to application with emollients.

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Currently there is very little evidence available to support the understanding of the mechanistic effects that may be occurring when emollients and TCSs are applied to the skin surface at similar times. Smoker and Voegeli (2014) succinctly reviewed the evidence base for combination therapy, concluding that ‘there is no substantive evidence to demonstrate the complex interplay between emollients and TCS and thereby underpin practice.’ It is, however, well established that an optimised formulation can enhance the amount of drug delivered to the skin, this can be achieved through careful selection of excipient with penetration enhancing effects or by creating saturated systems to enhance the thermodynamic activity of the drug in the formulation (Pellett et al., 1997; Santoyo & Ygartua, 2000; Whitefield & McKenzie, 1975). Thus, it is likely that an altered formulation on the surface of the skin, resulting from the application of multiple products at similar times, may impact on drug release from the formulation and delivery to the skin. Establishing the effects, if any, of applying combinations of TCSs and emollients on drug delivery to the skin will help inform on the current uncertainties surrounding the clinical use of these products.

Thus, the objectives of the work described in this Chapter were twofold: firstly, to screen the formulation effects of a range of commonly used emollient formulations when mixed with a TCS on TCS drug transport. Secondly, to establish whether the formulation effects observed impact on the percutaneous absorption and skin retention of the TCS. To achieve these objectives, Franz cell experiments across silicone membrane and full thickness human skin were conducted. A non-porous homogenous membrane was selected to understand the fundamental drug transport characteristics of the premixed formulations, a model which has been previously employed with success (Fiala et al., 2010; Oliveira et al., 2012b; Wood et al., 2012). Human skin was employed to evaluate whether the formulation changes observed in the premixed systems had the potential to be of clinical relevance. Mometasone furoate was employed as a model TCS, delivered from two marketed formulations: Elocon cream (0.1 % w/w mometasone furoate) and Elocon ointment (0.1 % w/w mometasone furoate). The emollients investigated were selected based on trends in emollient prescribing (Diprobace cream, Doublebase gel and Cetraben cream are within the top five prescribed emollients in the last five years; NHS Digital (2019)), the formulation type to allow for comparison of the

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performance of a cream and ointment formulation (Diprobase ointment as a comparator to Diprobase cream) or to observe the effect of excipients present within the formulations known to have penetration enhancing effects (Hydromol Intensive cream, Aquadrate cream, Doublebase gel).

2.2 Materials and methods

2.2.1 Materials

Micronised mometasone furoate (Ph Eur) was provided by MedPharm Ltd (Guildford, UK). Elocon cream (0.1 % w/w mometasone furoate), Elocon ointment (0.1 % w/w mometasone furoate), Diprobace cream, Diprobace ointment, Aquadrate cream, Hydromol Intensive cream, Cetraben cream and Doublebase gel were purchased from the University of Hertfordshire Campus Pharmacy (Hertfordshire, UK). Phosphate buffered saline (PBS) tablets, acetonitrile (HPLC grade), absolute ethanol (99 + %), titanium dioxide and white soft paraffin were acquired from Fisher Scientific (Leicestershire, UK). Aluminium starch octenylsuccinate (DryFlo[®]) was provided by AzkoNobel (Warrington, UK). Non-sterile, medical grade 0.002” silicone membrane was purchased from Bioplexus (Los Angeles, USA).

2.2.2 Analytical method development

2.2.2.1 Development of the HPLC methods for mometasone furoate quantification

Quantitative analysis of mometasone furoate in samples was achieved using an Agilent 1260 Infinity quaternary pump and high performance autosampler coupled to an Agilent 1260 multi wavelength UV/Vis detector set to 253 nm (Agilent Technologies, UK). Chromatographic analysis was performed using a reverse phase Hypersil[™] C₁₈ column (5 µm particle size, 250 mm x 4.6 mm; Phenomenex, UK) in conjunction with a SecurityGuard[™] guard cartridge system packed with a C₁₈ cartridge (4 mm x 3 mm; Phenomenex, UK), a sample injection volume of 20 µL and a constant flow rate of 1 mL min⁻¹. The column temperature was set to 21 ± 2 °C. The Agilent ChemStation software (Agilent Technologies, UK) was used for data acquisition. An isocratic elution method was developed to enable the suitable quantification of mometasone furoate in samples following *in vitro* drug transport experiments and a gradient elution method was developed for drug quantification in samples following *ex vivo* drug skin permeation and penetration experiments. The mobile phase composition for the isocratic elution of mometasone furoate was water (18.2 MΩ MilliQ; 45 %) and

HPLC grade acetonitrile (55 %). The mobile phase composition for the gradient elution of mometasone furoate is detailed in Figure 2-1. Under these conditions the elution times of mometasone furoate were 12.4 min and 19.6 min for the isocratic and gradient methods, respectively.

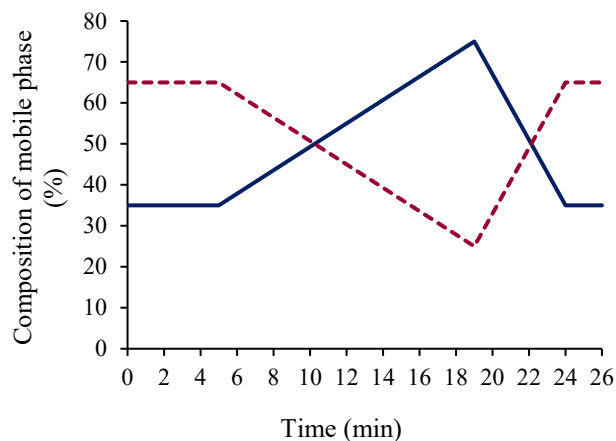


Figure 2-1: Graph illustrating the HPLC UV gradient elution profile of HPLC grade acetonitrile (—) and 18.2 MΩ MilliQ water (-----) employed for the quantification of mometasone furoate in samples.

2.2.2.2 Preparation of calibration standards

A 100 $\mu\text{g mL}^{-1}$ stock solution of mometasone furoate was prepared by weighing 10 mg of the drug into a 100 mL volumetric flask and making up to volume with the diluent, acetonitrile. A series of standards were prepared by appropriate dilution of the stock solution with the diluent; the concentration of the standards ranged from 0.05 $\mu\text{g mL}^{-1}$ to 100 $\mu\text{g mL}^{-1}$ and 0.01 $\mu\text{g mL}^{-1}$ to 100 $\mu\text{g mL}^{-1}$ for the isocratic and gradient elution methods, respectively. Drug quantification was achieved using the analytical methods detailed in Section 2.2.2.1 and calibration curves plotted for the detected range.

2.2.2.3 Determination of fitness for purpose of the analytical method

The HPLC methods were validated for linearity, precision and accuracy in accordance with the International Conference on Harmonization (ICH) guidelines (ICH, 2005). Linearity was determined by the correlation coefficient (R^2) for calibration curves constructed following the isocratic elution method analysis (concentration range 0.05 $\mu\text{g mL}^{-1}$ – 100 $\mu\text{g mL}^{-1}$) and the gradient elution method analysis (0.025 $\mu\text{g mL}^{-1}$ – 100 $\mu\text{g mL}^{-1}$) of mometasone furoate standards. The standard error for the

predicted y value for all x values in the regression (STEYX) was calculated and used in Equation 2-1 and Equation 2-2 to calculate the limit of detection (LOD) and limit of quantification (LOQ) of mometasone furoate, respectively.

$$\text{LOD} = \left(\frac{\text{STEYX}}{\text{SLOPE}} \right) \times 3.3$$

Equation 2-1

$$\text{LOQ} = \left(\frac{\text{STEYX}}{\text{SLOPE}} \right) \times 10$$

Equation 2-2

Determination of the precision of the analytical methods was achieved by intra-day and inter-day analysis. Intra-day precision was measured by 6 replicate injections of 5 $\mu\text{g mL}^{-1}$, 50 $\mu\text{g mL}^{-1}$ and 100 $\mu\text{g mL}^{-1}$ samples of standards of mometasone furoate prepared on the same day. Inter-day precision was assessed through the analysis of 6 replicate injections of 5 $\mu\text{g mL}^{-1}$, 50 $\mu\text{g mL}^{-1}$ and 100 $\mu\text{g mL}^{-1}$ samples prepared in triplicate on 3 separate days.

The accuracy of the analytical methods was tested by preparing triplicate samples of mometasone furoate in the diluent at three concentrations (low, medium and high) and quantifying using the respective HPLC UV methods. Accuracy was determined using Equation 2-3, where MC and TC denote the measured and theoretical drug concentration, respectively.

$$\% \text{ Accuracy} = \left(\frac{\text{MC}}{\text{TC}} \right) \times 100$$

Equation 2-3

2.2.3 Formulation selection

Two TCS products and six emollient formulations were selected for investigation based on trends in prescribing, formulation type and excipients with known penetration enhancing capacity. The full excipient list for the selected formulations is detailed in Table 2-1.

Table 2-1: The listed excipients for all formulations investigated. The topical corticosteroid products were Elocon cream and Elocon ointment, both containing 0.1 % w/w mometasone furoate. The six selected emollients were Aquadrate cream, Cetraben cream, Diprobase cream, Diprobase ointment, Doublebase gel and Hydromol Intensive cream. Data were obtained from the most recently published summary of product characteristics for the respective formulation.

Elocon cream	Elocon ointment	Aquadrate cream	Cetraben cream	Diprobase cream	Diprobase ointment	Doublebase gel	Hydromol Intensive cream
Mometasone furoate (0.1 % w/w)	Mometasone furoate (0.1 % w/w)	Isopropyl myristate	Glycerol	Phosphoric acid	White soft paraffin	Isopropyl myristate	Isopropyl myristate
		Urea (10 %)	Emulsifying wax	Sodium	Liquid paraffin	(15%)	Urea (10 %)
Hexylene glycol	Propylene glycol	White soft paraffin	Cetostearyl alcohol	dihydrogen		Glycerol	White soft paraffin
Phosphoric acid	stearate (2.0%)	Palmitic acid	Phenoxyethanol	phosphate		Liquid paraffin	Palmitic acid
Hydrogenated soybean lecithin	Hexylene glycol	Sorbitan laurate	Propylparaben	Sodium hydroxide		Sorbitan laurate	Sorbitan laurate
White wax	White beeswax	Arlatone G	Ethylparaben	Macrogol		Triethanolamine	Arlatone G
White soft paraffin	White soft paraffin	<i>(hydrogenated castor oil)</i>	Butylparaben	Cetostearyl Ether		Carbomer	<i>(hydrogenated castor oil)</i>
Aluminium starch octenylsuccinate	Purified water	Maize Starch	Methylparaben	(Cetomacrogol)		Phenoxyethanol	
		Syncrowax HR-C	Purified water	Cetostearyl alcohol		Purified water	Maize Starch
				Liquid paraffin			Syncrowax HR-C
				White soft paraffin			
				Chlorocresol			
				Purified water			

2.2.4 Franz cell assembly

2.2.4.1 Full thickness human scrotal skin preparation

Excised human scrotal skin was obtained with consent from gender reassignment surgeries following ethical approval from the South London Research Ethics Committee (ethics No. 10/H0807/51). Skin samples were removed from storage (- 20 °C) and left to thaw at ambient temperature, the subcutaneous fat was removed using a scalpel and samples were used immediately or stored at - 20 °C until required. Where skin samples were frozen prior to use, the skin was removed from storage and allowed to thaw at ambient temperature before being cut to size.

2.2.4.2 Franz cell assembly

Calibrated Franz cells (Soham Scientific, UK) with an average surface area of 1 cm² and average receiver volume of 3 mL were used to conduct drug transport investigations across silicone membrane and drug permeation and skin distribution studies employing human skin. On the day of the experiment(s), the full thickness human scrotal skin samples were cut to size (1.5 cm² x 1.5 cm²) and mounted, stratum corneum side facing upwards, between the donor and receiver chambers of Franz cells. Where Franz cell experiments were conducted on synthetic membrane, silicone membrane sheets (0.002'' thickness) were cut to size and mounted between the donor and receiver chambers of Franz cells. Receiver chambers were filled with the appropriate receiver fluid, selected based on the findings of the receiver fluid development studies, continuously stirred with a small Teflon coated magnetic flea and the sampling arm occluded to prevent evaporation of the solution. Assembled Franz cells were placed on a submersible stirrer plate in a pre-heated water bath set to 37 °C to achieve a membrane surface temperature of 32 °C and equilibrated for 30 min prior to dosing. Franz cells were briefly inverted to verify the integrity of the membrane barrier; following visual examination any samples allowing back diffusion of the receiver fluid into the donor chamber or an obvious drop in receiver fluid volume were replaced. Six replicate Franz cells were assembled for each formulation evaluated.

2.2.5 Franz cell method development

2.2.5.1 Selection of the receiver fluid systems for *in vitro* and *ex vivo* Franz cell experiments

To ensure adequate solubility of mometasone furoate in the receiver fluid, the solubility of mometasone furoate in PBS alone and PBS with absolute ethanol (10 %, 20 % or 30 %) was determined. To gain an appreciation of the potential for the solubility of mometasone furoate in the TCS formulation to be altered when mixed with the emollient formulations, an evaluation of the saturated solubility of mometasone furoate in the liquid excipients of the formulations was also investigated. The liquid excipients of all formulations were: water, glycerol, liquid paraffin, isopropyl myristate, castor oil and hexylene glycol.

Saturated solutions were prepared as follows: adequate amounts of mometasone furoate were added to the range of solutions until a suspension was formed (confirmed visually by the continued presence of drug particles in solution), samples were then stirred for 24 h at room temperature and filtered through Millex Millipore 0.22 µm syringe filters. All samples were appropriately diluted prior to drug quantification by HPLC UV analysis.

2.2.5.2 Selecting appropriate receiver fluid sampling time points for *in vitro* and *ex vivo* Franz cell experiments

A study was conducted to establish a sampling protocol to adequately profile drug transport across silicone membrane whilst maintaining sink conditions for the duration of the experiment. Franz cells (n=3) were assembled with silicone membrane and the receiver chamber filled with the receiver fluid system developed in Section 2.2.5.1 (PBS and ethanol; 70:30). The membrane was dosed with 500 mg of Elocon cream or Elocon ointment by weight. Samples (200 µl) of the receiver fluid were taken periodically up to 28 h and replaced with fresh preheated receiver fluid. With respect to Elocon cream, a sampling protocol was also required to investigate the permeation of mometasone furoate across human skin following the finite dosing of Elocon cream. To achieve this, skin samples were mounted in Franz cells (n=3) and the receiver chamber filled with the receiver fluid system developed in Section 2.2.5.1 (PBS and ethanol; 70:30). Skin samples were dosed with 10 µL of

Elocon cream and samples (200 μ l) of the receiver fluid were taken periodically up to 27 h and replaced with fresh preheated receiver fluid. The drug concentration at each time point was determined using the analytical methods summarised in Section 2.2.2.1 to establish whether sink conditions were maintained (a drug concentration of less than 10 % of the saturated solubility of mometasone furoate in the selected receiver fluid system).

2.2.5.3 Development of a drug extraction method for mometasone furoate from skin matrices

The suitability of acetonitrile as an extraction solvent for mometasone furoate from all matrices (skin surface and donor chamber, epidermal membrane, dermal membrane), following drug permeation experiments was investigated. Using a positive displacement pipette, 10 μ L of a 1 mg mL⁻¹ solution of mometasone furoate in acetonitrile, prepared as detailed in Section 2.2.2.2, was added to vials containing: cotton buds, tape strips, epidermal membranes, dermal membranes and an empty vial serving as the control. All vials were placed in a water bath set to 32 °C for 24 h. Following this period, 1 mL of acetonitrile was added to each vial, the vials were sonicated for 10 minutes and placed on a roller shaker for 18 h. Extraction solvents were removed entirely from the vials, filtered through 0.22 μ m PTFE filters and the drug was quantified by the gradient elution analytical method summarised in Section 2.2.2.1. Following data analysis, it was deemed necessary to conduct a second extraction to ensure the full recovery (100 ± 10 % of dose applied) of mometasone furoate. To enable ease of filtration of the skin surface residual formulation and tape strips, the extraction solvent volume was increased to 3 mL. Furthermore, to further facilitate drug extraction, a second 10 min sonication stage was incorporated following vial shaking for 18 h.

2.2.5.4 Determining drug - filter binding

Membrane binding studies were conducted to determine whether mometasone furoate had the potential to bind to PTFE filters during the drug extraction process. A saturated solution of mometasone furoate in acetonitrile was prepared as detailed in Section 2.2.5.1 and filtered through a 0.22 μ m PTFE filter. Aliquots of filtered and unfiltered solutions were diluted and quantified using the gradient elution analytical method summarised in Section 2.2.2.1.

2.2.5.5 Stability of mometasone furoate in the extraction solvent and receiver fluid systems

To ascertain the potential for mometasone furoate to degrade in the selected receiver fluid system during the Franz cell experimental period, drug stability in PBS and ethanol (70:30) was determined when stored at 37 °C for 24 h. To ascertain the potential for mometasone furoate to degrade in the selected receiver fluid system or extraction solvent during storage, the stability of mometasone furoate in two storage conditions was determined. The selected receiver fluid system and extraction solvent were PBS and ethanol (70:30) and acetonitrile, respectively, as determined following the findings of the receiver fluid system development and drug extraction method development (Section 2.2.5.1 and Section 2.2.5.3). A stock solution of 100 µg mL⁻¹ of mometasone furoate was prepared as detailed in Section 2.2.2.2 and aliquots stored at 2-8 °C and 25 °C for up to four weeks. At determined time points, samples were removed from storage and analysed using the gradient elution analytical method summarised in Section 2.2.2.1. To calculate drug stability at the relative conditions, the concentration of mometasone furoate at each time point was compared to freshly prepared samples at intervals up to 4 weeks.

2.2.6 *In vitro* silicone membrane Franz cell studies with Elocon cream

To investigate the effect of a mixed TCS and emollient system on drug transport across a synthetic membrane, an *in vitro* drug transport study was conducted using Elocon cream as a model TCS formulation. Franz cells were assembled with silicone membrane as detailed in Section 2.2.4.2. Informed by the findings of the receiver fluid system development studies (Section 2.2.5.1 and Section 2.2.5.5), the receiver chamber was filled with PBS and ethanol (70:30). Following the equilibration period, Franz cells were briefly removed from the water bath and the membrane was dosed with 500 mg of Elocon cream alone or 1 g of an Elocon cream and emollient mixture (1:1) prepared one hour in advance and applied to donor chambers by weight. The formulations were carefully spread over the membrane surface using a spatula to ensure contact with the membrane. The emollients used in this experiment were: Aquadrate cream, Cetraben cream, Diprobace cream, Diprobace ointment, Doublebase gel and Hydromol Intensive cream. Six replicate Franz cells were assembled for each emollient tested. Samples (200 µl) of the receiver fluid were taken periodically

up to 28 h and replaced with fresh preheated receiver fluid. Drug quantification in samples was achieved using the isocratic elution analytical method summarised in Section 2.2.2.1.

2.2.7 *In vitro* silicone membrane Franz cell studies with Elocon ointment

To investigate the effect of the TCS formulation on drug transport across silicone membrane from a mixed system, Elocon ointment was selected as a comparator product to Elocon cream. The experimental design was similar to that for Elocon cream with the following changes: silicone membrane mounted in Franz cells were dosed with either 500 mg of Elocon ointment alone or 1 g of an Elocon ointment and emollient mixture (1:1) prepared one hour in advance applied to donor chambers by weight. Samples (200 μ l) of the receiver fluid were taken periodically up to 26 h and replaced with fresh preheated receiver fluid. The emollients selected for investigation, synthetic membrane, receiver fluid system and analytical procedures remained unchanged and are detailed in full in Section 2.2.6.

2.2.8 *Ex vivo* human skin Franz cell study: Finite dosing of a pre-mixed TCS and emollient system

2.2.8.1 Studies investigating drug permeation across *ex vivo* human skin

Franz cells were assembled with human skin as detailed in Section 2.2.4.2. The receiver chamber was filled with PBS and ethanol (70:30), informed by the findings of the receiver fluid system development studies (Section 2.2.5.1 and Section 2.2.5.5). Following the equilibration period, Franz cells were briefly removed from the water bath and skin samples dosed with 10 μ L of Elocon cream or 20 μ L of an Elocon cream and emollient mixture (1:1), prepared one hour in advance and applied using a positive displacement pipette. To ensure contact with the membrane, the product was carefully spread over the surface of the skin using the tip of a capillary piston and the Franz cell returned to the water bath to commence the experiment. The selected emollients for investigation were: Aquadrate cream, Cetraben cream, Diprobace cream, Diprobace ointment, Doublebase gel and Hydromol Intensive cream. Samples (200 μ L) of the receiver fluid were taken at pre-determined

intervals up to 24 h and replaced with fresh preheated receiver fluid. Drug quantification was achieved using the gradient elution analytical method summarised in Section 2.2.2.1.

Scientist[®] 3.0 (Micromath Inc, Salt Lake City, UT, USA) was used to calculate the apparent partition (Kh) and diffusion (D/h^2) parameters when the Laplace transformation solution to Fick's second law, under finite dose conditions, was fit to the experimental permeation data sets (Equation 2-4), as previously described for the finite dose modelling of a permeant across human skin (Oliveira et al., 2012b).

$$\overline{Amount} = \frac{AP_1Q_0}{s \left[V \sqrt{\frac{s}{P_2}} \sinh \sqrt{\frac{s}{P_2}} + P_1A \cosh \sqrt{\frac{s}{P_2}} \right]}$$

Equation 2-4

The Laplace variable is denoted as 's' in Equation 2-4. Estimations of Kh (denoted as P_1) and D/h^2 (denoted as P_2) were determined based on the following experimental parameters: Diffusional surface area (A), amount of drug applied (Q_0), estimated membrane thickness (h) and the volume of formulation applied (V). The drug concentration in the formulation (set by Q_0 and V) was set to 0.1 % for the application of Elocon cream alone and 0.05 % for the applications of the Elocon cream and emollient mixture (1:1). Pseudo steady state drug flux (J_{ss}) and lag time (L_t) for drug permeation were estimated as previously described by Oliveira et al. (2012b) using Equation 2-5 and Equation 2-6, respectively.

$$J_{ss} = \frac{D}{h^2} \times Kh \times C_v$$

Equation 2-5

$$L_t = \frac{h^2}{6D} = \frac{1}{6P_2}$$

Equation 2-6

2.2.8.2 Studies investigating drug penetration to *ex vivo* human skin

Following the drug permeation across human skin studies, Franz cells were disassembled and drug content on the skin surface (residual formulation), the epidermis and dermis determined.

Residual formulations were removed from the donor chamber and the surface of the skin by three sequential wipes with cotton buds: an initial dry cotton bud was used to remove the residual formulation, then a second cotton bud soaked in the extraction diluent and a final dry cotton bud were used to swab the surface of the skin. The three cotton buds were placed in a vial for drug extraction. Two tape strips of the surface of the skin were taken and placed in a separate vial to complete recovery of the residual (unabsorbed) formulations.

The heat separation method detailed by Kligman and Christophers (1963) was employed to separate the epidermis from the dermis. Skin samples were mounted on glass slides and heated at 60 °C for 1 minute. The epidermis was removed from the dermis using tweezers and the separated layers placed in individual vials for drug extraction.

Quantification of mometasone furoate in the cotton buds, tape strips, epidermis and dermis was achieved using the extraction method developed in Section 2.2.5.3. All samples were analysed by the gradient elution analytical method summarised in Section 2.2.2.1.

2.2.9 Investigating the mechanistic effects on drug delivery to the skin from mixed Elocon cream and emollient formulations

2.2.9.1 Investigating the formulation design of Elocon cream

An *in vitro* Franz cell study across silicone membrane was conducted to determine the thermodynamic activity of mometasone furoate in Elocon cream (0.1 % w/w mometasone furoate). A 0.2 % w/w Elocon cream formulation was prepared as a comparator to investigate whether mometasone furoate was present as a suspension in the original formulation and thus at maximum thermodynamic activity. The 0.2 % w/w Elocon cream formulation was prepared by weighing 10 mg of mometasone furoate into a vial with 10 g of Elocon cream to achieve a final formulation strength of 0.2 % w/w mometasone furoate. The formulation was placed in a water bath set to 40 °C

and stirred overnight. One hour prior to dosing, the formulation was removed from the water bath and allowed to set at room temperature. Franz cells (n=6) were assembled with silicone membrane and equilibrated, as detailed in Section 2.2.4.2. Following equilibration, Franz cells were briefly removed from the water bath and the membrane was dosed with 1 g of Elocon cream (0.1 % w/w) or Elocon cream (0.2 % w/w) by weight. The receiver fluid system, sampling protocol, analytical procedures and data handling were as detailed in Section 2.2.6.

2.2.9.2 Polarised light microscopy of investigated formulations

Polarised light microscopy was employed to investigate the occurrence of crystalline structures in the formulations. Elocon cream alone, the emollient formulations alone and mixed systems of Elocon cream and emollients (1:1) were prepared on the same day as analysis. The emollients were: Aquadrate cream, Cetraben cream, Diprobase cream, Diprobase ointment, Doublebase gel and Hydromol Intensive cream. Samples were observed using a L3230 GX light microscope fitted with a polarising filter (GT Vision Ltd, Suffolk, UK). Images were captured using a x 20 objective lens, unless otherwise stated, using a GX CAM camera and GX Capture software (GT Vision Ltd, Suffolk, UK).

2.2.9.3 Hot stage microscopy of investigated formulations

Thermal analysis of crystalline structures observed in the premixed Elocon cream and emollient formulations was conducted using a L3230 GX light microscope equipped with a THMS 600 hot stage (Linkam Scientific, Surrey, UK). The emollients were: Aquadrate cream, Cetraben cream, Diprobase cream, Diprobase ointment, Doublebase gel and Hydromol Intensive cream. Micronised mometasone furoate, mometasone furoate crystals and samples of the premixed Elocon cream and emollient formulations (1:1) were mounted between two glass cover slides for analysis. Mometasone furoate crystals were prepared by process of solvent evaporation as follows: micronised mometasone furoate was stirred into a volatile solvent (methanol) in sufficient quantity to prepare a near saturated solution and the sample left uncovered at room temperature until crystal growth was observed. The temperature profile applied to the hot stage for thermal analysis of samples was as follows: an initial

temperature increase of 15 °C min⁻¹ from 0-180 °C, a ramp of 5 °C min⁻¹ from 180-260 °C then a cooling phase of 30 °C min⁻¹ to 0 °C.

2.2.9.4 Raman microscopy of investigated formulations

Raman microscopy of crystalline structures observed in the premixed formulations was performed using a Renishaw inVia Raman microscope (Renishaw, Gloucestershire, UK), calibrated for peak position and intensity using a silicon reference block. Samples of Elocon cream alone and Elocon cream in a pre-mixed system with emollients (1:1) were mounted on Raman grade calcium fluoride slides for spectral analysis. To establish whether solid excipients within the formulations would interfere with crystalline structure analysis, spectra were also collected for titanium dioxide, aluminium starch octenylsuccinate (DryFlo[®]) and white soft paraffin. Raman spectra were obtained using the x 100 long working distance magnification lens, a laser excitation wavelength of 785 nm, three accumulations per sample and an acquisition time of 10 s. Three replicate areas were scanned for each analysis and the single, most representative spectrum selected for presentation.

2.2.9.5 Moisture content analysis of the formulations

The moisture content of all formulations was determined using the Ohaus moisture analysis (Ohaus, USA). Samples (1 g) of Elocon cream, Aquadrate cream, Cetraben cream, Diprobace cream, Diprobace ointment, Doublebase gel and Hydromol Intensive cream were heated at 100 °C in intervals of 5 min until no further moisture loss was observed. Three replicate readings per formulation were performed and the average percent moisture content calculated.

2.2.10 Data treatment and statistical analysis

Following drug quantification in samples generated from *in vitro* drug transport and *ex vivo* drug permeation experiments, the concentration of mometasone furoate in the receiver fluid was corrected for previous sample removal and profiles constructed to present cumulative amount of drug permeated per unit area ($\mu\text{g cm}^{-2}$) over the exposure period. Linear regression was performed on infinite dose data sets to determine mean drug flux.

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Experimental data were expressed as mean ($n = 6$) \pm standard deviation (SD), unless otherwise stated. Statistical analysis was performed using Prism 8.0 (GraphPad, USA). The Shapiro Wilk test was employed to determine the normality of all data sets. Non-parametric analysis for multiple comparisons was performed using Kruskal-Wallis and a Mann–Whitney test applied for post hoc analysis. Parametric analysis for multiple comparisons was performed using analysis of variance (ANOVA) and Tukey’s post hoc test. Parametric analysis for two groups was performed using the unpaired t-test. Statistically significant differences were determined at a 95 % confidence interval ($p \leq 0.05$).

2.3 Results and Discussion

2.3.1 Analytical methods

An isocratic elution method was developed to enable the suitable quantification of mometasone furoate in samples following *in vitro* drug transport studies and *ex vivo* drug permeation and skin distribution studies. To enable the detection and quantification of lower drug concentrations in samples following *ex vivo* drug skin permeation and penetration experiments, a gradient elution method was also developed. Calibration standards were prepared over nominal concentration ranges and the sample chromatograms obtained following analysis with the isocratic and gradient elution HPLC UV methods are presented in Figure 2-2 a and Figure 2-2 b, respectively.

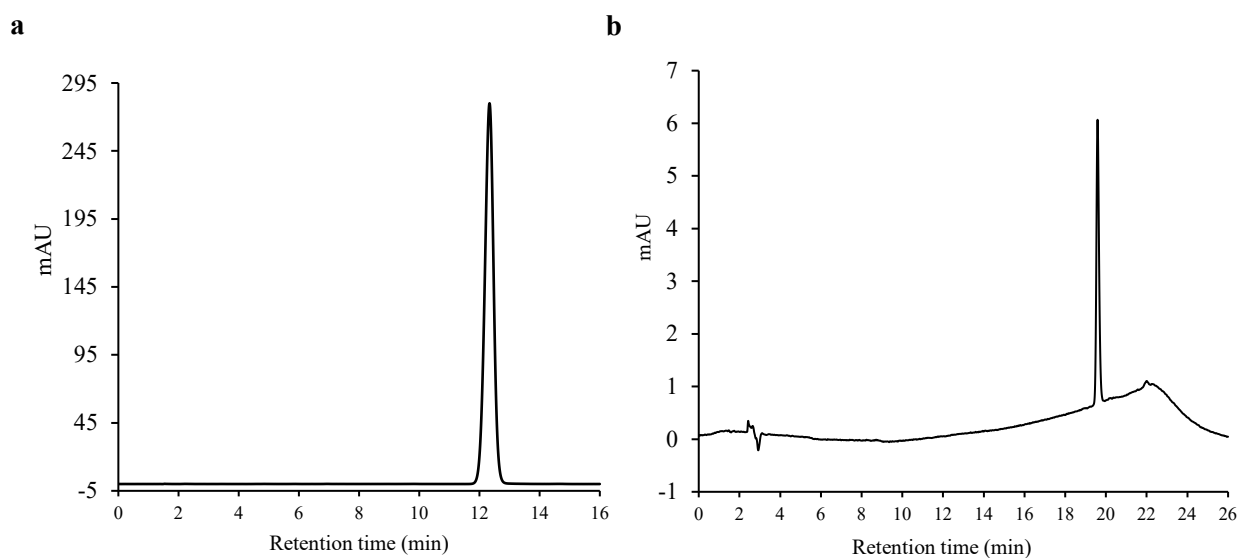


Figure 2-2: Sample chromatograms obtained at 253 nm following (a) the isocratic elution HPLC UV analysis of 100 µg mL⁻¹ of mometasone furoate solution and (b) the gradient elution HPLC UV analysis of 1 µg mL⁻¹ of mometasone furoate solution. The drug elution times are 12.41 min and 19.60 min, respectively.

During the process of drug extraction from the matrices, there is also the potential for UV absorbing skin endogenous compounds and excipients from the formulations to be extracted alongside the drug. As such the isocratic and gradient elution analytical methods were suitably developed to avoid co-elution of these components with the compound of interest. As an example for the method development for mometasone furoate quantification using the gradient elution HPLC UV method, the chromatogram generated from a sample of excipient extracts from the six employed emollients is presented in Figure 2-3. The elution times of components from the six emollients, skin endogenous compounds, cotton buds and scotch tape were determined to ensure no interference with the peaks of interest, when the analytical methods were employed. The resulting HPLC gradient methods enabled appropriate drug elution times with sufficient resolution from peaks of no interest to be achieved.

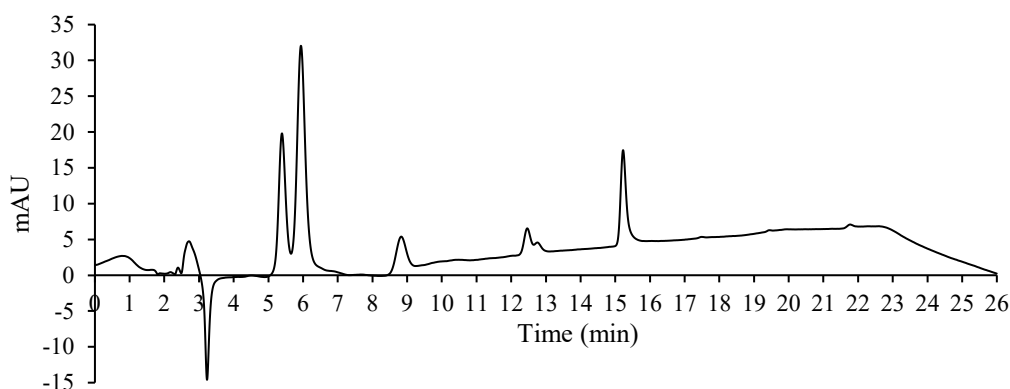


Figure 2-3: A chromatogram of excipient extracts from 6 emollients obtained at 253 nm from analysis using the gradient elution HPLC method for mometasone furoate quantification. No peaks are present in the 19 – 20 min window; there is no potential for the co – elution of excipient peaks with mometasone furoate.

Conducting drug permeation and penetration studies requires a suitably sensitive method for quantification of the compound of interest in samples (Snyder, Kirkland, & Glajch, 2012). To achieve this, calibration curves of mometasone furoate were constructed using the analytical methods developed to enable the quantification of mometasone furoate in samples following *in vitro* and *ex vivo* Franz cell studies and are presented in Figure 2-4.

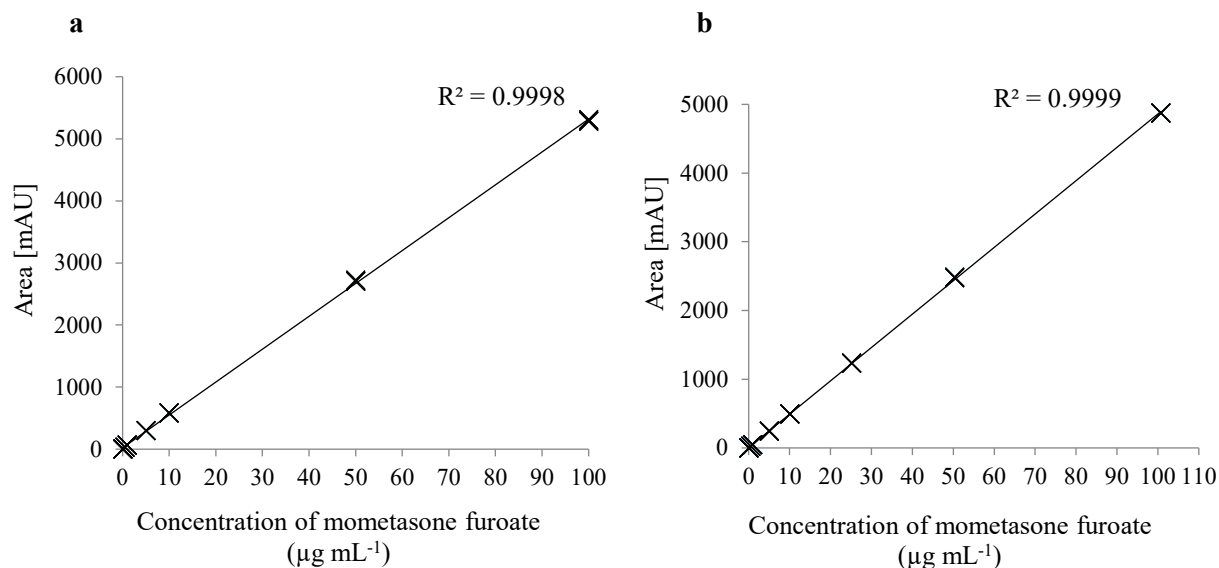


Figure 2-4: Calibration graphs for mometasone furoate standards obtained following analysis with (a) the isocratic elution HPLC method (concentration range $0.05 \mu\text{g mL}^{-1} - 100 \mu\text{g mL}^{-1}$) and (b) the gradient elution HPLC method ($0.025 \mu\text{g mL}^{-1} - 100 \mu\text{g mL}^{-1}$). Data show the concentration of six replicate injections for each concentration and the correlation coefficient (R^2).

The analytical methods were evaluated for linearity, precision and accuracy in accordance with the International Conference on Harmonization (ICH) guidelines (ICH, 2005). The linearity, accuracy and system suitability parameters for mometasone furoate are summarised in Table 2-2. The analytical methods implemented, as detailed in Section 2.2.2, were deemed to pass all specification criteria as defined by the ICH guidelines with suitable LOD and LOQ levels for the quantification of small amounts of mometasone furoate in samples following the *in vitro* drug transport and *ex vivo* drug permeation and penetration experiments.

Table 2-2: Summary of the parameters determined for the ‘fitness for purpose’ of the isocratic and gradient elution HPLC UV methods for quantification of mometasone furoate in samples.

Parameter	Mometasone furoate Isocratic method	Mometasone furoate Gradient method	Limit as per ICH guidance
Linearity (R ²)	0.9998	0.9999	> 0.999
Intra-day precision (% RSD) 5 µg mL ⁻¹ , 50 µg mL ⁻¹ and 100 µg mL ⁻¹	0.6, 0.3, 0.8	0.3, 0.5, 0.6	RSD ≤ 2 %
Inter-day precision (% RSD) 5 µg mL ⁻¹ , 50 µg mL ⁻¹ and 100 µg mL ⁻¹	0.7, 0.1, 0.6	0.5, 0.7, 0.4	RSD ≤ 2 %
Accuracy (%) 5 µg mL ⁻¹ , 50 µg mL ⁻¹ and 100 µg mL ⁻¹	99.4, 99.7, 99.8	99.6, 99.8, 99.7	100 ± 2 %
Limit of detection (µg mL⁻¹)	0.12	0.09	Report result
Limit of quantification (µg mL⁻¹)	0.36	0.26	Report result
Tailing factor	1.22	0.8	< 2

2.3.2 Franz cell method development

To determine whether PBS would confer a good receiver fluid system for *in vitro* drug transport and *ex vivo* drug permeation studies, thus maintaining sink conditions for the duration of the experiment, solubility studies with PBS and varying concentrations of absolute ethanol up to 30 % were conducted. The saturated solubility of mometasone furoate in the liquid excipients of Elocon cream and the six emollients were also determined. The findings are presented in Table 2-3.

Mometasone furoate demonstrated poor solubility in PBS alone and greatest solubility in a solution of PBS and ethanol (70:30). A 2 fold increase in the solubility of mometasone furoate in PBS solution was achieved in the presence of 20 % ethanol; further increasing the solvent composition to 30 % resulted in an additional 15 fold increase in the solubility of the drug. The inclusion of ethanol as a solubiliser in receiver fluid systems is well accepted, with guidance listing ethanol as an acceptable receiver fluid for *in vitro* skin absorption studies when used in concentrations up to 50 % (EMA, 2018; OECD, 2019). In addition to this guidance, Yang et al. (2015) reported a good correlation

between *ex vivo* skin penetration of estradiol and *in vivo* human studies when ethanol was included in the receiver fluid. The receiver fluid comprised of PBS and ethanol (70:30) demonstrated sufficient solubility (at least 7 fold greater than the maximum amount of drug anticipated to permeate skin) thus was selected to conduct the pilot Franz cell studies across silicone membrane or human skin.

Table 2-3: The saturated solubility of mometasone furoate in a range of solvent systems. Data are presented as the mean of three replicates and the range is denoted in brackets.

Solvent system	Solubility of mometasone furoate ($\mu\text{g mL}^{-1}$)
PBS	0.33 (0.08)
PBS + 10 % ethanol	0.40 (0.06)
PBS + 20 % ethanol	0.64 (0.07)
PBS + 30 % ethanol	9.25 (0.16)
Water	0* (0.00)
Glycerol	0.52 (0.14)
Liquid Paraffin	9.22 (2.51)
Isopropyl myristate	268.86 (17.25)
Castor oil	1206.64 (38.57)
Hexylene glycol	3285.73 (196.25)

*no drug detected on analysis with the gradient elution HPLC method

Water is a listed excipient for Elocon cream, Diprobace cream, Cetraben cream and Doublebase gel and was found to be a poor solubiliser for mometasone furoate (no drug detected on analysis). The saturated solubility of mometasone furoate in glycerol, an excipient also present in Cetraben cream and Doublebase gel, was also low at $0.52 \mu\text{g mL}^{-1}$. Hexylene glycol however, formulated in Elocon cream alone, demonstrated the greatest capacity for solubilising mometasone furoate. Comparatively, a 2.7 fold and 12.3 fold decrease in solubilising capacity was observed with castor oil and isopropyl myristate, respectively, when compared to hexylene glycol.

To closely replicate the clinical application of TCSs during the conduct of the *ex vivo* permeation and penetration Franz cell study, a finite dosing regime was adopted in line with current OECD

guidance (OECD, 2019). The current recommendations for the application of Elocon cream is that the products are applied as a thin film to the ‘affected areas of skin once daily’ (MSD, 2018). Given the above points, an exposure period of 24 h, reflecting the ‘in use’ conditions, was adopted.

Prior to the full scale Franz cell experiments with receiver fluid sampling at predetermined time points, a pilot experiment for the application of Elocon cream alone was conducted in order to establish whether sink conditions were likely to be maintained and to inform the sampling protocol for the full scale experiments. Following the application of an infinite dose of Elocon cream (0.1% w/w mometasone furoate) to silicone membrane and a finite dose of Elocon cream to human scrotal skin, the receiver fluid was sampled at time points up to 28 h and 27 h respectively. Cumulative drug permeation was calculated and the resultant permeation profiles are presented in Figure 2-5.

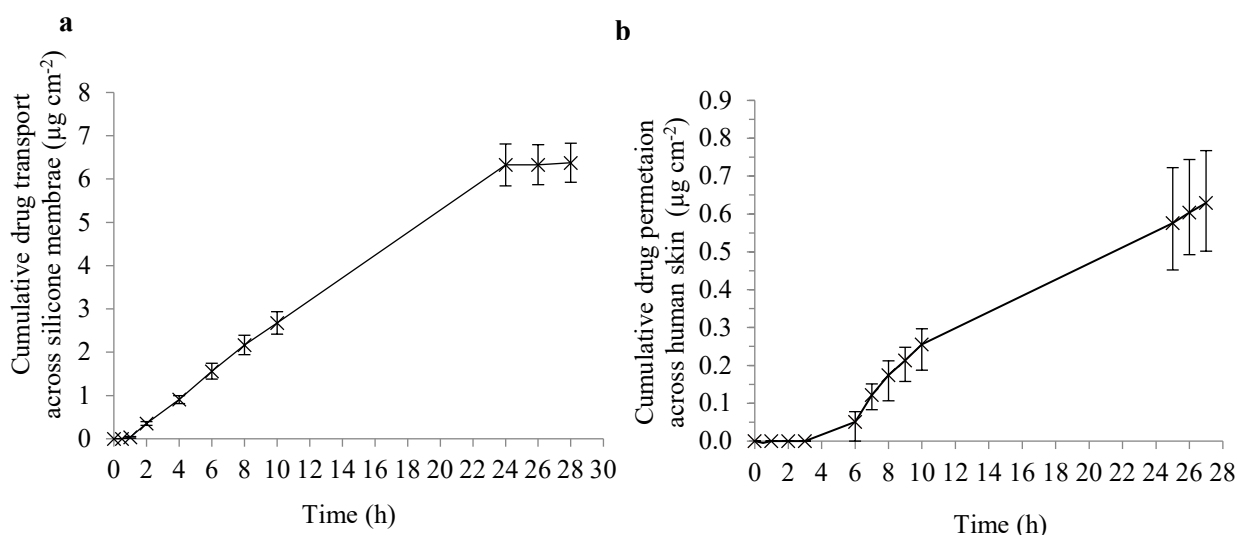


Figure 2-5 : Profiles show the cumulative amount of mometasone furoate (a) transport across silicone membrane over 28 h following an infinite dose of Elocon cream alone and (b) permeated across human skin over 27 h following a finite dose (10 µL) of Elocon cream alone. The receiver fluid system employed for both experiments was PBS and ethanol (70:30). Data are shown as the mean of three replicates; error bars denote the range of data points.

In the pilot Franz cell experiment employing silicone as the model membrane, mometasone furoate was detected in the receiver fluid from 30 min onwards (Figure 2-5a). The sampling time points were deemed appropriate to allow for drug flux to be calculated following the applications of both Elocon cream and Elocon ointment, given the number and spread of data points. To maintain sink conditions,

it is generally expected that the concentration of drug in the receiver fluid should not exceed 10 % of the drug's saturated solubility in the receiver fluid (Higuchi, 1960). On analysis, sink conditions were maintained for the duration of the experiment, with the drug concentration in the receiver fluid not exceeding more than 9 % of the saturated solubility in the receiver fluid at any timepoint, indicative of PBS and ethanol (70:30) being an appropriate receiver fluid for future silicone membrane experiments.

In the pilot Franz cell experiment employing human skin, mometasone furoate was not detected in the receiver fluid at time points up to 6 h. At 27 h, the average cumulative amount of drug in the receiver fluid was 6 % of the applied dose. The concentration of mometasone furoate in the receiver fluid did not exceed 2 % of the saturated solubility in the receiver fluid throughout the experiment, hence were indicative of sink conditions being maintained. Given the pilot experiment findings and the data from the solubility experiment (Figure 2-5b and Table 2-3, respectively), a solution containing PBS and ethanol (30 %) was selected as the receiver fluid to allow for a potential increase in drug permeation across skin when Elocon cream is mixed with particular emollients, compared to the skin permeation of mometasone furoate from Elocon cream alone.

An exposure period of 24 h was selected for Franz cell experiments. Given this time period, it was thought to be useful for time points close, and up to, 24 h be taken to determine if 'pseudo steady state' or 'donor depletion' conditions were met. From the profile presented in Figure 2-5b, an estimated lag time of 4.8 h for mometasone furoate was calculated from the linear portion of the graph between 6 h and 10 h. From these preliminary findings, the following sampling timepoints were selected: 1 – 4 h and 15 – 24 h.

During the *ex vivo* drug penetration study, the distribution of the drug within layers of the skin was investigated. To achieve this, a method to ascertain the suitability of acetonitrile as an extraction solvent was developed. On analysis, it was found that two sequential extractions were sufficient to recover 99 – 100 % of mometasone furoate from tape strips and the epidermis (Figure 2-6). An additional (third) extraction procedure was required to recover 98 – 99 % of mometasone furoate from the skin surface (cotton buds) and dermis. The OECD guidance for skin absorption studies

stipulate a mean recovery of $100 \pm 10\%$ of the applied dose (OECD, 2019); the validation procedure yielded percent drug recoveries within this range for all matrices. It was therefore deemed appropriate to adopt the triple extraction procedure for mometasone furoate and acetonitrile as the extraction solvent for future *ex vivo* drug penetration Franz cell experiments.

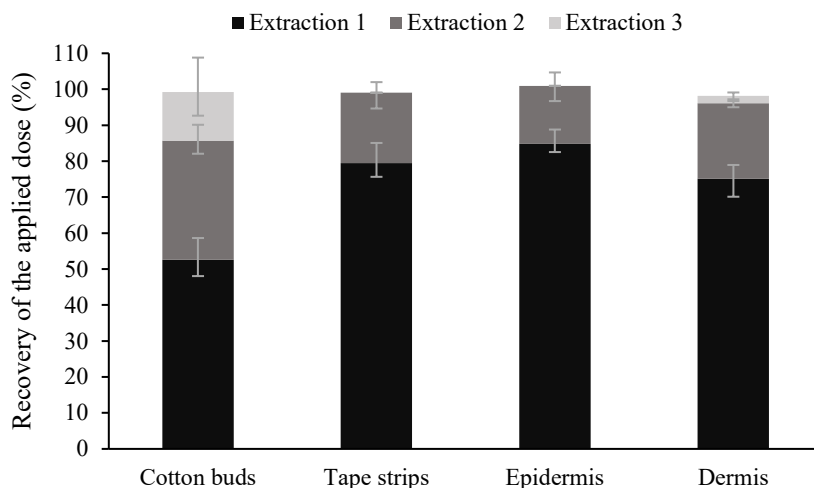


Figure 2-6: Graph shows the percent recovery of mometasone furoate from matrices following a $10\ \mu\text{L}$ dose of a standard solution of $1\ \text{mg mL}^{-1}$ of the drug. Data are presented as the mean of three replicates for each extraction. Error bars denote the range of values.

To ascertain the potential for mometasone furoate to degrade in the selected receiver fluid system during the Franz cell experimental period, drug stability in PBS and ethanol (70:30) was determined when stored at $37\ ^\circ\text{C}$ for 24 h. To ascertain the potential for mometasone furoate to degrade in the selected receiver fluid system (PBS and ethanol; 70:30) or extraction solvent (acetonitrile) during storage, the stability of mometasone furoate at $25\ ^\circ\text{C}$ and $2-8\ ^\circ\text{C}$ was determined. Mometasone furoate was stable in the receiver fluid system at the experimental temperature ($37\ ^\circ\text{C}$) over the experimental period (24 h). Mometasone furoate was stable in both solutions, at $25\ ^\circ\text{C}$ and $2-8\ ^\circ\text{C}$, for up to one month with a percent drug concentrations within $\pm 2\%$ of freshly prepared standards at the same concentration (Table 2-4).

Table 2-4: Stability of mometasone furoate in the receiver fluid and extraction solvent stored at 37 °C, 25 °C and 2 – 8 °C. Data are presented as the mean percent of the drug concentration compared to freshly prepared standards for three replicates. The range is presented in brackets.

	Time (days)	PBS and ethanol (70:30)	Acetonitrile
	0	100	100
Stability at 37 °C (% of T = 0)	1	99.27 (0.21)	-
	7	99.65 (0.18)	100.90 (0.19)
Stability at 25 °C (% of T = 0)	14	101.28 (2.98)	99.75 (0.09)
	21	98.92 (0.53)	99.23 (0.08)
	28	99.65 (0.88)	99.57 (0.41)
	7	99.98 (1.09)	98.97 (0.19)
Stability at 2 – 8 °C (% of T = 0)	14	99.85 (0.20)	99.95 (0.28)
	21	100.87 (0.04)	101.02 (0.23)
	28	101.28 (0.24)	99.56 (0.44)

The sampling protocols, receiver fluid systems and drug extraction methods developed and employed for the pilot *in vitro* and *ex vivo* Franz cell experiments were selected for full scale Franz cell experiments with silicone membrane and human scrotal skin performed at 37 °C.

2.3.3 *In vitro* silicone membrane Franz cell studies: Elocon cream and Elocon ointment premixed with emollients

To discern the differences in drug transport from a topical corticosteroid when mixed with a range of emollient formulations, an *in vitro* drug transport study across silicone membrane was conducted employing Elocon cream (0.1 % w/w mometasone furoate) as a model TCS and six emollients. The emollients selected were Aquadrate cream, Cetraben cream, Diprobace cream, Diprobace ointment, Doublebase gel and Hydromol Intensive cream. An infinite dosing model was employed and membranes were dosed with Elocon cream alone or Elocon cream and an emollient mixture (1:1). The resultant drug transport profiles are presented in Figure 2-7.

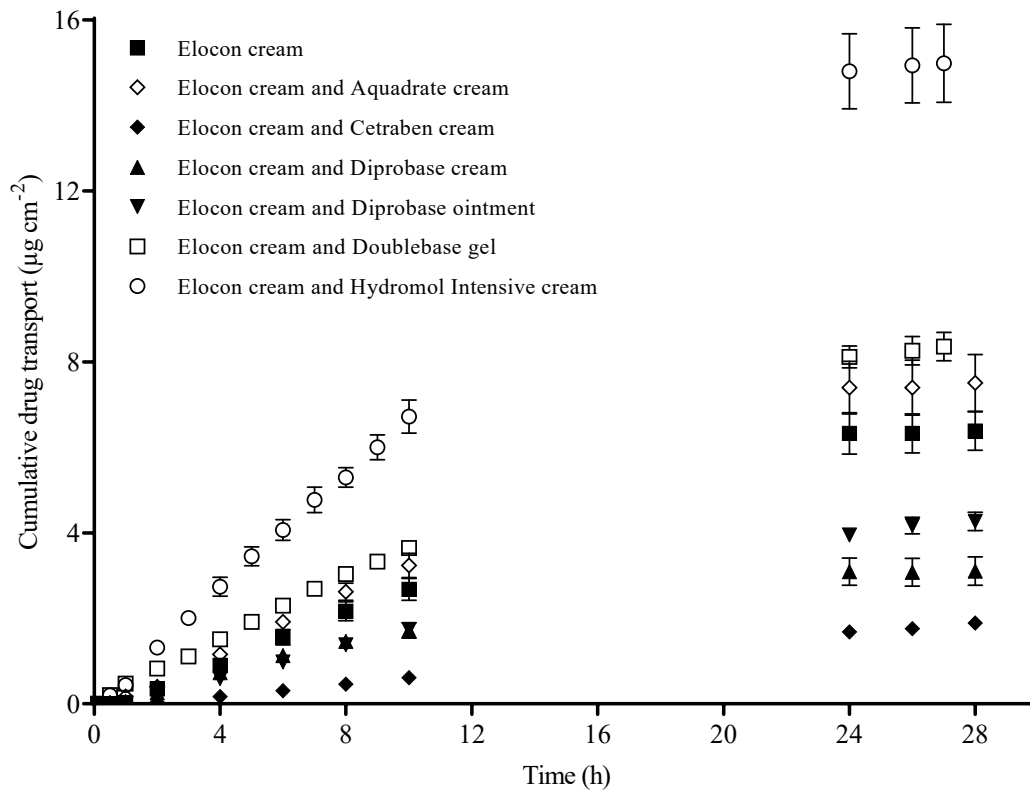


Figure 2-7: The cumulative amounts of mometasone furoate ($\mu\text{g cm}^{-2}$) transport across silicone membrane over 28 h from Elocon cream (■) when an infinite dose of the TCS was applied alone or in a pre-mixed system (1:1) with one of six emollients. The emollients were Aquadrate cream (◇), Cetraben cream (◆), Diprobace cream (▲), Diprobace ointment (▼), Doublebase gel (□) and Hydromol Intensive cream (○). Data shown as mean \pm SD (n=6).

By employing an infinite dosing system where the dose of the permeant (mometasone furoate) was in sufficient quantities such that donor depletion through drug transport was negligible, it would also be expected that the thermodynamic activity of the drug in the donor phase should remain constant over the experimental period. Under these conditions, the characteristic permeation profile would display an increase in cumulative drug transport with time, approaching a 'steady-state' rate of drug transport, indicative of the 'infinite' availability of the permeant in the donor phase.

On analysis, drug transport across silicone membrane following the application of pre mixed Elocon cream and emollient formulations profiled in similar patterns, with a drug transport increasing at a constant rate with time up to 10 h then reaching a plateau (evident from 24 h onwards in Figure 2-7). Infinite dose conditions were maintained in the donor chamber and sink conditions were maintained in the receiver fluid for all product combinations. Thus, the decrease in drug transport observed at the later time points is not likely to be indicative of drug depletion in the applied formulation or drug saturation in the receiver fluid. Possible explanations for this observation are drug depletion from formulation and membrane interface or solvent depletion from the formulation and membrane interface, resulting in an altered drug thermodynamic activity in the residual formulation.

Drug flux was calculated over the linear section of the cumulative drug permeated against time profiles (2 – 10 h; Figure 2-7). Linearity was determined as a correlation co-efficient (R^2) of 0.98 or greater over 5 or more time points and the findings are presented in Table 2-5. The application of a premixed system of Elocon cream and an emollient significantly altered drug flux in three out of six cases (with Hydromol Intensive cream, Diprobace cream and Cetraben cream) when compared to drug flux from Elocon cream alone ($p < 0.05$; Table 2-5). On analysis, the alteration did not reflect a simple dilution effect; rather, an emollients specific effect was observed with a 9.4 fold difference in drug flux depending on the particular emollient mixed with Elocon cream. Drug flux ranged from a 2.2 fold increase in the presence of Hydromol Intensive cream to a 4.3 fold decrease in the presence of Cetraben cream, when compared to drug flux from Elocon cream alone ($p < 0.05$). The magnitude of the difference and the emollient specific trend observed with drug flux was mirrored on analysis of the total amount of drug transport at 24 h (Q_{24}) when compared to the application of Elocon cream

alone. The total amount of drug transport at 24 h ranged from a 2.3 fold increase in the presence of Hydromol Intensive cream to a 3.8 fold decrease in the presence of Cetraben cream, when compared to Elocon cream alone ($p < 0.05$; Table 2-5).

Table 2-5: The mean drug flux calculated between 2h and 10 h ($\bar{J}_{2-10\text{ h}}$) and total drug transport across silicone membrane from Elocon cream alone or pre-mixed formulations (Q_{24}). The emollients were Aquadrate cream, Cetraben cream, Diprobace cream, Diprobace ointment, Doublebase gel and Hydromol Intensive cream. Data are shown as mean \pm SD (n=6). * denotes a significant difference when $\bar{J}_{2-10\text{ h}}$ or Q_{24} from Elocon cream alone was compared, respectively, to $\bar{J}_{2-10\text{ h}}$ or Q_{24} from pre-mixed formulations (one way ANOVA and Tukey's post hoc test; $p < 0.05$).

	$\bar{J}_{2-10\text{ h}}$ ($\mu\text{g cm}^{-2}\text{ h}^{-1}$)	Rank order (highest > lowest)	Q_{24} ($\mu\text{g cm}^{-2}$)	Rank order (highest > lowest)
Elocon cream	0.30 \pm 0.06	4	6.33 \pm 0.48	4
Elocon cream and Aquadrate cream	0.36 \pm 0.07	2	7.40 \pm 0.62	3
Elocon cream and Cetraben cream	0.07 \pm 0.02*	7	1.68 \pm 0.10 *	7
Elocon cream and Diprobace cream	0.18 \pm 0.04*	6	3.09 \pm 0.32 *	6
Elocon cream and Diprobace ointment	0.20 \pm 0.03	5	3.95 \pm 0.15 *	5
Elocon cream and Doublebase gel	0.36 \pm 0.02	2	8.12 \pm 0.26 *	2
Elocon cream and Hydromol Intensive cream	0.67 \pm 0.07*	1	14.80 \pm 0.88 *	1

To ascertain whether the emollient specific trends observed with Elocon cream were evident with other TCS formulation types, the effect of the application of the selected emollients with the equivalent strength TCS ointment formulation, Elocon ointment, was determined. The cumulative amount of mometasone furoate transport across silicone membrane following the applications of Elocon ointment alone and Elocon ointment pre-mixed with one of six emollients is presented in Figure 2-8.

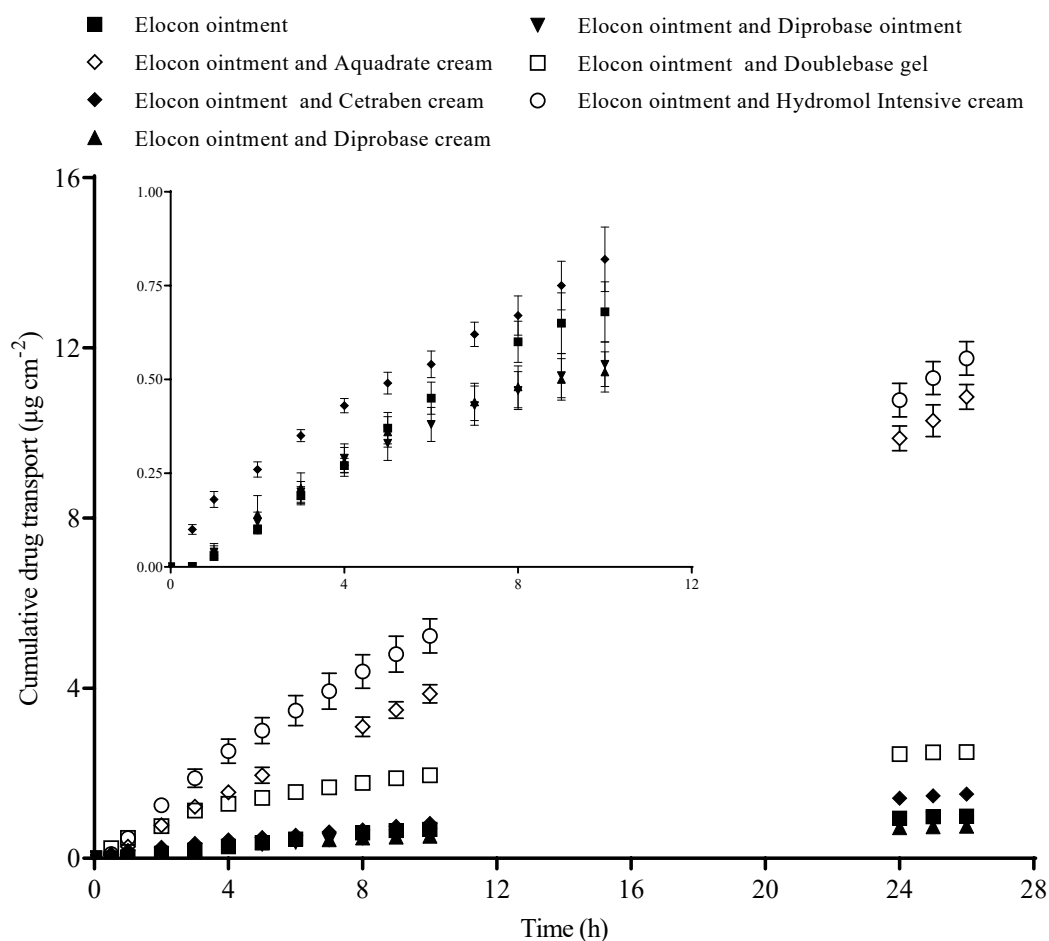


Figure 2-8: The cumulative amounts of mometasone furoate ($\mu\text{g cm}^{-2}$) transport across silicone membrane over 26 h from Elocon ointment (■) when an infinite dose of the TCS was applied alone or in a pre-mixed system (1:1) with one of six emollients. The emollients were Aquadrate cream (◇), Cetraben cream (◆), Diprobese cream (▲), Diprobese ointment (▼), Doublebase gel (□) and Hydromol Intensive cream (○). Data are shown as mean \pm SD (n=6).

Drug transport across silicone membrane was evident at early time points following the application of all formulations. Drug transport increased at a constant rate with time up to 10 h, after which the rate of transport slowed; this pattern was true for Elocon ointment applied alone and Elocon ointment applied with Aquadrate cream, Diprobese cream, Diprobese ointment and Cetraben cream. In the case of Hydromol Intensive cream and Aquadrate cream, the rate of drug transport continued to increase beyond 10 h, reflective of the classic infinite dose profile. Drug transport was approximately linear for all formulations at early time points (between 3 h and 10 h), thus this range was selected to calculate the change in drug flux across all formulations, presented in Table 2-6.

Table 2-6: The mean drug flux calculated between 3 h and 10 h ($\bar{J}_{3-10\text{ h}}$) and total drug transport across silicone membrane from Elocon ointment alone or pre-mixed formulations (Q_{24}). The emollients were Aquadrate cream, Cetraben cream, Diprobase cream, Diprobase ointment, Doublebase gel and Hydromol Intensive cream. Data are shown as mean \pm SD (n=6). * denotes a significant difference when $\bar{J}_{3-10\text{ h}}$ or Q_{24} from Elocon ointment alone was compared, respectively, to $\bar{J}_{3-10\text{ h}}$ or Q_{24} from pre-mixed formulations (one way ANOVA and Tukey's post hoc test; $p < 0.05$).

	$\bar{J}_{3-10\text{ h}}$ ($\mu\text{g cm}^{-2}\text{ h}^{-1}$)	Rank order (highest > lowest)	Q_{24} ($\mu\text{g cm}^{-2}$)	Rank order (highest > lowest)
Elocon ointment	0.07 ± 0.02	4	0.94 ± 0.23	5
Elocon ointment and Aquadrate cream	$0.34 \pm 0.12^*$	2	$8.72 \pm 2.87^*$	2
Elocon ointment and Cetraben cream	0.07 ± 0.03	4	$1.41 \pm 0.23^*$	4
Elocon ointment and Diprobase cream	0.05 ± 0.01	6	0.72 ± 0.17	7
Elocon ointment and Diprobase ointment	0.05 ± 0.01	6	0.90 ± 0.19	6
Elocon ointment and Doublebase gel	0.14 ± 0.03	3	$2.45 \pm 0.36^*$	3
Elocon ointment and Hydromol Intensive cream	$0.49 \pm 0.09^*$	1	$10.77 \pm 0.89^*$	1

The application of a premixed system of Elocon ointment and an emollient resulted in a significant increase in drug flux in two out of six cases. The change in drug flux ranged from a 6.6 fold increase following the application of Elocon ointment in the presence of Hydromol Intensive cream ($p < 0.05$) to a 4.9 fold increase in the presence of Aquadrate cream ($p < 0.05$) when compared to the application of Elocon ointment alone. The total amount of drug permeated at 24 h (Q_{24}) trended in a similar manner, with the total drug permeation from Elocon ointment ranging from an 11.5 fold increase in the presence of Hydromol Intensive cream to a 1.5 fold increase in the presence of Cetraben cream, compared to Elocon ointment alone (Table 2-6).

The pattern of an emollient specific trend in drug flux seen with Elocon cream was for the most part mirrored following application of the six emollients with the equivalent strength TCS ointment formulation, Elocon ointment, though to a different order of magnitude (Figure 2-9).

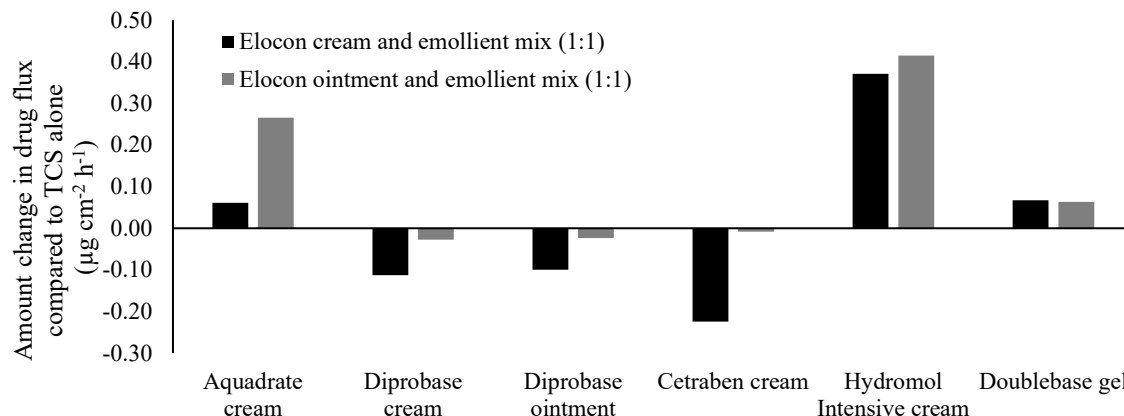


Figure 2-9: Amount change ($\mu\text{g cm}^{-2} \text{h}^{-1}$) in drug flux across silicone membrane from premixed TCS and emollient formulations relative to drug flux from Elocon cream alone ($0.30 \pm 0.06 \mu\text{g cm}^{-2} \text{h}^{-1}$) or Elocon ointment alone ($0.07 \pm 0.02 \mu\text{g cm}^{-2} \text{h}^{-1}$).

For both TCS formulations, an increase in drug flux was observed when Doublebase gel, Hydromol Intensive cream and Aquadrate cream were applied with the TCS. Dilution of a TCS should ideally occur with a diluent similar to the TCS base, to avoid complex formulation changes occurring in the TCS and altering the drug delivery profile. The investigated emollient products, however, are largely formulated with excipients dissimilar to the base of Elocon cream or Elocon ointment. Mixing these emollient formulations with the TCSs is likely to have altered drug solubility in the premixed formulation to differing extents, relative to the marketed product. In this case, drug thermodynamic activity in these premixed formulations may have altered relative to the marketed products, resulting in an increase in drug flux (Aquadrate cream, Doublebase gel and Hydromol Intensive cream) or a decrease in drug flux (Cetraben cream, Diprobace cream and Diprobace ointment) across silicone membrane.

It is, however, prudent to interpret drug transport data from synthetic membrane studies with an appreciation for potential solvent interactions with the membrane. Aquadrate cream, Doublebase gel and Hydromol Intensive cream are listed to contain proportions of IPM (Table 2-1). IPM is a lipophilic compound that has demonstrated high sorption into silicone membrane. The work of

Oliveira, Hadgraft and Lane (2012) proposed a model of solvent enhanced permeation whereby solvents such as IPM interact with silicone membrane promoting the partitioning of drugs into the membrane and causing these compounds to accumulate in 'pools' within the membrane. Furthermore, the effects of IPM on drug permeation across silicone membrane has been previously reported; Cross *et al.* (2001) found on application of hydrocortisone from a saturated solution of IPM that drug flux was increased by approximately 3.1 fold when compared to drug flux from a saturated solution of liquid paraffin (LP). Notably, creating a binary mixture of LP and IPM (1:1) resulted in an increase in drug flux to a lesser extent of approximately 2 fold when compared to drug flux from saturation solution of LP alone. As the topical emollients investigated in this study contain a substantially lower proportion of IPM than used in the reported studies (IPM composition in Doublebase gel for example is 15 % w/w; Table 2-1) the enhanced flux observed may not solely be attributable to IPM- membrane interactions for Hydromol Intensive cream, Aquadrate cream and Doublebase gel with altered drug solubility in the formulation and partitioning out of the formulation also contributing to the trends in altered drug transport.

Whilst there was a general trend in reduced drug flux from Elocon cream and Elocon ointment when mixed with Diprobace cream, Diprobace ointment and Cetraben cream, the magnitude of change was considerably smaller for Elocon ointment compared to Elocon cream. A potential explanation for this is that drug flux from the TCS ointment formulation alone was already significantly lower than that observed from the TCS cream formulation alone ($0.07 \pm 0.02 \mu\text{g cm}^{-2} \text{h}^{-1}$ and $0.30 \pm 0.06 \mu\text{g cm}^{-2} \text{h}^{-1}$ respectively; $p < 0.05$), masking the magnitude of the emollient specific decrease in drug flux. This may be attributable to lower initial drug thermodynamic activity in Elocon ointment compared to Elocon cream or the difference in viscosity of the formulations. Ointment type formulations inherently have a higher viscosity than cream based formulations and the impact of formulation type on drug diffusion through the vehicle has been previously investigated, with Fang *et al.* (1999) reporting a trend of decreased clobetasol propionate release from various bases as viscosity increased. Similarly, Christensen *et al.* (2011) found drug release across a nylon membrane from a marketed 1 % w/w hydrocortisone ointment formulation to be lower than the equivalent strength

cream formulation, hypothesising that the high viscosity of the ointment base was hindering drug release. An inverse relationship exists between the viscosity of a formulation and the diffusion coefficient, as described by the Stokes – Einstein equation (Miller, 1924) and this factor, coupled to the decrease in drug concentration in the premixed formulations, may have contributed to the reduction in drug transport across silicone membrane when compared to Elocon ointment alone. Complementary drug release studies may be conducted to confirm this theory.

The studies conducted thus far were with an aim to screen TCS and emollient formulation effects on drug transport. Whilst a porous freely permeable membrane such as regenerated cellulose membrane would enable the evaluation of drug diffusion in the absence of partitioning, back transport of the organic receiver fluid into the donor chamber may compromise the integrity of the semi solid preparations and alter drug release characteristics from the formulation (Nakano & Patel, 1970; Stolar et al., 1960; Walters & Dekker, 2002), thus silicone membrane was employed to elucidate relative changes in drug flux. In light of the findings where the potential for an altered drug flux was greatest with Elocon cream, coupled to the greater frequency of prescribing for Elocon cream in clinical practice (NHS Digital, 2019), it was deemed suitable to carry forward this TCS to investigate the impact of mixed TCS and emollient formulations on drug delivery to *ex vivo* human skin. Furthermore, it was anticipated that this would enable a more accurate appreciation for the broader application of these findings to the clinical use of multiple topical products at similar times.

2.3.4 *Ex vivo* human skin Franz cell study: Finite dosing of a pre-mixed TCS and emollient system to the skin

To establish whether the potential changes observed across silicone membrane are of clinical relevance, an *ex vivo* drug permeation and skin distribution study was conducted employing human skin. A finite dose of Elocon cream alone or Elocon cream in a premixed system with the one of the six emollient was applied to human scrotal skin and drug permeation and penetration was evaluated. It was deemed beneficial to investigate skin distribution of the drug as topical corticosteroids exert their clinical effect by binding to macromolecules present in the cytoplasm of keratinocytes and fibroblasts, thus the cellular targets are present within the viable epidermis and dermis (Ponec et al., 1981).

The absolute recovery of mometasone furoate from Elocon cream, following the application of Elocon cream alone or a pre-mixed formulation of Elocon cream and an emollient, ranged from 97–105 % of the applied dose for all experiments conducted, falling within the OECD defined acceptable criteria of a ± 10 % deviation from the dose applied (OECD, 2019). The distribution of mometasone furoate in all matrices and the receiver fluid when Elocon cream was applied alone or with one of six emollients is presented in Figure 2-10.

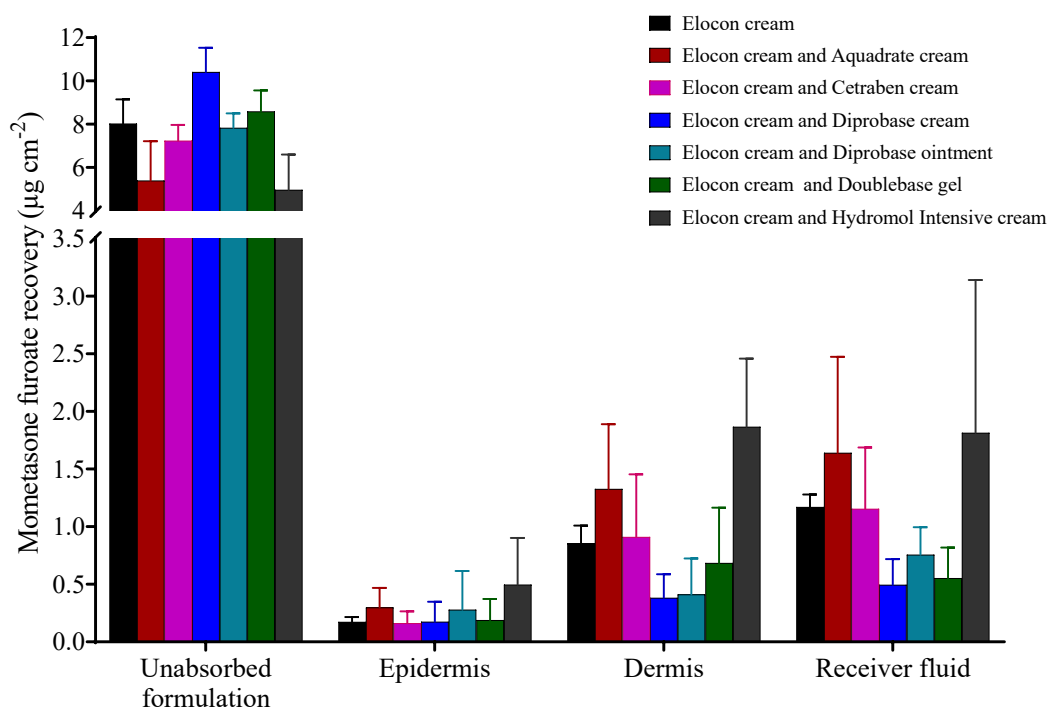


Figure 2-10: Figures show mometasone furoate recovery ($\mu\text{g cm}^{-2}$) after 24 h of application from the unabsorbed formulation, human skin (epidermal and dermal layers) and receiver fluid following the application of a finite dose of Elocon cream alone or a 1:1 pre-mix of Elocon cream and one of six emollients to the skin. The emollients were Aquadrate cream, Cetraben cream, Diprobase cream, Diprobase ointment, Doublebase gel and Hydromol Intensive cream. Data shown as mean + SD (n=6).

The distribution of mometasone furoate in the epidermis, dermis and receiver fluid followed a similar trend for all emollients when applied in combination with Elocon cream and was largely evident in the following order of magnitude: unabsorbed drug > receiver fluid > dermis > epidermis. For clarity, the total drug absorption (total drug content in the epidermis, dermis and receiver fluid) was used for statistical analysis as an indication of the change in absolute mometasone furoate absorption from Elocon cream alone when compared to the finite application of a pre-mixed system of Elocon cream and an emollient to human skin (Table 2-7).

Table 2-7: The total amount of mometasone furoate absorbed (epidermis, dermis and receiver fluid; $\mu\text{g cm}^{-2}$) following the application of a finite dose of Elocon cream alone or a 1:1 premixed mixture of Elocon cream and one of six emollients to the skin. The emollients were Aquadrate cream, Cetraben cream, Diprobace cream, Diprobace ointment, Doublebase gel and Hydromol Intensive cream. Data shown as mean \pm SD (n=6). * denotes a significant difference when total drug absorption to the skin ($\mu\text{g cm}^{-2}$) for each emollient was compared to total drug absorption to the skin from Elocon cream alone (Kruskal-Wallis and Mann-Whitney test; $p < 0.05$).

Formulation	Total drug delivery to skin ($\mu\text{g cm}^{-2}$)	Rank order (highest > lowest)
Elocon cream	2.20 \pm 0.24	4
Elocon cream and Aquadrate cream	3.26 \pm 1.15	2
Elocon cream and Cetraben cream	2.23 \pm 0.37	3
Elocon cream and Diprobace cream	1.05 \pm 0.36 *	7
Elocon cream and Diprobace ointment	1.45 \pm 0.54 *	5
Elocon cream and Doublebase gel	1.42 \pm 0.57 *	6
Elocon cream and Hydromol Intensive cream	4.18 \pm 1.49 *	1

Total drug delivery to the skin was significantly altered when Elocon cream was applied with four out of six emollients (Hydromol Intensive cream, Diprobace ointment, Doublebase gel and Diprobace cream; $p < 0.05$). The application of Hydromol Intensive cream with Elocon cream resulted in a 2 fold, significant increase in the total amount of mometasone furoate delivered to the skin when compared to the total drug delivery from Elocon cream alone ($p < 0.05$). In the presence of Diprobace cream, Doublebase gel and Diprobace ointment, total drug delivery significantly decreased when compared to Elocon cream alone, with 2 fold, 1.5 fold and 1.5 fold decreases respectively ($p < 0.05$; Table 2-7).

The data suggest that applying an emollient in a pre-mixed system with Elocon cream can significantly alter drug delivery to the skin compared to the application of the TCS alone. The extent to which drug delivery to the skin was altered, compared to Elocon cream alone, was governed by

the particular emollient employed. To further elucidate the mechanistic effects occurring when particular emollients were mixed with Elocon cream, drug permeation across human skin was evaluated. The cumulative amount of mometasone furoate permeated across human skin over 24 h, following the application of Elocon cream alone or in a premixed system with one of six emollients is presented in Figure 2-11.

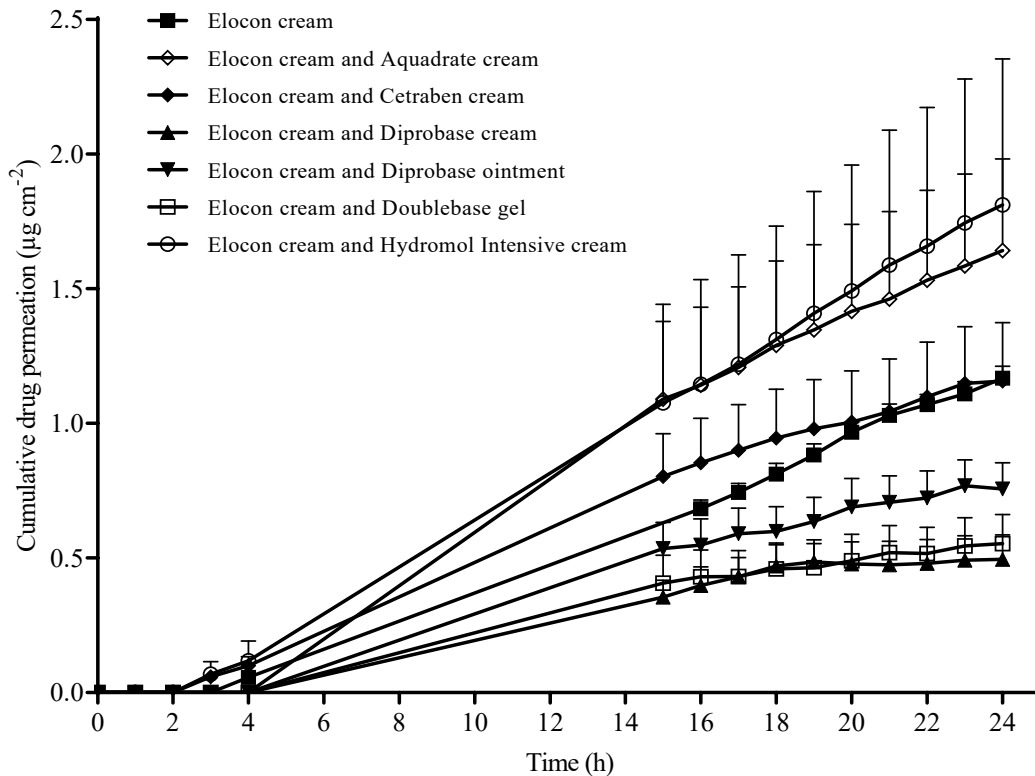


Figure 2-11: The cumulative amount of mometasone furoate ($\mu\text{g cm}^{-2}$) permeated across human scrotal skin from Elocon cream when a finite dose of Elocon cream was applied alone or in a 1:1 premixed system with one of six emollients. The emollients were Aquadrate cream (\diamond), Cetraben cream (\blacklozenge), Diprobace cream (\blacktriangle), Diprobace ointment (\blacktriangledown), Doublebase gel (\square) and Hydromol Intensive cream (\circ). Data are shown as mean + SEM ($n=6$).

Chapter 2

A plot of drug permeation across *ex vivo* human skin against time produced permeation profiles typical of finite dose experiments (Hahn et al., 2012). Drug permeation across human skin was consistently low at the early time points (0–4 h) for all TCS and emollient formulation combinations. Consistent with the findings of drug transport study across silicone membrane, an emollient specific effect was observed where drug permeation from Elocon cream across human skin varied with the particular emollient applied. In the presence of Doublebase gel, Diprobase cream and Diprobase ointment, drug permeation reached a plateau at earlier time points compared to the application of Elocon cream alone. This was accompanied by lower amounts of total drug permeation at 24 h (Q_{24}) compared to Elocon cream alone. Conversely, in the presence of Hydromol Intensive cream and Aquadrate cream, drug permeation from Elocon cream appeared not to reach a plateau over the entire experimental period and resulted in greater total drug permeation in comparison to the application of Elocon cream alone. In all cases, however, donor depletion was not observed with the percent of the applied dose permeating skin after 24 h ranging from 5.03% in the presence of Diprobase cream to 18.31 % in the presence of Hydromol Intensive cream.

To elucidate the mechanistic effects occurring when the TCS is applied with an emollient to skin an evaluation of the normalised partition (Kh) and diffusion co-efficient (D/h^2) across skin is required. For finite dose experiments resulting in non-steady-state diffusion profiles, where the approximation of Kh and D/h^2 by Fick's first law is not appropriate, further mathematical treatment of the data set is often necessary (Anissimov & Watkinson, 2013; Mitragotri et al., 2011). In such cases drug permeation is governed by Fick's second law, a partial differential equation, which once solved can allow a mechanistic evaluation of emollient specific effects on drug permeation across skin from Elocon cream. One approach for solving Fick's second law is through use of the Laplace transformation (Crank, 1975) and Oliveira et al. (2012b) have previously documented the suitability of this technique for the finite dose modelling of a permeant across human skin using Equation 2-4. As such, the permeation profiles presented in Figure 2-11 were modelled using Equation 2-4 to obtain the apparent partition and diffusion co-efficients for mometasone furoate across human skin.

When defining the parameters for the modelling of the permeation profiles, the model was fit to the data set under the assumption that the drug concentration in a premixed system with an emollient was half that of Elocon cream alone, to reflect a simple 1:1 dilution of the TCS. This approach enabled an evaluation of whether proportionate reductions in the permeation parameters were observed on introduction of an emollient to the TCS, when compared to the application of Elocon cream alone.

A representative plot of drug permeation when Elocon cream was applied with Doublebase gel is presented in Figure 2-12 with the respective model fitting obtained using Scientist[®] 3.0 (Micromath Inc, Salt Lake City, UT, USA). The estimated permeation parameters are presented in Table 2-8.

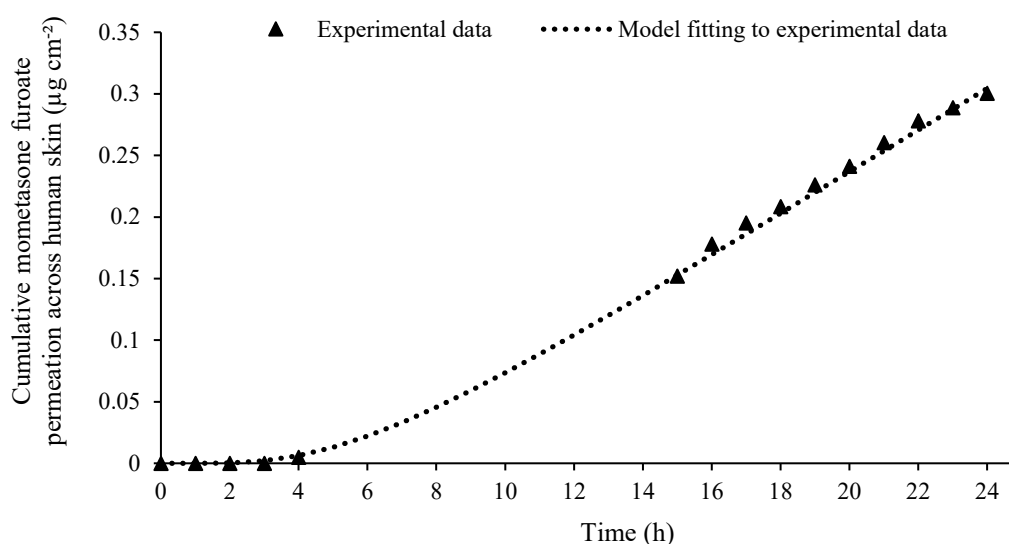


Figure 2-12: A representative profile of mometasone furoate permeation across human skin following the finite application of Elocon cream and Doublebase gel over 24 h. The experimental data points (▲) and respective model fitting (····) are shown for a single replicate.

Table 2-8: Estimated apparent diffusion co-efficient (D/h^2), lag time (L_t), apparent partition co-efficient (Kh), total drug permeation at 24 h (Q_{24}) and pseudo steady state drug flux (J_{ss}) obtained from the nonlinear modelling of the permeation data presented in Figure 2-12. Data are shown as mean \pm SD ($n = 6$). * denotes a significant difference when D/h^2 , L_t , Kh , Q_{24} and J_{ss} values for premixed formulations were compared to the respective permeation parameters for Elocon cream alone (Kruskal-Wallis and Mann-Whitney test; $p < 0.05$).

	Applied dose of formulation (equivalent C_v)	D/h^2 (cm)	L_t (h)	Kh (h^{-1})	Q_{24} ($\mu\text{g cm}^{-2}$)	J_{ss} ($\mu\text{g cm}^{-2} h^{-1}$)	Rank (J)
Elocon cream	10 μL (0.1 %)	2.59E-02 \pm 2.08E-03	6.48 \pm 0.53	3.51E-03 \pm 6.85E-05	1.17 \pm 0.11	9.08E-06 \pm 6.32E-07	3
Elocon cream and Aquadrate cream		3.26E-02 \pm 1.17E-02	5.65 \pm 1.62	7.59E-03* \pm 1.02E-03	1.64 \pm 0.83	1.29E-05 \pm 6.96E-06	2
Elocon cream and Cetraben cream		8.00E-02* \pm 2.96E-02	2.63* \pm 1.59	1.95E-03* \pm 8.03E-05	1.16 \pm 0.53	7.88E-06 \pm 3.11E-06	4
Elocon cream and Diprobase cream	10 μL of TCS mixed with 10 μL of emollient	3.05E-02 \pm 7.80E-03	5.79 \pm 1.24	2.51E-03* \pm 1.48E-04	0.50* \pm 0.22	3.88E-06* \pm 1.25E-06	6
Elocon cream and Diprobase ointment	(0.05 %)	3.08E-02 \pm 7.30E-03	5.78 \pm 1.61	3.58E-03 \pm 1.32E-04	0.76* \pm 0.24	5.54E-06* \pm 1.43E-06	5
Elocon cream and Doublebase gel		4.40E-02* \pm 1.38E-02	4.14* \pm 1.17	1.58E-03* \pm 9.80E-05	0.55* \pm 0.27	3.534E-06* \pm 1.29E-06	7
Elocon cream and Hydromol Intensive cream		2.93E-02 \pm 1.41E-02	5.33 \pm 1.62	9.42E-03* \pm 1.44E-03	1.81 \pm 1.33	1.46E-05 \pm 9.41E-06	1

The change in drug flux largely followed the trends observed with total drug delivery to the skin, where drug flux from the TCS increased up to 1.6 fold in the presence of Hydromol Intensive cream and decreased up to 2.6 fold in the presence of Doublebase gel, compared to drug flux from Elocon cream alone. The changes in drug flux were attributed to an altered apparent partition coefficient, apparent diffusion coefficient or a combination of both parameters.

A significant decrease in drug flux was observed when Elocon cream was applied in the presence of Diprobace cream, Diprobace ointment and Doublebase gel where drug flux was reduced 2.3 fold, 1.6 fold and 2.6 fold, respectively compared to Elocon cream alone (Table 2-8; $p < 0.05$). The decrease in drug flux may have arisen from a decrease in the degree of drug saturation in the mixed formulations, relative to the individual marketed product. The extent of change is likely to have been influenced by the initial degree of drug saturation in Elocon cream (saturated or sub-saturated), the nature of the emollient excipients introduced into the formulation (acting in a solvent or antisolvent capacity) and the relative proportions of these excipients. Thus a full interpretation of the findings first requires an understanding of the degree of saturation of mometasone furoate in the TCS formulation alone, to ascertain whether Elocon cream is performing at maximum thermodynamic activity, and then an appreciation for the altered degree of drug saturation in the presence of emollients, to facilitate an assessment of the change in drug thermodynamic activity, if any.

In practicality, determining the saturated solubility of the drug in marketed formulations is challenging without knowledge of the exact composition of the TCS and emollient formulations. Silicone membrane has demonstrated an ability to discriminate changes in drug thermodynamic activity across different formulations (Davis & Hadgraft, 1991; Flynn & Smith, 1972; Pellett et al., 1997). Thus, in an attempt to determine whether Elocon cream was indeed behaving as a saturated semi-solid formulation, excess drug was added to Elocon cream to prepare a 0.2 % w/w strength formulation and the resultant drug flux across silicone was compared to that of the equivalent marketed product (0.1 % w/w Elocon cream). The drug transport profiles are presented in Figure 2-13.

	J_{2-10h} ($\mu\text{g cm}^{-2} \text{h}^{-1}$)	Q_{24} ($\mu\text{g cm}^{-2}$)
Elocon cream (0.1 % w/w mometasone furoate)	0.30 ± 0.06	6.33 ± 0.48
Elocon cream (0.2 % w/w mometasone furoate)	0.27 ± 0.05	5.88 ± 0.37

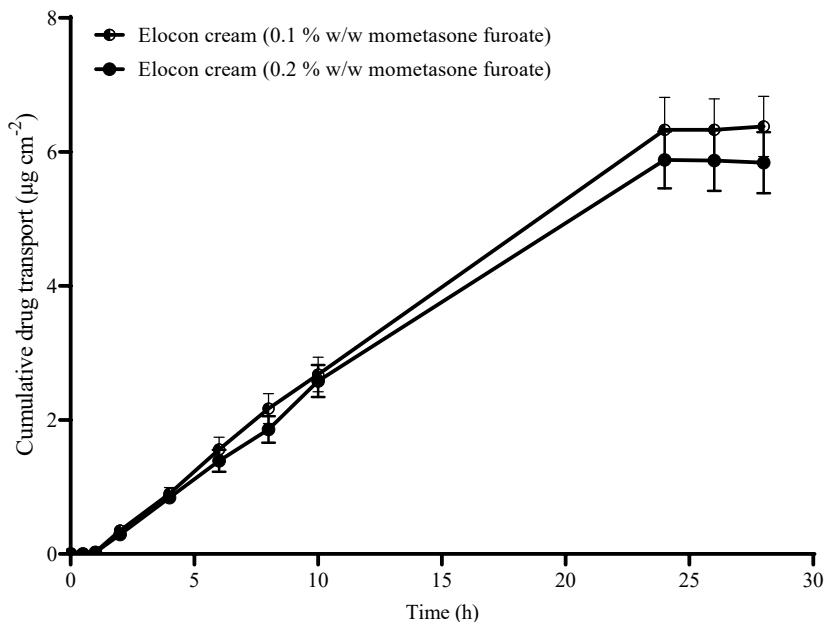


Figure 2-13: The cumulative amounts of mometasone furoate ($\mu\text{g cm}^{-2}$) transport across silicone membrane over 28 h from 0.1 % w/w Elocon cream and 0.2 % w/w Elocon cream following the application of an infinite dose of the formulations. Data are shown as mean \pm SD ($n = 6$). * denotes a significant difference when J_{2-10h} or Q_{24} from 0.1 % w/w Elocon cream was compared, respectively, to 0.2 % w/w Elocon cream (unpaired t-test; $p < 0.05$).

On analysis, no significant difference in drug flux, calculated between 2 h and 10 h, was observed between the formulations ($p > 0.05$) suggesting that mometasone furoate is likely to be saturated, or nearly saturated, in the TCS base. Lippold and Schneemann (1984) reported a similar trend *in vivo*, finding that increasing the concentration of betamethasone-17-benzoate in a suspension type ointment resulted no further increase in the skin blanching response in human volunteers.

Given these findings, it is then beneficial to interpret the modelled permeation data with consideration to the behaviour of semi-solid formulations where the drug is held in suspension, thus at maximum thermodynamic activity. Following the application of such a formulation, drug release is initially dictated by the diffusion of the dissolved drug from the area of formulation closest to the membrane, resulting in a decrease in the concentration of dissolved drug in this region over time.

However, the presence of excess drug particles in the same region serves to maintain a constant concentration gradient by subsequently dissolving and diffusing towards the formulation and membrane interface, thereby maintaining maximum thermodynamic activity.

Thus, in cases where drug flux across skin was reduced (Diprobace cream, Diprobace ointment, Doublebase gel and Cetraben cream), it is likely that this was partially attributed to a reduction in the degree of drug saturation in the premixed formulation, compared to Elocon cream alone. In the case of Diprobace cream, the reduction in drug flux across human skin was attributed to a 1.4 fold significant decrease in the apparent partition co-efficient for mometasone furoate when compared to the application of Elocon cream alone ($p < 0.05$). Similarly, in the presence of Doublebase gel, where drug flux decreased by 2.6 fold compared to Elocon cream alone, a significant reduction in the apparent partition co-efficient for mometasone furoate was observed when compared to Elocon cream alone ($p < 0.05$). Diprobace cream and Doublebase gel are emollient formulations with listed proportions of water; an excipient which has demonstrated poor solubilising capabilities for mometasone furoate (Table 2-3). Introduction of an antisolvent to a saturated system, such as Elocon cream, may have caused drug crystallisation in the premixed formulation and consequently reduced the dose available for absorption. The effect of particle size on the rate of dissolution and consequently drug delivery to the skin was evidenced by Barrett et al. (1965) who reported decreased percutaneous absorption of fluocinolone acetonide from a vehicle when the drug was present in crystalline form compared to the micronised form. Furthermore, reduced drug permeation following the application of supersaturated formulations has been attributed to drug crystallisation on and in the skin (Santos et al., 2010, 2012). It was postulated that simultaneous to a reduction in the degree of drug saturation in the premixed formulation when Elocon cream was mixed with Diprobace cream or Doublebase gel, water was acting in an antisolvent capacity to further reduce drug thermodynamic activity in the formulation. To provide an indication of the water content of the emollient and TCS formulations, an analysis of the moisture content of individual formulations was conducted and the findings are presented in Table 2-9.

Table 2-9: The percent moisture content of all investigated formulations. Data are shown as the mean of three replicates. The range is denoted in brackets.

Formulation	Moisture content (%)
Elocon cream	14.60 (0.86)
Aquadrate cream	0.78 (0.04)
Cetraben cream	36.09 (8.62)
Diprobace cream	60.40 (1.75)
Diprobace ointment	1.02 (0.03)
Doublebase gel	23.18 (4.04)
Hydromol Intensive cream	0.85 (0.06)

The percent moisture content ranged from 0.78 % for Aquadrate cream to 60.40 % for Diprobace cream. Elocon cream displayed a 14.60 % moisture content on analysis. The study revealed that Diprobace cream, Doublebase gel and Cetraben cream have notably higher proportions of water than Elocon cream and thus when these emollients were mixed they may have reduced drug solubility in the premixed formulation compared to drug solubility in Elocon cream alone. Polarised light microscopy was subsequently employed to identify whether crystalline structures were indeed present in formulations when Elocon cream was mixed with these emollients. Representative images are presented in Figure 2-14. Crystalline structures were observed when Elocon cream was mixed with Doublebase gel and Diprobace cream and analysed one hour following preparation, presented in Figure 2-14 C and D respectively. The needle like crystal morphology observed in the premixed formulations resembled the morphology of pure mometasone furoate crystals (Figure 2-14 A). These structures were absent from the Elocon cream formulation alone (Figure 2-14 B) and emollient formulations alone (data not shown) and suggests drug crystal formation occurs following the mixing of Elocon cream and Diprobace cream or Doublebase gel.

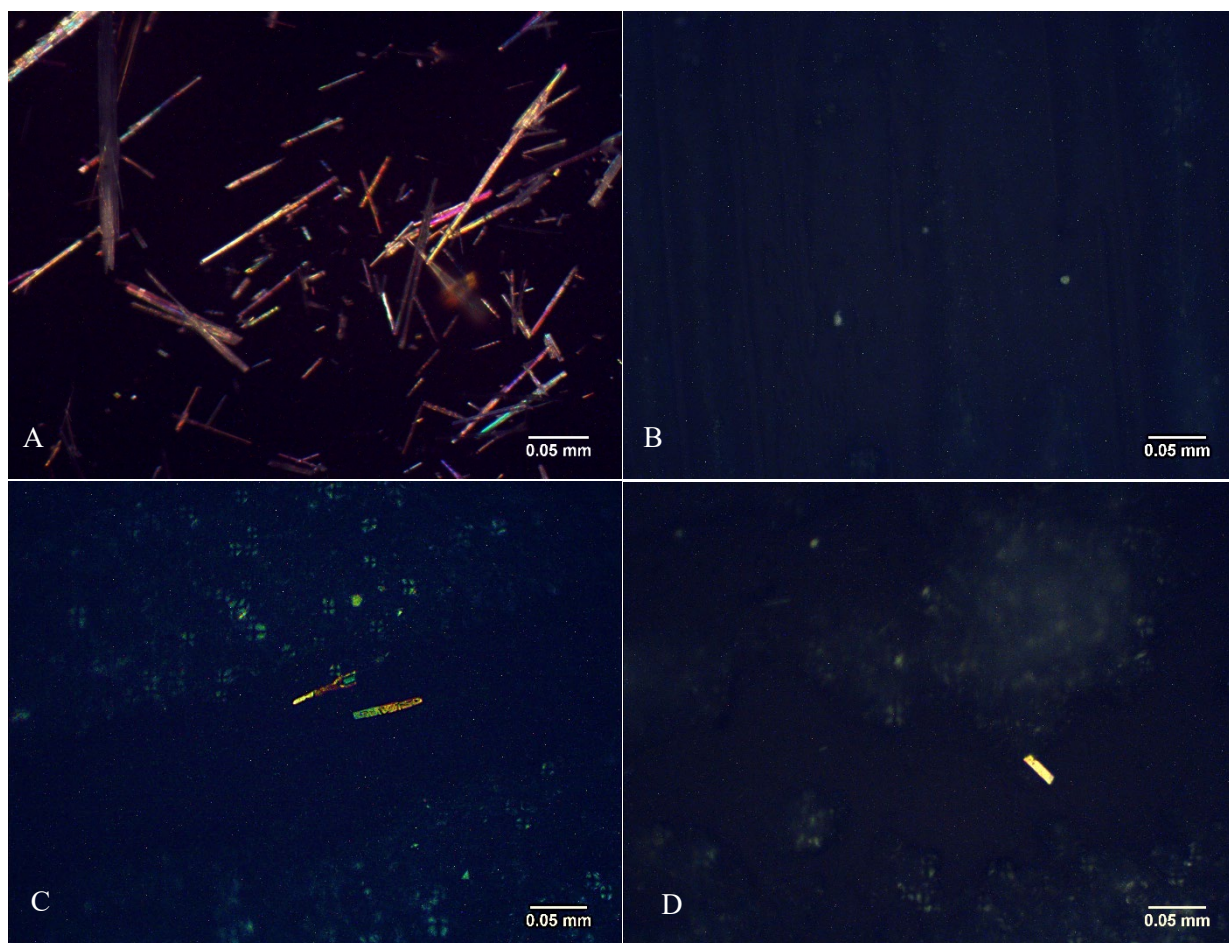


Figure 2-14: Polarised light microscopy images (x 20 magnification) of (A) crystalline mometasone furoate, (B) Elocon cream alone (C) Elocon cream premixed with Doublebase gel and (D) Elocon cream premixed with Diprobace cream.

To characterise the melting range of the crystalline structures observed, HSM was employed. As an example of the thermal behaviour of crystalline structures in the mixed formulations, Figure 2-15 shows the melting phase of a crystalline structure observed in Elocon cream when mixed with Diprobace cream. The crystalline structure exhibited a melting range of 185.7 °C to 200 °C, a lower value than that observed for pure mometasone furoate (218 °C to 220 °C). The lower and broader melting range compared to the pure crystalline melting range may be attributable to partial dissolution of the drug into the surrounding environment as solubility increases, as a function of temperature. The broader melting temperature ranges are also typically observed for impure solids, a likely scenario considering that mometasone furoate crystals are forming in a complex environment resulting from the mixing of the TCS and emollient products (Staveley, 2016). To confirm these findings, Raman microscopy of the crystalline structures in Elocon cream mixed with Diprobace

cream or Doublebase gel was conducted. The obtained spectra are presented in Figure 2-16 and confirm the presence of mometasone furoate crystals in the pre mixed systems of Elocon cream with Diprobase cream or Doublebase gel. Crystalline structures were absent from formulations of Elocon cream mixed with Hydromol Intensive cream, Aquadrate cream, Cetraben cream and Diprobase ointment when observed by polarised light microscopy.

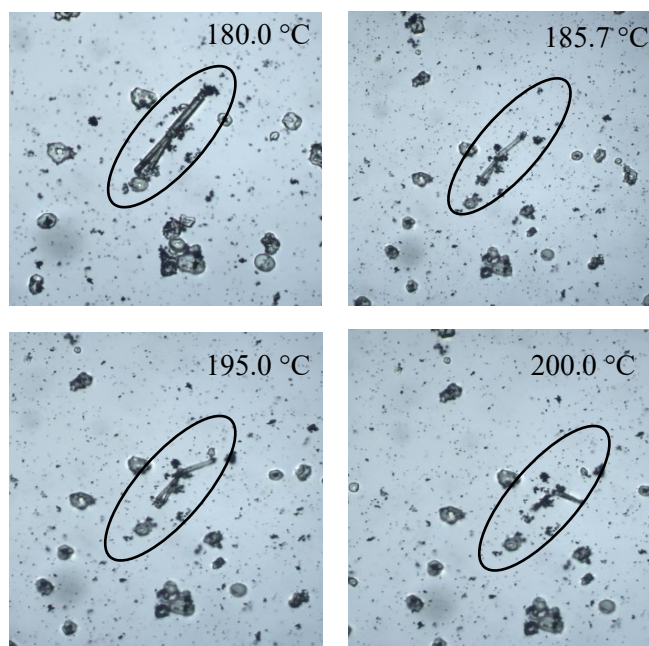


Figure 2-15: Light microscopy images (x 20 magnification) obtained following the thermal analysis (hot stage microscopy) of crystalline structures observed in the premixed Elocon cream and Diprobase cream formulation. The melting range of the crystalline structure was 185.7 °C to 200 °C.

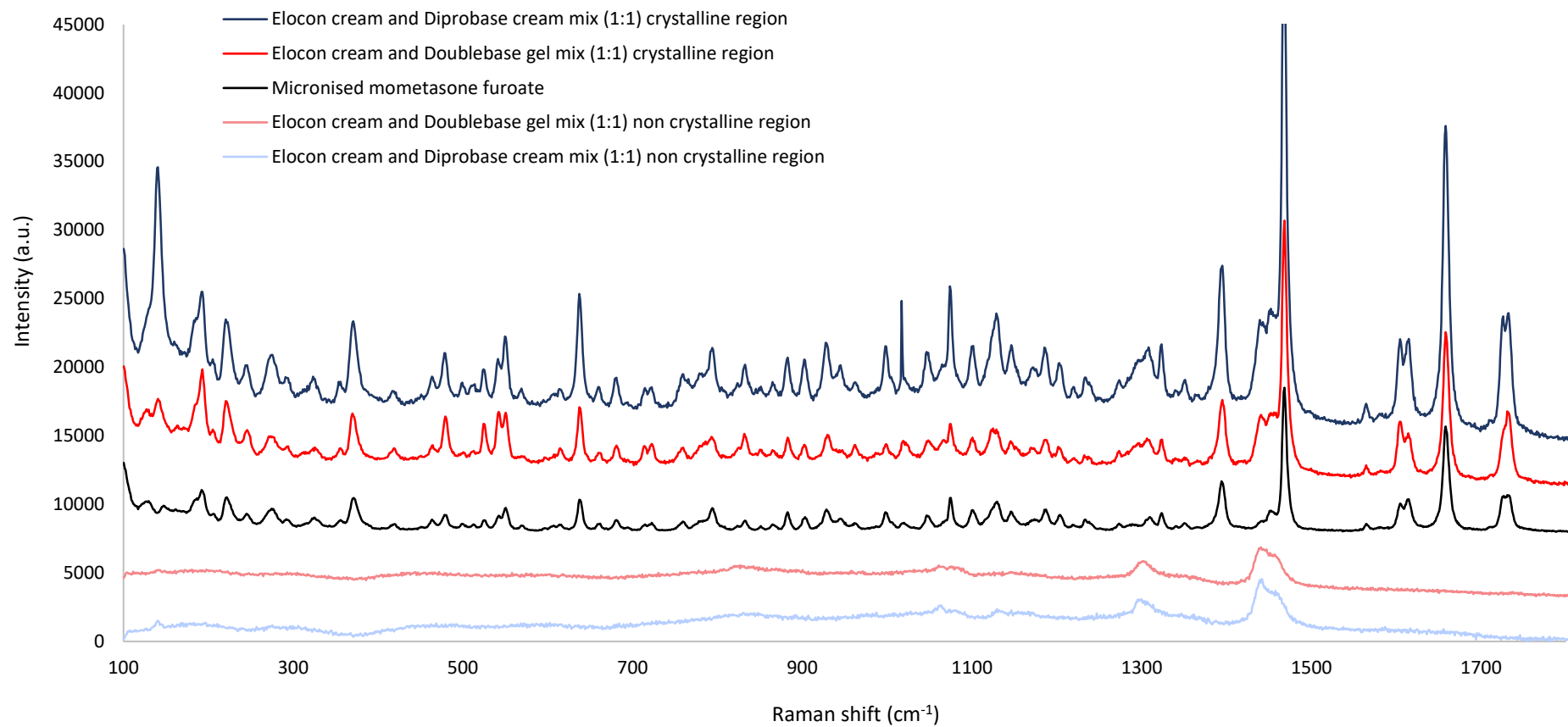


Figure 2-16: Raman spectra obtained from micronized mometasone furoate, crystalline and non-crystalline regions of premixed Elocon cream and Diprobase cream (1:1) or Elocon cream and Doublebase gel (1:1). Spectra were obtained at x100 magnification, a laser excitation wavelength of 785 nm, three accumulations per sample and an acquisition time of 10 s.

It is noteworthy that though Doublebase gel resulted in a significant decrease in total drug delivery to the skin and flux across the skin, findings attributed to the formation of drug crystals in the premixed formulation, non-linear modelling of the permeation profile identified a significant 1.7 fold increase in the apparent diffusion co-efficient when compared to Elocon cream alone ($p < 0.05$), suggesting that complex interactions of the mixed formulation excipients with the stratum corneum may be occurring. Doublebase gel is listed to contain IPM (15 % w/w), an aliphatic ester with demonstrated potential to increase skin permeation of TCSs (Eichner et al., 2017). IPM has been shown to disrupt stratum corneum lipid packing, increase fluidisation of the lipids thus enhance drug diffusion through the stratum corneum (Leopold & Lippold, 1995; Sato et al., 1988) in addition to the earlier reported enhancing effects across silicone membrane. Doublebase gel is also formulated with glycerol, a hygroscopic excipient which has been shown to interact with intercellular lipids of the stratum corneum to favour a liquid crystalline state and enhance the water holding capacity of the stratum corneum (Batt et al., 1988; Froebe et al., 1990). Hydration of the stratum corneum typically lends to greater drug permeation, though the exact mechanism of action is yet to be elucidated (Hadgraft & Lane, 2005). Thus, the extent to which total drug delivery to the skin, and drug flux across the skin, was altered in the presence of Doublebase gel was likely to be a conflated effect of drug crystallisation in the pre-mixed formulation and emollient excipients potentially acting with penetration enhancing effects.

In the presence of Cetraben cream, the permeation parameters displayed a similar trend to Doublebase gel, with a significant 1.8 fold decrease in the apparent partition co-efficient being observed, accompanied by a significant 3.1 fold increase in the apparent diffusion co-efficient ($p < 0.05$), though no significant change in drug flux was evident when compared to Elocon cream alone ($p > 0.05$). Cetraben cream is also listed to contain a proportion of glycerol, which may explain the observed increase in the apparent diffusion co-efficient. Additionally, during the microscopic analysis of Cetraben cream clear phase separation was observed when mixed with Elocon cream (data not shown), indicative of the emollient excipients reducing the miscibility of the oil and water phases in the premixed formulation (Florence & Whitehill, 1981). These observations highlight the

complexity of the heterogenous emollient and TCS systems investigated and help to identify the multitude of formulation effects that may be occurring when emollient products were mixed with Elocon cream (Figure 2-17).

When Diprobase ointment was applied with Elocon cream however, drug flux and total drug delivery to the skin were significantly decreased by 1.6 fold and 1.5 fold, respectively, in the absence of an altered apparent partition co-efficient and diffusion co-efficient, when compared to Elocon cream alone. Thus far, findings of reduced drug flux were centred on the notion that excipients within the emollient formulation can act in an antisolvent capacity for mometasone furoate, essentially reducing the solubility of the drug in the premixed formulation, encouraging drug crystallisation and altering the expected drug partitioning from the formulation into the skin. However, in the absence of excipients in Diprobase ointment with a substantial antisolvent capacity or the presence of drug crystals when mixed with Elocon cream, a potential explanation for the observed reduction in flux could be the reduced degree of drug saturation in the vehicle, as a result of dilution of the TCS formulation from 0.1 % w/w to 0.05 % w/w. In such a situation, incorporating a relatively simple emollient base of liquid paraffin and white soft paraffin, similar to that of Elocon cream, could result in the reduced drug thermodynamic activity in the applied formulation.

It is also important to consider the change in thermodynamic activity of any CPEs in Elocon cream when mixed with Diprobase cream, Diprobase ointment, Doublebase gel and Cetraben cream. Elocon cream is formulated with hexylene glycol, an excipient which can act to favour the partitioning of the drug out of the vehicle and into the skin (Barry, 1987; Mollgaard & Hoelgaard, 1983). Thus, simultaneous to the reduction in drug thermodynamic activity when the TCS was mixed with an emollient, a reduction in the thermodynamic activity of hexylene glycol in the pre mixed formulation may have contributed to the reduction in drug delivery to the skin observed with Diprobase cream, Diprobase ointment, Cetraben cream and Doublebase gel.

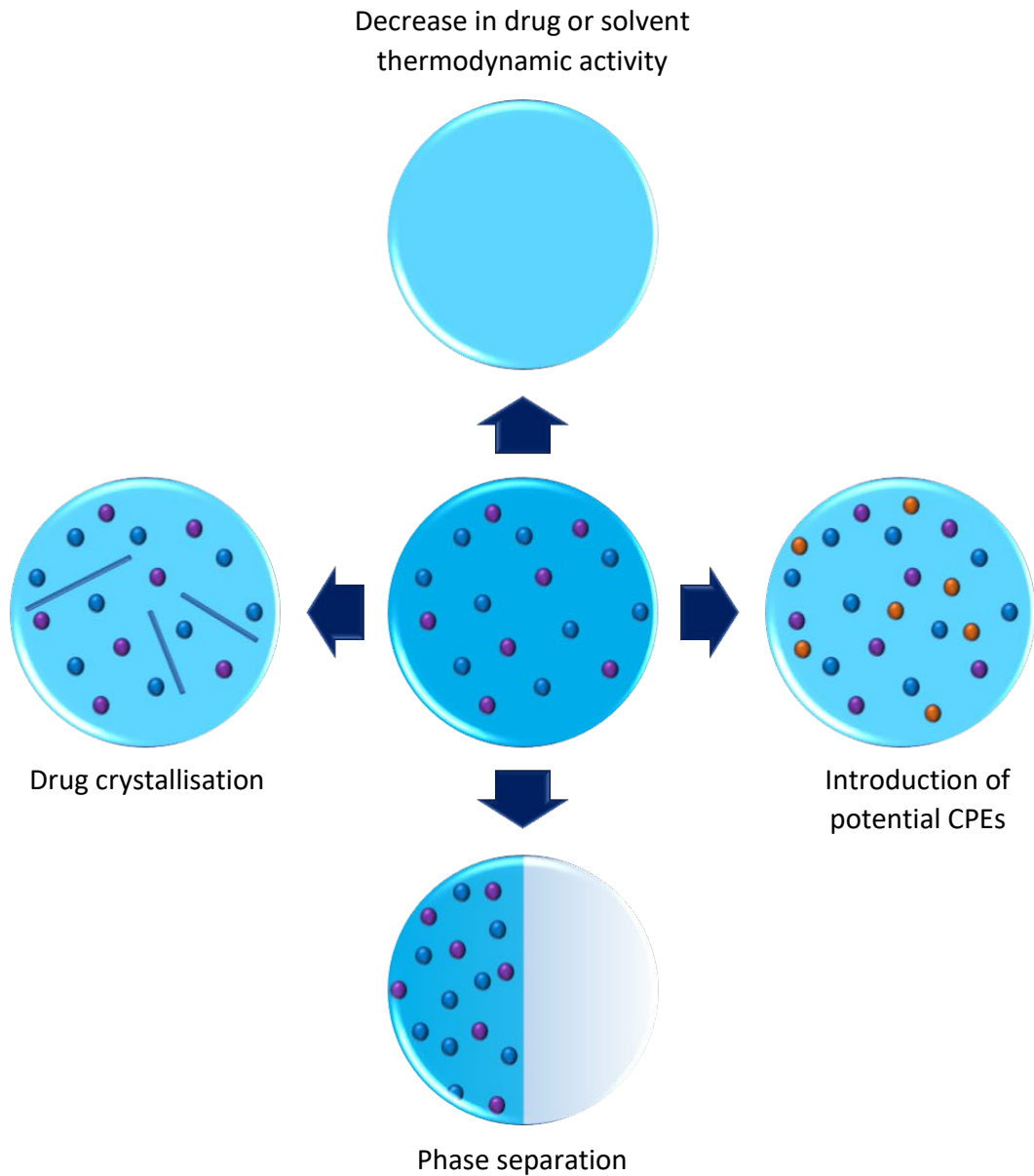


Figure 2-17: Schematic of the potential changes to the TCS formulation on introduction of an emollient to Elocon cream. Depending on the formulation design of the products, the altered drug delivery profile from the mixed product may arise a result of one or several changes to the formulation.

In all cases thus far, the apparent partition co-efficient of mometasone furoate from Elocon cream was significantly reduced when mixed with an emollient (Cetraben cream, Diprobace cream and Doublebase gel) or remained unchanged (Diprobace ointment) when compared to Elocon cream alone. When Aquadrate cream and Hydromol Intensive cream, were mixed with Elocon cream, a significant 2.2 fold and 2.7 fold increase in the apparent partition co-efficient was observed, with a largely unchanged apparent diffusion co-efficient, when compared to Elocon cream alone. This alteration appeared to increase drug flux by 1.6 fold in the presence of Hydromol Intensive cream ($p > 0.05$) and is likely to have resulted in the significant increase in total drug delivery to the skin when compared to Elocon cream alone ($p < 0.05$; Table 2-8). Hydromol Intensive cream and Aquadrate cream contain proportions of IPM, as with Doublebase gel, however, did not exhibit drug crystallisation on microscopic analysis. Thus, in the absence of drug crystals in the premixed formulation, it is likely that IPM acted with penetration enhancing capacity to increase drug delivery to the skin. Indeed, this is supported by the observed increase in apparent partition co-efficient in presence of Aquadrate cream and Hydromol Intensive cream, when compared to Elocon cream alone. Furthermore, Hydromol Intensive cream delivered the largest amount of drug to the skin with a significant 2 fold increase compared to the application of Elocon cream alone. It is worth considering that Hydromol Intensive cream and Aquadrate cream are also formulated with 10 % urea, a hygroscopic agent with the potential to enhance skin penetration of the TCS (Beastall et al., 1986; Feldmann, 1974). Whilst it is understood that urea acts to marginally increase the water content in the stratum corneum, the exact mechanism of action of water on enhancing skin permeation is less clear, especially when considering the permeation of lipophilic molecules such as mometasone furoate. One proposed mechanism is that in the presence of water, lacunar domains (sites of corneodesmosome degradation) present within the lipid bilayers expand to form a continuous 'pore pathway' presenting a route for drug delivery (Elias et al., 2002; Menon et al., 2003). It is therefore possible that the enhanced drug delivery to the skin when Elocon cream was applied with Hydromol Intensive cream or Aquadrate cream is not only attributable to IPM in the formulation or the absence of excipients with antisolvent capacity when compared to emollients such as Diprobace cream, for example, but also to the incorporation of urea into the pre-mixed formulation.

2.4 Conclusion

To develop an understanding of the impact on drug delivery when TCS and emollient formulations are applied to the surface of the skin at similar times, the work presented in this Chapter evaluated the transport of mometasone furoate across silicone membrane from Elocon cream (0.1 % w/w mometasone furoate) and Elocon ointment (0.1 % mometasone furoate), then the impact on drug permeation and skin distribution when Elocon cream was applied alone and in a premixed system with selected emollients. The findings of this Chapter suggest that mixing TCSs with emollients can impact significantly on the expected TCS formulation performance, drug partitioning and delivery to the skin. On investigation of two model TCS formulations (Elocon cream and Elocon ointment), drug transport across silicone membrane following the application with a range of emollients trended in an emollient specific manner. With respect to Elocon cream for example, drug flux across silicone membrane ranged from a 2 fold increase in the presence of Hydromol Intensive cream to a 4 fold decrease in the presence of Cetraben cream when compared to drug flux from Elocon cream alone. These findings, when coupled with the data generated following *ex vivo* drug permeation and skin distribution studies, were indicative of altered apparent partitioning of the drug from the formulation as well as a change in the expected skin absorption of the drugs. Notably, the apparent partitioning of the drug from the mixed formulations into skin ranged from a 2.2 fold decrease when Elocon cream was applied with Doublebase gel to a 2.7 fold increase when Elocon cream was applied with Hydromol Intensive cream. Reduced partitioning was attributed to drug crystallisation in the formulation in some cases and creation of a sub saturated system in others. Conversely, in the presence of Hydromol Intensive cream, it was hypothesised that the inclusion of excipients with penetration enhancing capacity, namely IPM and urea, increased drug partitioning from the formulation into the skin.

Clinically, introduction of emollient excipients to the TCS which may act to reduce partitioning by altering the solubility of the drug in the mixed formulation, or enhance penetration, by interacting with the membrane, has the potential to alter the expected drug delivery profile to skin *in vivo* compared to the application of the TCS alone. In a clinical scenario where multiple products are

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applied to the surface of the skin with very short time intervals, there may not be sufficient time for the individual products to be fully absorbed resulting of the mixing of the formulations on the surface of the skin. Thus, the aim of employing a premixed system of TCS and emollients in this Chapter was to create an environment applicable to a clinical scenario and the data presented serves to highlight the complexity of the factors impacting drug delivery when multiple products are applied to the skin at similar times.

Chapter Three:

Evaluating the impact of altering the order of, and time interval between, the applications of Dermovate cream and emollients on the percutaneous absorption and skin retention of clobetasol propionate

3.1 Introduction

The work presented in Chapter 2 demonstrated emollient specific effects on drug delivery to the skin from Elocon cream. To determine whether similar trends are evident with other TCSs, clobetasol propionate was selected for investigation. Dermovate cream is the most potent TCS currently available (0.05 % w/w clobetasol propionate; UK very potent classification) and treatment with this TCS is only initiated in cases unresponsive to treatment with lower potency classes because of the increased risk of associated side effects (Eichenfield et al., 2014; Feldman, 2005; National Institute for Health and Care Excellence, 2018a; Wollenberg et al., 2016). Hypothalamus pituitary adrenal (HPA) axis suppression is one of the reported side effects of topically applied corticosteroids (Hengge et al., 2006) and highly potent steroids, such as clobetasol propionate, have demonstrated increased ability to suppress adrenal function following topical application to the skin of atopic dermatitis patients (Carruthers et al., 1975; Ellison et al., 2000). For example, the application of only 2 g per day of 0.05 % clobetasol propionate cream to diseased skin has been reported to decrease morning cortisol levels after only a few days (Ohman et al., 1987; Olsen & Cornell, 1986). In addition, the use of very potent TCSs is associated with an increased risk of local side effects such as skin atrophy, characterised by skin thinning and loss of elasticity (Castela et al., 2012). Dilution of a very potent TCS may lower the risk of side effects, such as HPA suppression (Wolkerstorfer et al., 2000), however the extent to which efficacy may also be reduced is unpredictable and made additionally challenging if the diluent used is dissimilar to the TCS base (Refai & Müller-Goymann, 2002; Ryatt et al., 1982; Wiedersberg et al., 2008). The work reported in Chapter 2 is indicative of an altered Elocon cream formulation on the surface of the skin when the product was applied with an emollient. In some cases, this resulted in a significant increase, or decrease, in drug delivery to *ex vivo* human skin. Dermovate cream is formulated with propylene glycol and Doublebase gel, Hydromol Intensive cream and Aquadrate cream contain proportions of excipients with penetration enhancing capabilities (IPM and urea). Sato et al. (1988) demonstrated the synergistic effects of propylene glycol and IPM on the delivery of nicorandil from suspension formulations, reporting a 70 fold enhancement of nicorandil across rat skin when delivered from a

binary solution of IPM (10 %) and propylene glycol compared to delivery from propylene glycol alone. Should similar effects be observed when the very potent TCS, clobetasol propionate, is applied to the skin at similar times to an emollient, the findings are likely to be relevant to the current clinical recommendations for the use of TCSs and emollients.

Prescribers typically select topical products based on a number of factors such as formulation type, patient preference, ease of product use and convenience (Del Rosso & Friedlander, 2005; Feldman et al., 2008; Rapp et al., 1997). Patient preference has been found to be an important factor for adherence to treatment. For example, a survey of product use amongst patients with psoriasis revealed that 38 % of patients desired less frequent applications of TCSs, including those on once-daily regimes, and non-compliance in 11 % of patients was attributed to the greasy feel of formulations (van de Kerkhof et al., 1998). Thus, a wide range of TCS and emollient combinations are likely to be used in practice and each combination has the potential to behave differently when applied to the skin surface at similar times.

The clinical guidance about the co-application of TCSs and emollients reflects a lack of consensus between healthcare bodies, with recommendations made on the basis of clinical opinion rather than evidence-based findings (Voegeli, 2017). Furthermore, little consideration is paid to the practicalities of applying TCSs and emollients on a daily basis, an important factor as patients, or their carers, typically allocate shorter amounts of time to product applications than recommended (Jemec et al., 2006; Loden, 2005; Ring et al., 2012). In such situations, without an understanding of the benefits or drawbacks of particular regimes, adherence to seemingly complex and time-consuming application regimes is likely to be low (Smoker & Voegeli, 2014). Understandably, this has resulted in uncertainties amongst healthcare professionals and patients about the safest way in which TCSs and emollients can be used together to ensure clinical efficacy, adherence to treatment and patient safety (Batchelor et al., 2013). Given these considerations, it is important to appreciate the formulation effects that may be occurring on the surface of the skin when different combinations of products are applied at similar times.

Chapter 3

To develop an evidence base to begin to address the current uncertainties, the objectives of the work presented in this Chapter were to evaluate the impact on drug delivery to *ex vivo* human skin when the order of, and time interval between, product applications were altered. To achieve these objectives, *in vitro* and *ex vivo* Franz cell experiments were conducted. The selected TCSs were Dermovate cream (0.05 % w/w clobetasol propionate) and Dermovate ointment (0.05 % w/w clobetasol propionate). The emollients investigated were as selected during the work performed in Chapter 2 to allow for direct comparisons (Aquadrone cream, CetraBEN cream, Diprobase cream, Diprobase ointment, Doublebase gel and Hydromol Intensive cream). To allow for an initial analysis of the effect of introducing an emollient to the TCS formulation on drug delivery to the skin, a premixed TCS and emollient system was employed. Furthermore, this model should allow for an understanding of altered drug delivery to the skin when products are applied in quick succession. To then investigate the impact of altering the order of, and time interval between, product applications on drug delivery to *ex vivo* skin four application regimes were selected with an aim to reflect some of the currently recommended practices: the application of a TCS before an emollient (with a five minute or thirty minute interval) and the application of a TCS after an emollient (with a five minute or thirty minute interval).

3.2 Materials and methods

3.2.1 Materials

Micronised clobetasol propionate (Ph Eur) was provided by MedPharm Ltd (Guildford, UK). Dermovate cream (0.05 % w/w clobetasol propionate), Dermovate ointment (0.05 % w/w clobetasol propionate), Aquadrate cream, Cetraben cream, Diprobace cream, Diprobace ointment, Doublebase gel and Hydromol Intensive cream were acquired from the University of Hertfordshire Campus Pharmacy (Hertfordshire, UK). Phosphate buffered saline (PBS) tablets, acetonitrile (HPLC grade) and absolute ethanol (99 + %) were acquired from Fisher Scientific (Leicestershire, UK). Non-sterile, medical grade 0.002” silicone membrane was acquired from Bioplexus (Los Angeles, USA).

3.2.2 Analytical method development

3.2.2.1 Development of the HPLC methods for clobetasol propionate quantification

Quantitative analysis of clobetasol propionate in samples was achieved using an Agilent 1260 Infinity quaternary pump and high performance autosampler coupled to an Agilent 1260 multi wavelength UV/Vis detector set to 235 nm (Agilent Technologies, UK). Chromatographic analysis was performed using a reverse phase Hypersil™ C18 column (5 µm particle size, 250 mm x 4.6 mm; Phenomenex, UK) in conjunction with a SecurityGuard™ guard cartridge system packed with a C18 cartridge (4 mm x 3 mm; Phenomenex, UK), a sample injection volume of 20 µL and a constant flow rate of 1 mL min⁻¹. The Agilent ChemStation software (Agilent Technologies, UK) was used for data acquisition. The mobile phase composition was acetonitrile (HPLC grade) and water (18.2 MΩ MilliQ). Clobetasol propionate eluted at 15.9 min under the following gradient conditions: 35 % acetonitrile from 0 min to 5 min, 35–95 % acetonitrile from 5 min to 17 min, 95–35 % acetonitrile from 17 min to 19 min, 35 % acetonitrile from 19 min to 22 min.

3.2.2.2 Preparation of calibration standards

A 100 µg mL⁻¹ stock solution of clobetasol propionate was prepared by weighing 10 mg of the drug into a 100 mL volumetric flask and making up to volume with the diluent, acetonitrile. A series of standards

were prepared by appropriate dilution of the stock solution with the diluent; the concentration of the standards ranged from 0.05 $\mu\text{g mL}^{-1}$ to 100 $\mu\text{g mL}^{-1}$. Drug quantification was achieved using the analytical methods detailed in Section 3.2.2.1 and calibration curves plotted for the detected range.

3.2.2.3 Determination of fitness for purpose of the analytical method

The HPLC method was validated for linearity, precision and accuracy in accordance with the International Conference on Harmonization (ICH) guidelines (ICH, 2005). Linearity was determined by the correlation coefficient (R^2) for concentrations ranging from 0.05 $\mu\text{g mL}^{-1}$ to 100 $\mu\text{g mL}^{-1}$. The standard error for the predicted y value for all x values in the regression (STEYX) was calculated and used, as previously described, in Equation 2-1 and Equation 2-2 to calculate the limit of detection (LOD) and limit of quantification (LOQ) of clobetasol propionate, respectively (Section 2.2.2.3).

Determination of the precision of the analytical method was achieved by intra-day and inter-day analysis. Intra-day precision was measured by 6 replicate injections of 5 $\mu\text{g mL}^{-1}$, 50 $\mu\text{g mL}^{-1}$ and 100 $\mu\text{g mL}^{-1}$ samples of standards of clobetasol propionate prepared on the same day. Inter-day precision was assessed through the analysis of 6 replicate injections of 5 $\mu\text{g mL}^{-1}$, 50 $\mu\text{g mL}^{-1}$ and 100 $\mu\text{g mL}^{-1}$ samples prepared in triplicate on 3 separate days.

The accuracy of the analytical methods was tested by preparing triplicate samples of clobetasol propionate in the diluent at three concentrations (low, medium and high) and quantifying using the gradient HPLC UV method. Accuracy was determined as previously described using Equation 2-3 (Section 2.2.2.3).

3.2.3 Formulation selection

Dermovate cream and Dermovate ointment were selected as model TCS formulations for investigation. The full excipient list for the selected TCS formulations is detailed in Table 3-1. The emollients selected were Aquadrate cream, Cetraben cream, Diprobase cream, Diprobase ointment, Doublebase gel and Hydromol Intensive cream. The full emollient excipient list is as previously presented in Chapter 2 (Table 2-1; Section 2.2.3).

Table 3-1: The listed excipients for Dermovate cream and Dermovate ointment. Data were obtained from the most recently published summary of product characteristics for the respective formulation.

Dermovate cream (0.05 % w/w)	Dermovate ointment (0.05 % w/w)
Clobetasol propionate	Clobetasol propionate
Propylene glycol	Propylene glycol
Glycerol monostearate	Sorbitan sesquioleate
Sodium citrate	White soft paraffin
Citric acid monohydrate	
Arlacel 165 (glycerol monostearate)	
Cetostearyl alcohol	
Beeswax substitute 6621	
Chlorocresol	
Purified water	

3.2.4 Microscopic analysis of Dermovate cream and Dermovate ointment premixed with emollients

Light microscopy was employed to observe the occurrence, or absence, of crystalline structures in the premixed TCS and emollient formulations. Dermovate cream or Dermovate ointment alone, the emollient formulations alone and mixed TCS and emollient systems (1:1) were prepared on the same day as analysis. The emollients were: Aquadrate cream, Cetraben cream, Diprobase cream, Diprobase ointment, Doublebase gel and Hydromol Intensive cream. Samples were observed using a L3230 GX light microscope (GT Vision Ltd, Suffolk, UK). Where crystalline structures were observed, images were captured using a x 20 objective lens, using a GX CAM camera and GX Capture software (GT Vision Ltd, Suffolk, UK).

3.2.5 Franz cell assembly

Full thickness human scrotal skin was prepared as detailed in Section 2.2.4.1. Franz cells were assembled as detailed in Section 2.2.4.2.

3.2.5.1 Franz cell method development

3.2.5.2 Selection of the receiver fluid systems for *in vitro* and *ex vivo* Franz cell experiments

To ensure adequate solubility of clobetasol propionate in the receiver fluid, the solubility of clobetasol propionate in PBS alone and PBS with absolute ethanol (10 %, 20 % or 30 %) was determined. To gain an appreciation of the potential for the solubility of clobetasol propionate in the TCS formulation to be altered when mixed with the emollient formulations, an evaluation of the saturated solubility of clobetasol propionate in the liquid excipients of the formulations was also investigated. The liquid excipients of all formulations were: water, glycerol, liquid paraffin, isopropyl myristate, castor oil, sorbitan sesquioleate and propylene glycol.

Saturated solutions were prepared as follows: adequate amounts of clobetasol propionate were added to the range of solutions until a suspension was formed (confirmed visually by the continued presence of drug particles in solution), samples were then stirred for 24 h at room temperature and filtered through Millex Millipore 0.22 µm syringe filters. All samples were appropriately diluted in mobile phase prior to drug quantification by HPLC UV analysis.

3.2.5.3 Determining drug - filter binding

Membrane binding studies were conducted to determine whether clobetasol propionate had the potential to bind to PTFE filters during the drug extraction process. A saturated solution of clobetasol propionate in acetonitrile was prepared as detailed in Section 3.2.5.2. The experiment was conducted as detailed in Section 2.2.5.4 and drug quantification achieved using the gradient elution analytical method summarised in Section 3.2.2.1.

3.2.5.4 Selecting appropriate receiver fluid sampling time points for *in vitro* and *ex vivo* Franz cell experiments

A study was conducted to establish a sampling protocol to adequately profile clobetasol propionate transport across silicone membrane whilst maintaining sink conditions for the duration of the experiment. Franz cells (n=3) were assembled with silicone membrane and the receiver chamber filled with the receiver fluid system developed in Section 3.2.5.2 (PBS and ethanol; 70:30). The membrane

was dosed with 500 mg of Dermovate cream or Dermovate ointment by weight. Samples (200 μ l) of the receiver fluid were taken periodically up to 26 h and replaced with fresh preheated receiver fluid. With respect to Dermovate cream, a sampling protocol was also required to investigate the permeation of clobetasol propionate across human skin following the finite dosing of Dermovate cream. To achieve this, skin samples were mounted in Franz cells (n=3) and the receiver chamber filled with the receiver fluid system developed in Section 3.2.5.2 (PBS and ethanol; 70:30). Skin samples were dosed with 10 μ L of Dermovate cream and samples (200 μ l) of the receiver fluid were taken periodically up to 24 h and replaced with fresh preheated receiver fluid. The drug concentration at each time point was determined using the analytical methods summarised in Section 3.2.2.1 to establish whether sink conditions were maintained.

3.2.5.5 Development of drug extraction method for clobetasol propionate from skin matrices

The suitability of acetonitrile as an extraction solvent for clobetasol propionate from all matrices (skin surface and donor chamber, epidermal membrane, dermal membrane), following drug permeation experiments was investigated. Using a positive displacement pipette, 10 μ L of a 1 mg mL⁻¹ solution of clobetasol propionate in acetonitrile, prepared as detailed in Section 3.2.2.2 was added to vials containing: cotton buds, tape strips, epidermal membranes, dermal membranes and an empty vial serving as the control. All vials were placed in a water bath set to 32 °C for 24 h. Following this period, 1 mL of acetonitrile was added to each vial, the vials were sonicated for 10 minutes and placed on a roller shaker for 18 h. Extraction solvents were removed entirely from the vials, filtered through 0.22 μ m PTFE filters and drug quantified by the gradient elution analytical method summarised in Section 3.2.2.1.

3.2.5.6 Stability of clobetasol propionate in the extraction solvent and receiver fluid systems

To ascertain the potential for clobetasol propionate to degrade in the selected receiver fluid system during the Franz cell experimental period, drug stability in PBS and ethanol (70:30) was determined when stored at 37 °C for 24 h. To ascertain the potential for clobetasol propionate to degrade in the selected receiver fluid system or extraction solvent during storage, the stability of clobetasol propionate

in these solutions was determined, when stored at 2-8 °C and 25 °C. A stock solution of 100 µg mL⁻¹ of clobetasol propionate was prepared as detailed in Section 3.2.2.2 and the experiment was conducted as described in Section 2.2.5.5. Samples were analysed using the gradient elution analytical method summarised in Section 3.2.2.1.

3.2.6 *In vitro* silicone membrane Franz cell studies with Dermovate cream

To investigate the effect of a mixed TCS and emollient system on drug transport across a synthetic membrane, an *in vitro* Franz cell study was conducted using Dermovate cream as a model TCS formulation. Franz cells were assembled with silicone membrane as detailed in Section 2.2.4.2. Informed by the findings of the receiver fluid system development studies, the receiver chamber was filled with PBS and ethanol (70:30) and Franz cells were equilibrated in a water bath set to 37 °C. Following the equilibration period, Franz cells were briefly removed from the water bath and the membrane was dosed with 500 mg of Dermovate cream alone or 1 g of a Dermovate cream and emollient mixture (1:1) applied to the donor chambers by weight. The formulations were carefully spread over the membrane surface using a spatula to ensure contact with the membrane. The emollients used in this experiment were: Aquadrate cream, Cetraben cream, Diprobace cream, Diprobace ointment, Doublebase gel and Hydromol Intensive cream. Six replicate Franz cells were assembled for each emollient investigated. Samples (200 µl) of the receiver fluid were taken periodically up to 26 h and replaced with fresh preheated receiver fluid. Drug quantification in samples was achieved using the gradient elution analytical method summarised in Section 3.2.2.1.

3.2.7 *In vitro* silicone membrane Franz cell studies with Dermovate ointment

To investigate the effect of the TCS formulation on drug transport across silicone membrane from a mixed system, Dermovate ointment was selected as a comparator product to Dermovate cream. The experimental design was similar to that described in Section 3.2.6 with the following changes: silicone membrane mounted in Franz cells were dosed with either 500 mg of Dermovate ointment alone or 1 g of a Dermovate ointment and emollient mixture (1:1) applied to donor chambers by weight. The

emollients selected for investigation, receiver fluid system, sampling time points, analytical method and data handling remained unchanged.

3.2.8 *Ex vivo* human skin Franz cell study: Finite dosing of a premixed TCS and emollient system

3.2.8.1 Studies investigating drug permeation across *ex vivo* human skin

Franz cells were assembled with human skin as detailed in Section 2.2.4.2. The receiver chamber was filled with a PBS and ethanol (70:30), informed by the findings of the receiver fluid system development studies (Section 3.2.5.2 and Section 3.2.5.6). Following the equilibration period, Franz cells were briefly removed from the water bath then skin samples were dosed with 10 μL of Dermovate cream or 20 μL of a Dermovate cream and emollient mixture (1:1), prepared one hour in advance and applied using a positive displacement pipette. To ensure contact with the membrane, the product was carefully spread over the surface of the skin using the tip of a capillary piston and the Franz cell returned to the water bath to commence the experiment. The selected emollients for investigation were: Aquadrate cream, Cetraben cream, Diprobase cream, Diprobase ointment, Doublebase gel and Hydromol Intensive cream. Samples (200 μL) of the receiver fluid were taken at pre-determined intervals up to 24 h and replaced with fresh preheated receiver fluid. Drug quantification was achieved using the gradient elution analytical method summarised in Section 3.2.2.1.

Scientist[®] 3.0 (Micromath Inc, Salt Lake City, UT, USA) was used to calculate the apparent partition (Kh) and diffusion (D/h^2) parameters when the Laplace transformation solution to Fick's second law, under finite dose conditions, was fit to the experimental permeation data sets (Equation 2-4), as previously described in Section 2.2.8.1 (Chapter 2).

The drug concentration in the formulation was set to 0.05 % for the application of Dermovate cream alone and 0.025 % for the applications of the premixed Dermovate cream and emollient mixture (1:1). Pseudo steady state drug flux (J_{ss}) and lag time (L_t) for drug permeation were estimated as previously described in Equation 2-5 and Equation 2-6 respectively (Chapter 2).

3.2.8.2 Studies investigating drug penetration to *ex vivo* human skin

Following the drug permeation across human skin studies, Franz cells were disassembled and drug content on the skin surface (residual formulation), the epidermis and dermis determined as previously described in Section 2.2.8.2 (Chapter 2). Quantification of clobetasol propionate in the cotton buds, tape strips, epidermis and dermis was achieved using the extraction method developed in Section 3.2.5.5. All samples were analysed by the gradient elution analytical method summarised in Section 3.2.2.1.

3.2.9 *Ex vivo* human skin study with Dermovate cream and emollients: Investigating the effect of altering the order and timing of the application of Dermovate cream with emollients on drug delivery to the skin

3.2.9.1 Studies investigating drug permeation across *ex vivo* human skin

Franz cells were assembled with human skin as detailed in Section 2.2.4.2. The receiver chamber was filled with PBS and ethanol (70:30), informed by the findings of the receiver fluid system development studies. Following the equilibration period at 37 °C, Franz cells were briefly removed from the water bath and skin samples dosed with 10 µL of Dermovate cream alone or 10 µL of Dermovate cream followed, after a five minute interval, by 10 µL of an emollient. To observe the effect of altering the order of product application on clobetasol propionate absorption to the skin, the reverse application regimen was employed where skin samples were dosed with 10 µL of an emollient followed, after a five min interval, with 10 µL of Dermovate cream. To investigate the impact of altering the time interval between products applications on drug absorption to the skin, experiments were conducted as detailed above but with a thirty minute time interval instead of a five minute time interval between product applications. A summary of the application protocols employed is presented in Figure 3-1.

To ensure contact with the membrane, the product was carefully spread over the surface of the skin using the tip of a capillary piston and the Franz cell returned to the water bath to commence the experiment. The emollients selected for investigation were Doublebase gel, Diprobase ointment and Hydromol Intensive. Samples (200 µL) of the receiver fluid were taken at pre-determined intervals up

to 24 h and replaced with fresh preheated receiver fluid. Drug quantification was achieved using the gradient elution analytical method summarised in Section 3.2.2.1.

Scientist[®] 3.0 (Micromath Inc, Salt Lake City, UT, USA) was used to calculate the apparent partition (Kh) and apparent diffusion (D/h^2) parameters using Equation 2-4. The drug concentration in the formulation was set to 0.05 % for the application of Dermovate cream alone and all application regimes. The pseudo steady state flux (J_{ss}) and lag time (L_t) for drug permeation were estimated as previously described in Equation 2-5 and Equation 2-6 respectively.

3.2.9.2 Studies investigating drug penetration in *ex vivo* human skin

Following the drug permeation across human skin studies, Franz cells were disassembled and drug content on the skin surface (residual formulation), the epidermis and dermis determined. Removal of the residual formulation and heat separation of the epidermis and dermis was achieved by adopting the protocol described in Section 3.2.8.2. Quantification of clobetasol propionate in the cotton buds, tape strips, epidermis and dermis was achieved using the extraction method developed in Section 3.2.5.5. All samples were analysed by the gradient elution analytical method summarised in Section 3.2.2.1.

3.2.10 Data treatment and statistical analysis

The concentration of clobetasol propionate in the receiver fluid was corrected for previous sample removal and profiles constructed to present cumulative amount of drug permeated per unit area ($\mu\text{g cm}^{-2}$) over the exposure period. Linear regression was performed on infinite dose data sets to determine mean drug flux.

Experimental data were expressed as mean ($n = 6$) \pm standard deviation (SD), unless otherwise stated. Statistical analysis was performed using Prism 8.0 (GraphPad, USA). The Shapiro Wilk test was employed to determine the normality of all data sets. Non-parametric analysis for multiple comparisons was performed using Kruskal-Wallis and a Mann–Whitney test applied for post hoc analysis. Parametric analysis for multiple comparisons was performed using analysis of variance (ANOVA) and Tukey's post hoc test. Statistically significant differences were determined at a 95 % confidence interval ($p \leq 0.05$).

Application of premixed TCS and emollient products		
	<i>In vitro</i> Franz cell experiments	<i>Ex vivo</i> Franz cell experiments
Membrane	Silicone	Human skin
Dosing model	Infinite	Finite
Application protocol	Dermovate cream premixed with: Aquadrate cream Diprobace cream Diprobace ointment Cetraben cream Doublebase gel Hydromol Intensive	Dermovate cream premixed with: Aquadrate cream Diprobace cream Diprobace ointment Cetraben cream Doublebase gel Hydromol Intensive

Altered order of, and time interval between, product applications to <i>ex vivo</i> human skin				
	Application protocol 1	Application protocol 2	Application protocol 3	Application protocol 4
Finite application of product 1	Dermovate cream	Diprobace ointment Doublebase gel Hydromol Intensive	Dermovate cream	Diprobace ointment Doublebase gel Hydromol Intensive
Time interval	5 minutes		30 minutes	
Finite application of product 2	Diprobace ointment Doublebase gel Hydromol Intensive	Dermovate cream	Dermovate cream	Diprobace ointment Doublebase gel Hydromol Intensive

Figure 3-1: Summary of the application protocols employed to investigate the altered drug transport across silicone membrane and drug permeation and penetration to human skin in the presence of emollients.

3.3 Results and discussion

3.3.1 Analytical methods

A gradient elution method was developed to enable the suitable quantification of clobetasol propionate in samples following *in vitro* and *ex vivo* Franz cell experiments. Calibration standards were prepared over a nominal concentration range and analysed by the HPLC UV method detailed in Section 3.2.2.1. The elution time of components from the six emollients, skin endogenous compounds, cotton buds and scotch tape were also determined to ensure no interference with the peak of interest. The resulting HPLC gradient method provided a suitable drug elution time with sufficient resolution from peaks of no interest to be achieved. A sample chromatogram of clobetasol propionate generated following analysis is presented in Figure 3-2.

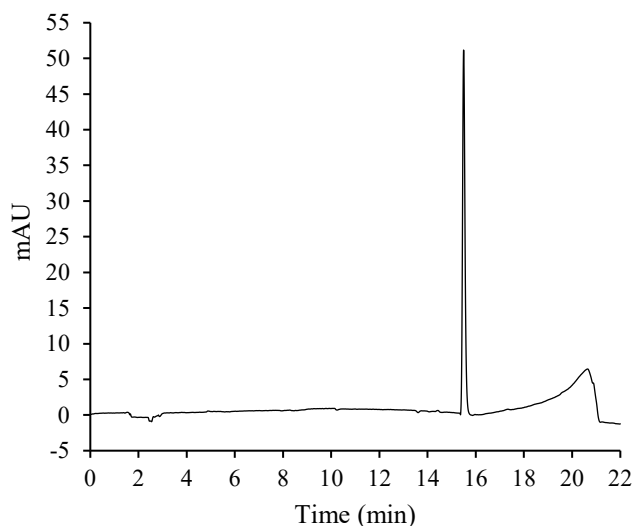


Figure 3-2: Sample chromatogram obtained at 235 nm following the gradient elution HPLC UV analysis of a 100 $\mu\text{g mL}^{-1}$ of clobetasol propionate solution. The drug elution time for clobetasol propionate was 15.9 min.

A calibration curve was constructed using the developed HPLC method to enable the quantification of clobetasol propionate in samples following *in vitro* and *ex vivo* Franz cell studies and is presented in Figure 3-3.

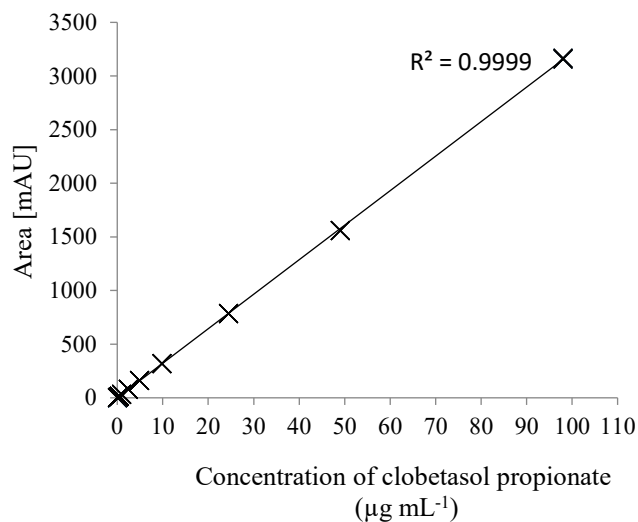


Figure 3-3: Calibration curve for clobetasol propionate standards obtained following analysis with the gradient elution HPLC UV method over the concentration range of 0.05 µg mL⁻¹ to 100 µg mL⁻¹. Data show the concentration of six replicate injections for each concentration and the correlation coefficient (R²).

The analytical methods were evaluated for linearity, precision and accuracy in accordance with the International Conference on Harmonization (ICH) guidelines (ICH, 2005). The linearity, accuracy and system suitability parameters for clobetasol propionate are summarised in Table 3-2. The analytical method implemented, as detailed in Section 3.2.2.1, was deemed to pass the specification criteria as defined by the ICH guidelines with suitable LOD and LOQ levels for the quantification of small amounts of clobetasol propionate in samples following the *in vitro* drug transport and *ex vivo* drug permeation and penetration experiments.

Table 3-2: Summary of the parameters determined for the ‘fitness for purpose’ of the gradient elution HPLC UV methods for quantification of clobetasol propionate in samples.

Parameter	Clobetasol propionate gradient method	Limit as per ICH guidelines
Linearity (R^2)	0.9999	> 0.999
Intra-day precision (% RSD) 5 $\mu\text{g mL}^{-1}$, 50 $\mu\text{g mL}^{-1}$ and 100 $\mu\text{g mL}^{-1}$	0.2, 0.4, 0.5	RSD \leq 2 %
Inter-day precision (% RSD) 5 $\mu\text{g mL}^{-1}$, 50 $\mu\text{g mL}^{-1}$ and 100 $\mu\text{g mL}^{-1}$	0.4, 0.5, 0.7	RSD \leq 2 %
Accuracy (%) 5 $\mu\text{g mL}^{-1}$, 50 $\mu\text{g mL}^{-1}$ and 100 $\mu\text{g mL}^{-1}$	98.7, 99.2, 99.7	100 \pm 2 %
Limit of detection ($\mu\text{g mL}^{-1}$)	0.10	Report result
Limit of quantification ($\mu\text{g mL}^{-1}$)	0.30	Report result
Tailing factor	1.0	< 2

3.3.2 Microscopic analysis of Dermovate cream and Dermovate ointment premixed with emollients

Light microscopy was employed to observed structural changes in the premixed TCS and emollient formulations when compared to Dermovate cream or Dermovate ointment alone. On analysis, no visible drug particles or crystals were observed on analysis of the TCSs alone and all TCS and emollient mixtures (images not shown).

3.3.3 Franz cell method development

The saturated solubility of clobetasol propionate in a range of receiver fluid systems was determined to ascertain whether sink conditions were likely to be maintained for the duration of the experimental period. Four receiver fluid systems of PBS and varying concentrations of ethanol were investigated. The saturated solubility of clobetasol propionate in the liquid excipients of Dermovate cream, Dermovate ointment and the six emollients were also determined. The findings are presented in Table 3-3.

Table 3-3: The solubility of clobetasol propionate in various solvent systems. Data are presented as mean of three replicates. The range is denoted in brackets.

Solvent	Solubility ($\mu\text{g mL}^{-1}$)
PBS	0*
PBS + 10 % ethanol	1.16 (0.23)
PBS + 20 % ethanol	12.30 (0.40)
PBS + 30 % ethanol	63.96 (3.48)
Water	0*
Liquid paraffin	1.45 (0.38)
Glycerol	79.94 (8.14)
Isopropyl myristate	1468.40 (64.61)
Sorbitan sesquioleate	5032.02 (4.03)
Propylene glycol	8426.42 (128.36)
Castor oil	10035.09 (321.73)

*No drug detected on analysis with the HPLC method

Clobetasol propionate was not soluble in PBS alone (below the limit of detection) and demonstrated greatest solubility in a system comprising of PBS and ethanol (70:30). Water is a listed excipient for Dermovate cream, Diprobace cream, Cetraben cream and Doublebase gel and demonstrated no solubilising capability for clobetasol propionate (below the limit of detection). Similarly, liquid paraffin, an excipient present in Diprobace cream, Diprobace ointment and Doublebase gel, solubilised clobetasol propionate to a limited extent. Castor oil, an excipient present in Hydromol Intensive cream and Aquadrate cream demonstrated the greatest solubilising capacity for clobetasol propionate. This was followed by propylene glycol, an excipient formulated in Dermovate cream and Dermovate ointment where the saturated solubility was $8426 \mu\text{g mL}^{-1}$. Sorbitan sesquioleate and isopropyl myristate were less capable of solubilising clobetasol propionate with a 1.7 and 5.7 fold decrease in solubilising capacity, respectively, when compared to propylene glycol.

To maintain sink conditions, it is generally accepted that the concentration of drug in the receiver fluid should not exceed 10 % of the drug's saturated solubility in the receiver fluid (Higuchi, 1960). As clobetasol propionate solubility in PBS and ethanol (70:30) was greater than the maximum amount anticipated to be delivered to the receiver fluid, this receiver fluid system was selected for further investigation.

A pilot drug transport study across silicone membrane and drug permeation study across human skin was conducted to ensure adequate drug solubility in PBS and ethanol (70:30) and maintenance of sink conditions over the entire experimental period. Furthermore, this study was conducted to inform the sampling protocol for the full scale *in vitro* and *ex vivo* experiments. Following the application of an infinite dose of Dermovate cream (0.05 % w/w clobetasol propionate) to silicone membrane and a finite dose of Dermovate cream to human scrotal skin, the receiver fluid was sampled at time points up to 26 h and 24 h respectively. Cumulative drug permeation was calculated and the resultant permeation profiles are presented Figure 3-4.

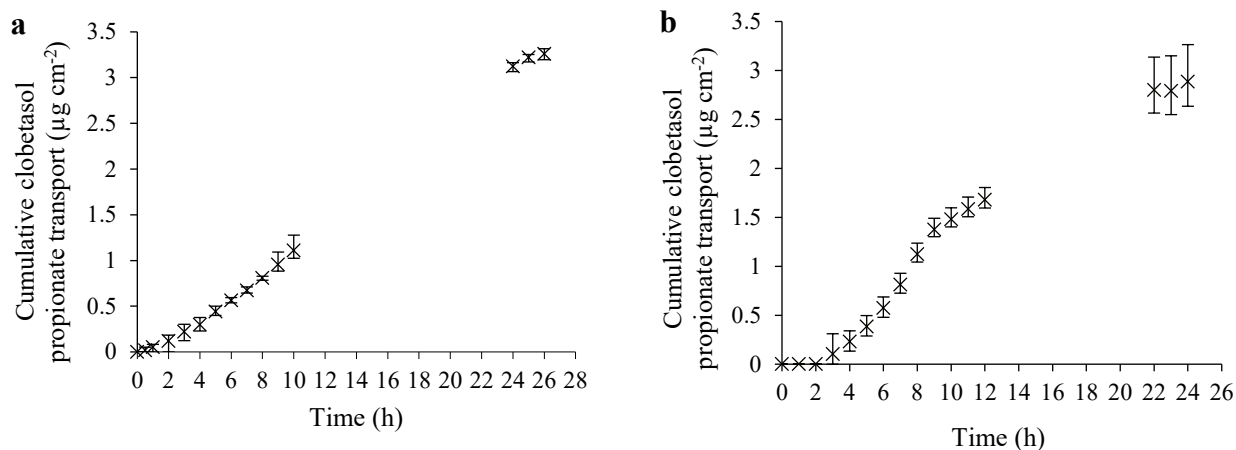


Figure 3-4: Permeation profiles show the cumulative amount of clobetasol propionate transport across (a) silicone membrane over 26 h following an infinite dose of Dermovate cream alone or (b) human skin over 24 h following a finite dose (10 µL) of Dermovate cream alone. The receiver fluid employed for both experiments was PBS and ethanol (70:30). Data are shown as the mean of three replicates; error bars denote the range of data points.

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In the pilot Franz cell experiment employing silicone as the model membrane, clobetasol propionate was detected in the receiver fluid from 30 min onwards (Figure 3-4a). The sampling time points were deemed appropriate to allow for drug transport to be calculated following the applications of both Dermovate cream and Dermovate ointment, given the number and spread of data points. On analysis, sink conditions were maintained for the duration of the experiment as the concentration of clobetasol propionate in the receiver fluid did not exceed 5 % of the drug saturated solubility in the receiver fluid, indicative of a PBS and ethanol mixture (70:30) being appropriate for future silicone membrane experiments.

In the pilot Franz cell experiment employing human skin, clobetasol propionate was detected in the receiver fluid from 3 h onwards. At 24 h, the average cumulative amount of drug in the receiver fluid was approximately 60 % of the applied dose. The concentration of clobetasol propionate in the receiver fluid did not exceed 1.2 % of the saturated solubility in the receiver fluid throughout the experiment, hence were indicative of sink conditions being maintained. Given the findings of the pilot experiment and the data from the solubility experiment (Figure 3-4b and Table 3-3 respectively), it appeared that a receiver fluid system with 20 % ethanol would be sufficient for the application of Dermovate cream alone. However, to allow for a potential increase in drug permeation when Dermovate cream was mixed with emollients, as observed from the work presented in Chapter 2, a solution containing PBS and ethanol (70:30) was selected as the receiver fluid thus ensuring that sink conditions would not be violated. Given the number and spread of data points, the sampling time points employed for the pilot *ex vivo* experiment were considered appropriate to allow for drug flux to be calculated following the application of Dermovate cream, thus was adopted for future *ex vivo* human skin Franz cell experiments.

During the *ex vivo* drug penetration study, drug distribution in the layers of the skin was investigated. To achieve this a method to ascertain the suitability of acetonitrile as an extraction solvent was developed. Drug recovery following the dosing of a spiked formulation to cotton buds, tape strips, epidermis and dermis was assessed. On analysis, two sequential extractions were sufficient to recover 98–103 % of clobetasol propionate from all matrices investigated (Figure 3-5), thus acetonitrile was an appropriate extraction solvent to employ.

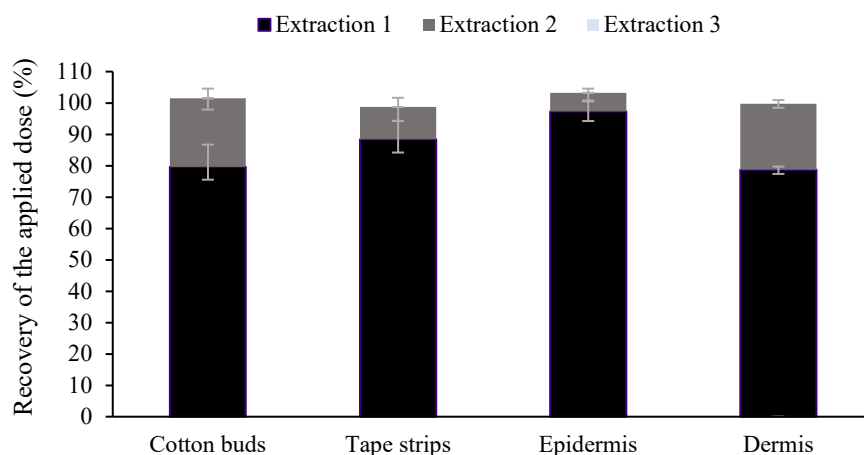


Figure 3-5: Graph shows the percent recovery of clobetasol propionate from matrices following a 10 μL dose of a standard solution of 1 mg mL^{-1} of the drug. Data are presented as the mean of three replicates for each extraction. Error bars denote the range of values.

To determine whether clobetasol propionate was stable in the receiver fluid system during the experimental period, the stability of clobetasol propionate in the receiver fluid system (PBS and ethanol; 70:30) was determined over 24 hours. To ascertain the potential for clobetasol propionate to degrade in the selected receiver fluid system or extraction solvent (acetonitrile) during storage, the stability of clobetasol propionate in three storage conditions was determined. Clobetasol propionate was stable in the receiver fluid system at the experimental temperature (37 °C) over the experimental period (24 h). Clobetasol propionate was also stable in both solutions, at 25 °C and 2–8 °C, for up to one month with percent drug concentrations within $\pm 2\%$ of freshly prepared standards at the same concentration (Table 3-4).

Table 3-4: Stability of clobetasol propionate in the receiver fluid and extraction solvent stored at 37 °C, 25 °C and 2 – 8 °C. Data are presented as the mean percent of the drug concentration compared to freshly prepared standards for three replicates. The range is denoted in brackets.

	Time (days)	PBS and ethanol (70:30)	Acetonitrile
	0	100	100
Stability at 37 °C (% of T = 0)	1	99.42 (0.27)	-
	7	98.94 (0.57)	99.78 (0.29)
Stability at 25 °C (% of T = 0)	14	99.52 (0.15)	99.54 (0.26)
	21	99.28 (0.42)	101.25 (0.14)
	28	98.99 (0.46)	100.89 (0.31)
Stability at 2 – 8 °C (% of T = 0)	7	100.08 (0.17)	99.76 (0.36)
	14	99.86 (0.15)	99.82 (1.39)
	21	99.23 (0.22)	98.99 (0.46)
	28	100.58 (0.31)	100.20 (0.25)

The sampling protocols, receiver fluid systems and drug extraction methods developed and employed for the pilot *in vitro* and *ex vivo* Franz cell experiments were selected for full scale Franz cell experiments with silicone membrane and human scrotal skin performed at 37 °C.

3.3.4 *In vitro* silicone membrane Franz cell studies: Dermovate cream and Dermovate ointment premixed with emollients

The findings from Chapter 2 are indicative of an altered partitioning of mometasone furoate from Elocon cream in the presence of particular emollients. To evaluate whether the trends observed were true for other commonly used TCSs, experiments with another, highly potent, TCS were conducted.

In vitro drug transport data were generated employing silicone membrane and an infinite dosing regimen to screen the effect of applying selected emollients in a premixed system with Dermovate cream on drug transport. The six selected emollients were as previously employed: Aquadrate cream, Cetraben cream, Diprobace cream, Diprobace ointment, Doublebase gel and Hydromol Intensive cream. The drug transport profiles are presented in Figure 3-6.

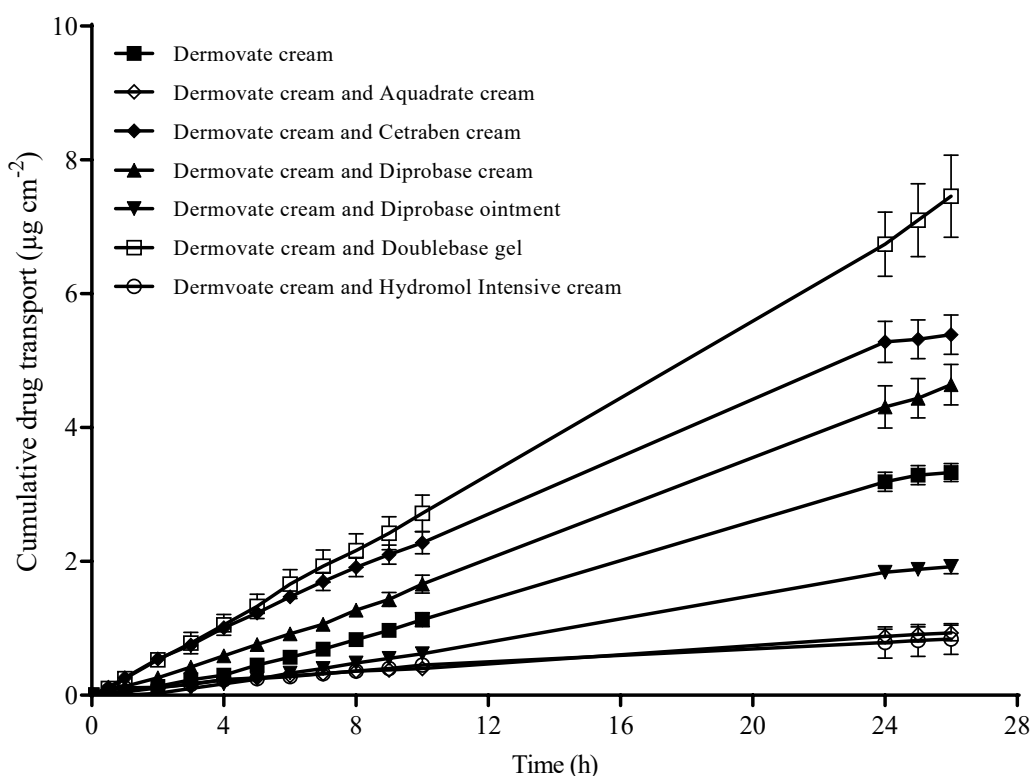


Figure 3-6: The cumulative amounts of clobetasol propionate ($\mu\text{g cm}^{-2}$) transport across silicone membrane over 26 h from Dermovate cream (■) when an infinite dose of the TCS was applied alone or in a pre-mixed system (1:1) with one of six emollients. The emollients were Aquadrate cream (◇), Cetraben cream (◆), Diprobace cream (▲), Diprobace ointment (▼), Doublebase gel (□) and Hydromol Intensive cream (○). Data shown as mean \pm SD (n=6).

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Drug transport across silicone membrane was evident at early time points following the application of all Dermovate cream and emollient formulations. Drug transport increased at a constant rate with time up to 10 h, after which the rate of transport slowed; this pattern was true for Dermovate cream applied alone and Dermovate cream applied with Aquadrate cream, CetraBen cream, Diprobase cream, Diprobase ointment and Hydromol Intensive cream. In the case of Doublebase gel, the rate of drug transport continued to increase beyond 10 h, reflective of the classic infinite dose profile. Drug transport was approximately linear for all formulations at early time points (between 3 h and 10 h), thus this range was selected to calculate the change in drug flux across all formulations.

Clobetasol propionate flux, calculated between 3 h and 10 h, was significantly altered in the presence of five emollients when compared to drug flux following the application of Dermovate cream alone ($p < 0.05$; Table 3-5). A 9.5 fold difference in drug flux was observed depending on the particular emollient applied with Dermovate cream, ranging from a 2.2 fold increase in the presence of Doublebase gel to a 4.3 fold decrease in the presence of Aquadrate cream when compared to Dermovate cream alone ($p < 0.05$).

Table 3-5: The mean drug flux calculated between 3 h and 10 h ($\bar{J}_{3-10\text{ h}}$) and total drug transport across silicone membrane from Dermovate cream alone or premixed formulations (Q_{24}). The emollients were Aquadrate cream, Cetraben cream, Diprobace cream, Diprobace ointment, Doublebase gel and Hydromol Intensive cream. Data shown as mean \pm SD (n=6). * denotes a significant difference when $\bar{J}_{3-10\text{ h}}$ or Q_{24} from Dermovate cream alone was compared, respectively, to $\bar{J}_{3-10\text{ h}}$ or Q_{24} from premixed formulations (one way ANOVA and Tukey's post hoc test; $p < 0.05$).

	$\bar{J}_{3-10\text{ h}}$ ($\mu\text{g cm}^{-2}\text{ h}^{-1}$)	Rank order (highest > lowest)	Q_{24} ($\mu\text{g cm}^{-2}$)	Rank order (highest > lowest)
Dermovate cream	0.13 \pm 0.02	4	3.19 \pm 0.14	4
Dermovate cream and Aquadrate cream	0.03 \pm 0.01*	7	0.88 \pm 0.11*	6
Dermovate cream and Diprobace cream	0.17 \pm 0.02	3	4.31 \pm 0.31*	3
Dermovate cream and Diprobace ointment	0.08 \pm 0.01*	5	1.84 \pm 0.09*	5
Dermovate cream and Cetraben cream	0.22 \pm 0.02*	2	5.28 \pm 0.31*	2
Dermovate cream and Hydromol Intensive	0.04 \pm 0.01*	6	0.79 \pm 0.23*	7
Dermovate cream and Doublebase gel	0.28 \pm 0.02*	1	6.74 \pm 0.48*	1

To observe whether this trend was evident when the same six emollients were premixed with the equivalent strength ointment TCS formulation, drug transport across silicone membrane following the applications of Dermovate ointment alone and Dermovate ointment premixed with an emollient was investigated. Drug transport across silicone membrane following the application of Dermovate ointment alone and Dermovate ointment premixed with emollients profiled in a similar pattern to Dermovate cream, with drug transport evident at early time points and increasing with time up to 8 hours then plateauing (Figure 3-7). Average drug flux was calculated from the approximately linear portion of the graph between 3 and 8 h ($R^2 > 0.96$) and the findings are presented in Table 3-6.

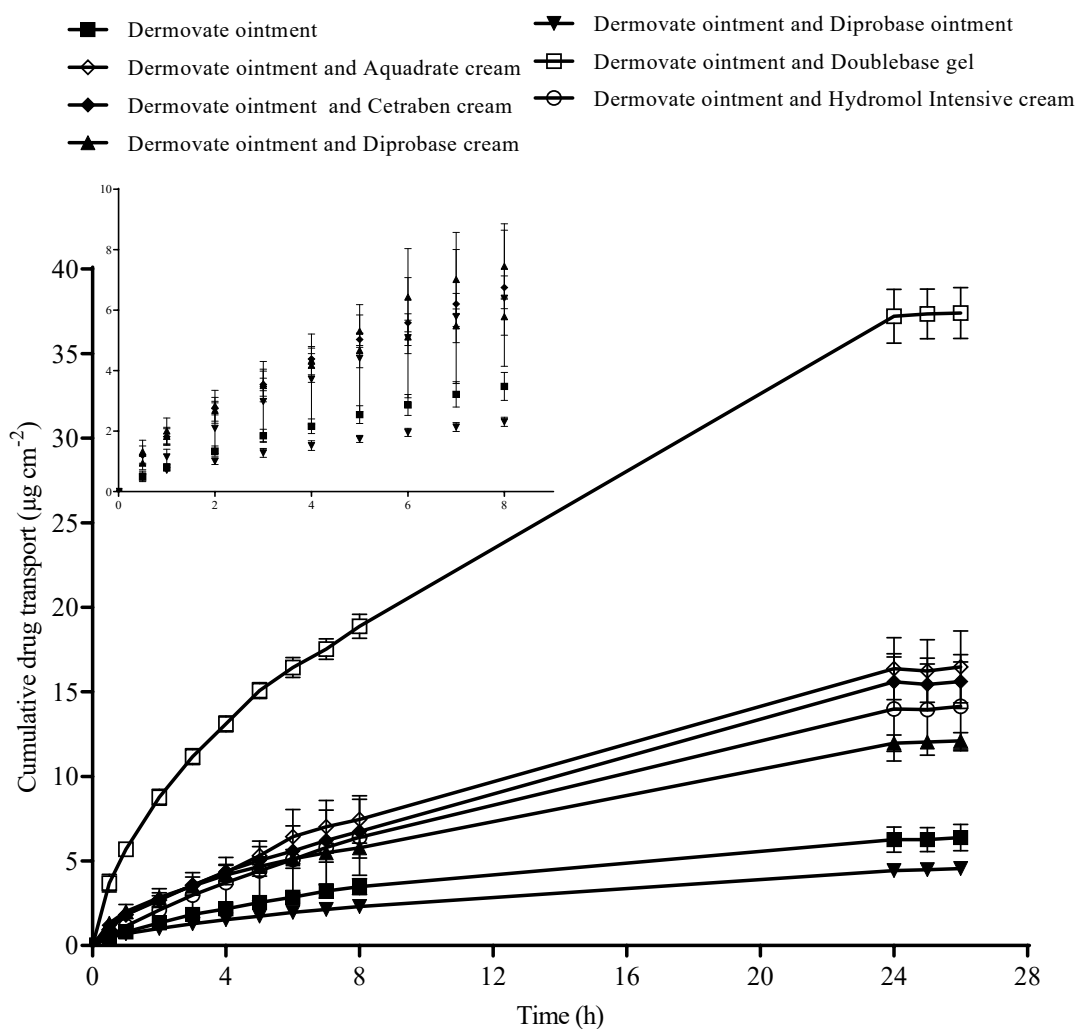


Figure 3-7: The cumulative amounts of clobetasol propionate ($\mu\text{g cm}^{-2}$) transport across silicone membrane over 26 h from Dermovate ointment (■) when an infinite dose of the TCS was applied alone or in a premixed system (1:1) with one of six emollients. The emollients were Aquadrate cream (◇), Cetraben cream (◆), Diprobate cream (▲), Diprobate ointment (▼), Doublebase gel (□) and Hydromol Intensive cream (○). Data shown as mean \pm SD (n=6).

Table 3-6: The mean drug flux calculated between 3h and 8 h ($\bar{J}_{3-8\text{ h}}$) and total drug transport across silicone membrane (Q_{24}) from Dermovate ointment alone or Dermovate ointment pre mixed with emollients. The emollients were Aquadrate cream, Cetraben cream, Diprobace cream, Diprobace ointment, Doublebase gel and Hydromol Intensive cream. Data shown as mean \pm SD (n=6). * denotes a significant difference when $\bar{J}_{3-8\text{ h}}$ or Q_{24} from Dermovate cream alone was compared, respectively, to $\bar{J}_{3-8\text{ h}}$ or Q_{24} from pre mixed formulations (one way ANOVA and Tukey's post hoc test; $p < 0.05$).

	$\bar{J}_{3-8\text{ h}}$ ($\mu\text{g cm}^{-2}\text{h}^{-1}$)	Rank order (highest > lowest)	Q_{24} ($\mu\text{g cm}^{-2}$)	Rank order (highest > lowest)
Dermovate ointment	0.25 \pm 0.09	6	6.26 \pm 0.75	6
Dermovate ointment and Aquadrate cream	0.62 \pm 0.11*	2	16.38 \pm 1.84*	2
Dermovate ointment and Diprobace cream	0.39 \pm 0.03*	5	11.97 \pm 0.48*	5
Dermovate ointment and Diprobace ointment	0.17 \pm 0.03	7	4.43 \pm 0.25	7
Dermovate ointment and Cetraben cream	0.61 \pm 0.11*	3	15.59 \pm 1.67*	3
Dermovate ointment and Hydromol Intensive	0.54 \pm 0.09*	4	13.99 \pm 3.07*	4
Dermovate ointment and Doublebase gel	1.28 \pm 0.12*	1	37.21 \pm 1.59*	1

Average clobetasol propionate flux was significantly altered in the presence of five emollients when compared to average drug flux following the application of Dermovate ointment alone ($p < 0.05$; Table 3-6). The change in drug flux ranged from a 5.1 fold increase in the presence of Doublebase gel to a 1.6 fold increase in the presence of Diprobace cream, when compared to Dermovate ointment alone. The emollient effect on drug flux across silicone membrane from Dermovate ointment was largely consistent with the trends observed with Dermovate cream (Figure 3-8). The greatest increase in drug flux from both TCS formulations was observed in the presence of Doublebase gel ($p < 0.05$).

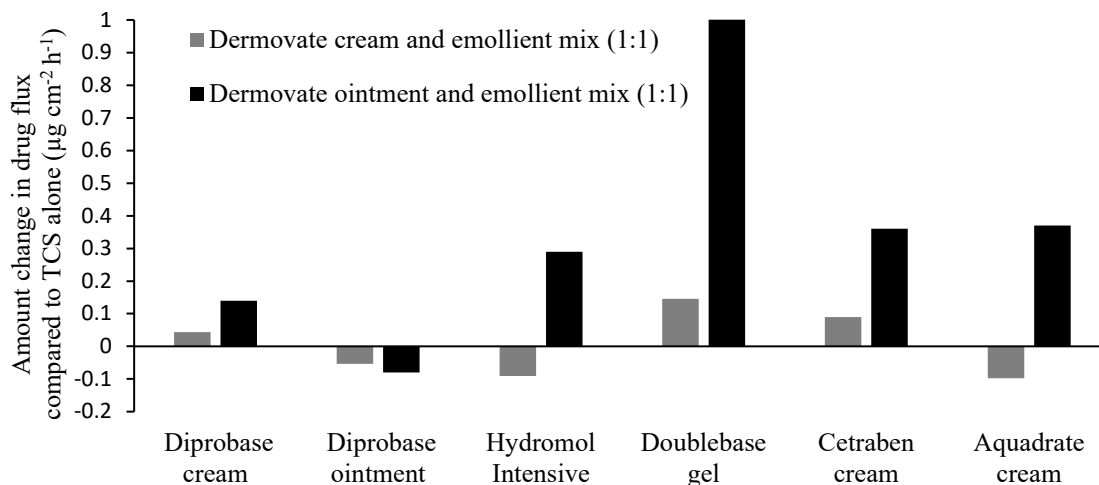


Figure 3-8: Amount change ($\mu\text{g cm}^{-2}\text{h}^{-1}$) in drug flux across silicone membrane from premixed TCS and emollient formulations relative to drug flux from Dermovate cream alone ($0.13 \pm 0.02 \mu\text{g cm}^{-2}\text{h}^{-1}$) or Dermovate ointment alone ($0.25 \pm 0.02 \mu\text{g cm}^{-2}\text{h}^{-1}$).

The significant increase in drug flux across silicone membrane when the TCS formulations were mixed with Doublebase gel could be partially attributable to the presence of IPM (15%) in the emollient formulation demonstrating solvent-membrane interactions, thus increasing drug flux from the formulation. IPM is a lipophilic compound that has demonstrated high sorption into silicone membrane. The work of Oliveira, Hadgraft and Lane (2012) proposed a model of solvent enhanced permeation whereby solvents such as IPM interact with silicone membrane promoting the partitioning of drugs into the membrane and causing these compounds to accumulate in ‘pools’ within the membrane. These findings are in agreement with the over estimation of drug flux across silicone membrane as a result of oil-membrane interactions observed with Elocon cream and Doublebase gel in Chapter 2 and the reported high degree of sorption and interaction of IPM with silicone membrane (Dias et al., 2007; McAuley et al., 2010).

Drug flux across silicone membrane significantly decreased when Diprobases ointment was premixed with Dermovate cream ($p < 0.05$) but was unchanged when mixed with Dermovate ointment ($p > 0.05$). On observation of the premixed TCS formulation with Diprobases ointment, the formulation appeared homogenous with no visible drug particles. Though it is difficult to definitively discern the mechanism of altered drug flux, it was observed during formulation preparation that the addition of Diprobases ointment to Dermovate cream appeared to increase the viscosity of the premixed

formulation compared to all other premixed formulations. An inverse relationship exists between the viscosity of a formulation and the diffusion co-efficient, as described by the Stokes–Einstein equation (Miller, 1924) and this factor, coupled to the decrease in drug concentration in the premixed formulation, may have reduced drug transport across silicone membrane when compared to Dermovate cream alone. Complementary drug release studies may be conducted to confirm this theory.

Mixing an emollient with a TCS formulation has the potential to significantly alter drug flux across silicone membrane, as observed from the *in vitro* data presented. The magnitude and direction in which drug transport is altered varies depending on the emollient employed and this data set offers a basis for understanding the potential formulation effects occurring when mixing TCSs and emollients. To discern the emollient effect on drug permeation across human skin, *ex vivo* drug permeation and penetration Franz cell experiments were conducted. Dermovate cream was selected as the TCS for further investigation given the greater prescribing frequency of the cream formulation, thus wider spread implications should drug delivery to the skin be altered significantly. Comparison of the *in vitro* and *ex vivo* data from Chapter 2 indicated that the drug transport trends observed across silicone membrane largely correlated with the drug permeation across skin data following the applications of premixed TCS and emollient systems. On this basis, the silicone membrane data allowed for the screening of a large number of emollients from which a smaller set could be selected for further investigation. Thus, it was considered suitable to carry forward three of the six selected emollient formulations for *ex vivo* investigations. Doublebase gel and Hydromol Intensive cream were selected as emollients with the potential to enhance and reduce drug delivery to the skin, respectively. Diprobase ointment (comprised of liquid paraffin and white soft paraffin) was selected to discern the effect on drug delivery to the skin when Dermovate cream was mixed with a relatively simple emollient ointment base.

3.3.5 *Ex vivo* human skin Franz cell study: Investigating the effect of the application protocol on drug delivery to the skin from Dermovate cream

The use of *in vitro* silicone membrane experiments has been shown to be beneficial in differentiating between the performance of different formulations containing the same drug (Oliveira et al., 2011; Raghavan et al., 2000; Watkinson et al., 2009), however such experiments are not always indicative of how the formulations may perform across human skin (Herkenne et al., 2007). To establish whether the potential changes observed across silicone membrane are of clinical relevance, an *ex vivo* Franz cell study was conducted employing human skin. A finite dose of the three selected emollients was applied in a premixed system with Dermovate cream and drug permeation and penetration to human skin was evaluated. Further to this, in order to elucidate the potential variations in drug delivery when products are applied according to multiple application protocols in a clinical setting, the effect of employing four currently recommended TCS and emollient application protocols on drug delivery to the skin was evaluated (altered order of, and time intervals between, product applications).

The absolute recovery of clobetasol propionate from Dermovate cream ranged 95–104 % of the applied dose for all experiments conducted, falling within the OECD defined acceptable criteria (OECD, 2019). The distribution of clobetasol propionate in all matrices and the receiver fluid when applied in a premixed system with either Diprobace ointment, Doublebase gel or Hydromol Intensive cream or when employing one of the four application protocols is presented in Figure 3-9. For each application protocol employed, drug distribution was largely evident in the following order of magnitude: unabsorbed drug > receiver fluid > dermis > epidermis. For clarity the total drug absorption (total drug content in the epidermis, dermis and receiver fluid) was used for statistical analysis as an indication of the change in absolute clobetasol propionate absorption in the presence of one of the three emollients when applied according to multiple application regimes (Figure 3-10).

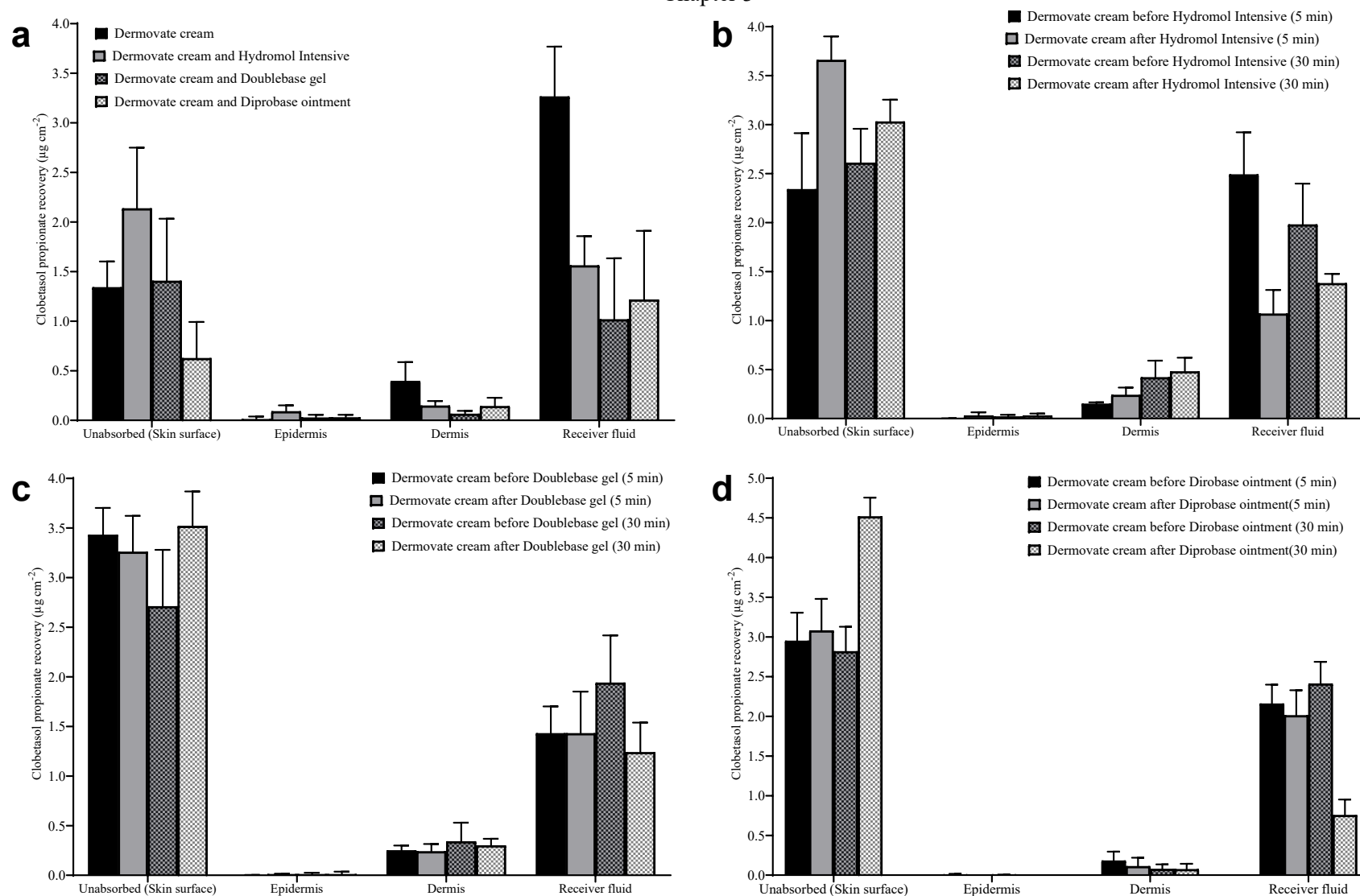


Figure 3-9: Drug distribution in the unabsorbed formulation, epidermis, dermis and receiver fluid ($\mu\text{g cm}^{-2}$) recovered from Dermovate cream following the finite application of (a) Dermovate cream alone and Dermovate cream premixed with an emollient, or Dermovate cream applied with (b) Hydromol Intensive, (c) Doublebase gel or (d) Diprobase ointment according to multiple application protocols. Data are shown as mean + SD (n=6).

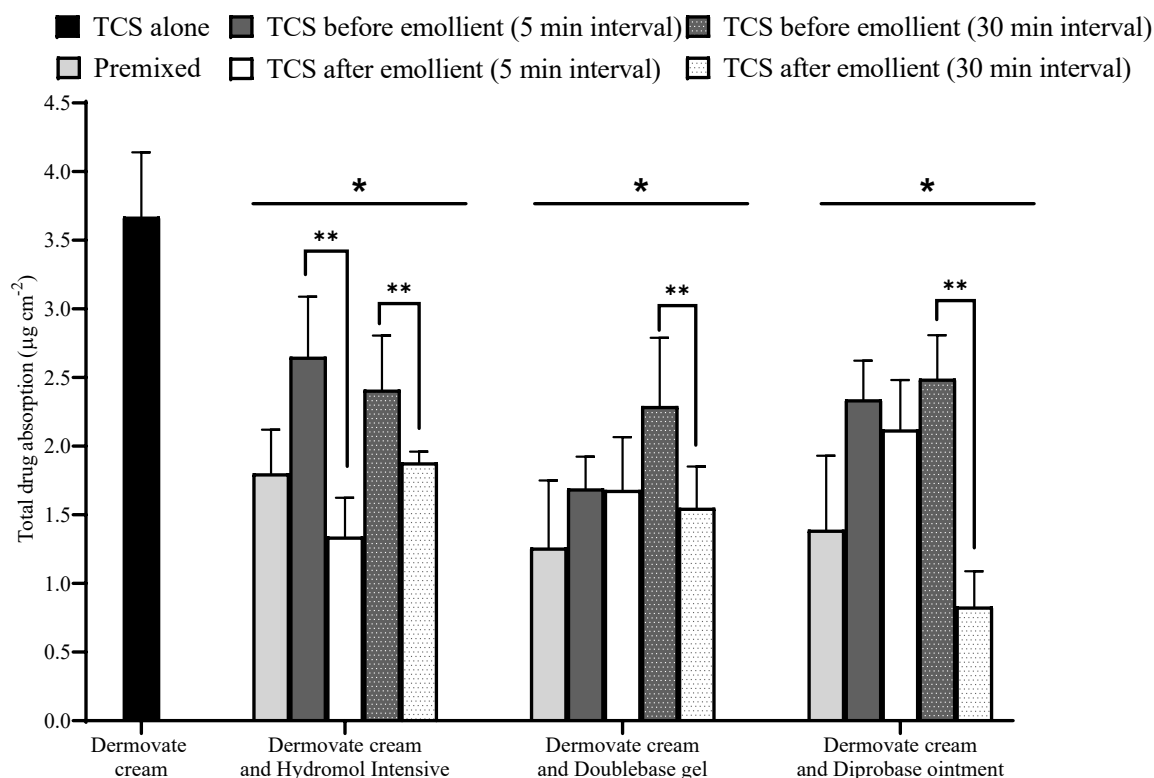


Figure 3-10: Total clobetasol propionate delivered to the skin (epidermis, dermis and receiver fluid; $\mu\text{g cm}^{-2}$) after 24 h following the application of a finite dose of: Dermovate cream alone, Dermovate cream premixed with an emollient, Dermovate cream before an emollient (with a 5 min or 30 min interval) or Dermovate cream after an emollient (with a 5 min or 30 min interval). The emollients were Doublebase gel, Diprobace ointment or Hydromol Intensive. Data are shown as the mean + SD ($n=6$). * denotes a significant difference when compared to the total drug recovered from Dermovate cream alone. ** denotes a significant difference when the application of the TCS before the emollient was compared to the application of the TCS after the emollient, for a particular time interval and emollient (Kruskal-Wallis and Mann-Whitney test; $p < 0.05$).

In all cases, total drug delivery to the skin significantly decreased following the application of an emollient and Dermovate cream compared to the application of Dermovate cream alone ($p < 0.05$; Figure 3-10). The decrease in drug delivery to the skin ranged from 1.4 fold when Dermovate cream was applied five minutes before Hydromol Intensive cream to 4.4 fold when Dermovate cream was applied thirty minutes after Diprobace ointment. Furthermore, in several cases the order in which products were applied impacted the magnitude to which drug delivery to the skin was reduced when compared to Dermovate cream alone. As a general trend, applying Dermovate cream after an emollient resulted in significantly less drug delivery to the skin compared to the application of

Dermovate cream before the emollient when employing the same time interval between product applications. The decrease in drug delivery ranged from 1.3 fold when comparing Dermovate cream applied thirty minutes before and after Hydromol Intensive cream, to 3 fold when comparing Dermovate cream applied thirty minutes before and after Diprobace ointment ($p < 0.05$). This trend held for all application regimes with the exception of Dermovate cream applied five minutes before Doublebase gel or Diprobace ointment compared respectively to Dermovate cream applied five minutes after Doublebase gel or Diprobace ointment ($p > 0.05$).

The data suggest that (i) applying an emollient at similar times to Dermovate cream significantly reduces drug delivery to the skin, (ii) applying the TCS after an emollient can cause significantly less drug delivery to the skin compared to the reverse order of application and (iii) leaving time intervals up to thirty minutes between product applications may not be sufficient to mitigate emollient effects on TCS drug delivery to the skin. These findings contradict current clinical recommendations that an emollient should be applied 15 – 30 minutes before the application of a TCS (National Institute for Health and Care Excellence, 2018b; Primary Care Dermatology Society, 2019) or the opinion that that the application of emollients and TCSs should be separated by thirty minutes, with the order of product application being unimportant (Moncrieff et al., 2013). To further elucidate the mechanistic effects occurring when the various application protocols were employed, drug permeation across human skin was evaluated. The cumulative amount of clobetasol propionate permeated across human skin over 24 h following the application of Dermovate cream alone and Dermovate cream with an emollient according to the application protocols is presented in Figure 3-11.

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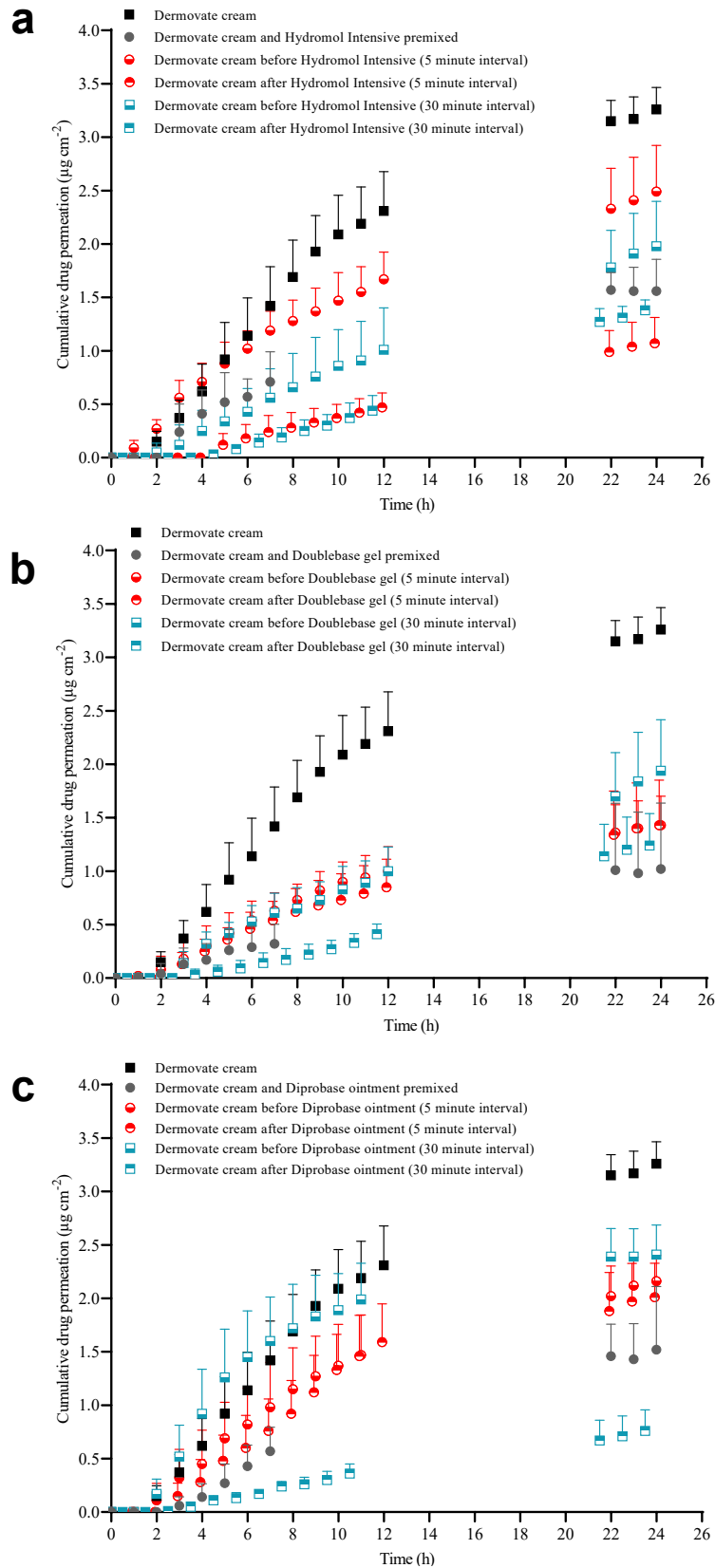


Figure 3-11: The cumulative amount of clobetasol propionate ($\mu\text{g cm}^{-2}$) permeated across human scrotal skin from Dermovate cream when a finite dose of Dermovate cream was applied alone (■), in a premixed system (●), 5 minutes before an emollient (◐), 5 minutes after an emollient (◑), 30 minutes before an emollient (◒) or 30 minutes after an emollient (◓). The emollients are (a) Hydromol Intensive cream, (b) Doublebase gel and (c) Diprobace ointment. Data are shown as mean + SD (n= 6).

Drug permeation across human skin was evident from early timepoints (2 h onwards) for all application protocols with drug concentration increasing with time. Total drug permeation (Q_{24}) was greatest following the application of Dermovate cream alone, with drug permeation increasing with time up to 12 h, then appearing to plateau at the later timepoints. In total, 65 % of the applied dose permeated the skin over 24 h following the application of Dermovate cream alone. In comparison, applying Dermovate cream in combination with either Diprobace ointment, Doublebase gel or Hydromol Intensive cream resulted in significantly lower amounts of total drug permeation at 24 h compared to Dermovate cream alone with considerable variations in the permeation profiles being observed. The percent of the applied dose permeating human skin after 24 h ranged from 14 % when Dermovate cream was applied thirty minutes after Diprobace ointment to 50 % when Dermovate cream was applied five minutes before Hydromol Intensive cream. In some cases, drug permeation profiled in a similar manner to Dermovate cream alone, plateauing at later time points as observed when Dermovate cream was applied five minutes before Diprobace ointment (Figure 3-11 C). In other cases, drug permeation appeared to be continuing at 24 h, as observed when Dermovate cream was applied five minutes before Hydromol Intensive cream (Figure 3-11 A). Whilst the collection of data beyond 24 h may provide insight into whether permeation rates for particular TCS and emollient combinations eventually match that of Dermovate cream, to more closely mimic the once daily clinical application period for a TCS it was considered practical to set the experimental duration to 24 h. The permeation profiles presented in Figure 3-11 were modelled using Equation 2-4 (Chapter 2) to obtain the normalised apparent partition and diffusion co-efficient for clobetasol propionate across human skin. The model fittings were similar to the representative modelled profile presented in Chapter 2 (Figure 2-11) and are therefore not presented again. The estimated permeation parameters are presented in Table 3-7.

Table 3-7: Estimated apparent diffusion co-efficient (D/h^2), lag time (L_t), apparent partition co-efficient (Kh), total drug permeation at 24 h (Q_{24}) and pseudo steady state drug flux (J_{ss}) obtained from the nonlinear modelling of the permeation data presented in Figure 3-11. Data are shown as mean \pm SD ($n = 6$). * Denotes a significant difference when D/h^2 , L_t , Kh , Q_{24} and J_{ss} values were compared to the respective permeation parameters for Dermovate cream alone. ** Denotes a significant difference when J_{ss} values were compared to the premixed formulation within the same emollient group ((Kruskal-Wallis and Mann-Whitney test; $p < 0.05$)).

Time interval	Product 1	Product 2	D/h^2 (cm)	L_t (h)	Kh (h^{-1})	Q_{24} (μgcm^{-2})	J_{ss} ($\mu\text{gcm}^{-2}\text{h}^{-1}$)
	Dermovate cream		7.26E-02 $\pm 1.23\text{E-}02$	2.36 ± 0.36	1.46E-02 $\pm 1.64\text{E-}03$	3.26 ± 0.21	5.40E-05 $\pm 1.55\text{E-}05$
	Premixed Dermovate cream and Hydromol Intensive		1.54E-01* $\pm 3.19\text{E-}02$	1.13* ± 0.22	2.60E-03* $\pm 1.34\text{E-}04$	1.56 * ± 0.30	1.01E-05* $\pm 2.42\text{E-}06$
5 minutes	Dermovate cream	Hydromol Intensive	1.44E-01* $\pm 2.82\text{E-}02$	1.20* ± 0.25	6.02E-03* $\pm 4.45\text{E-}04$	2.49* ± 0.43	4.34E-05** $\pm 8.55\text{E-}06$
	Hydromol Intensive	Dermovate cream	3.11E-02* $\pm 5.56\text{E-}03$	5.57* ± 1.20	6.31E-03* $\pm 3.63\text{E-}04$	1.07* ± 0.24	9.75E-06* $\pm 1.52\text{E-}06$
30 minutes	Dermovate cream	Hydromol Intensive	8.28E-02 $\pm 2.01\text{E-}02$	2.15 ± 0.56	3.17E-03* $\pm 3.02\text{E-}04$	1.98* ± 0.42	1.34E-05* $\pm 4.29\text{E-}06$
	Hydromol Intensive	Dermovate cream	1.93E-02* $\pm 2.68\text{E-}03$	8.81* ± 1.22	1.66E-02 $\pm 1.39\text{E-}03$	1.38* ± 0.10	1.60E-05* $\pm 2.61\text{E-}06$
	Premixed Dermovate cream and Doublebase gel		8.76E-02 $\pm 3.64\text{E-}02$	2.48 ± 1.47	2.66E-03* $\pm 3.41\text{E-}04$	1.02 * ± 0.62	5.86E-06* $\pm 2.78\text{E-}06$
5 minutes	Dermovate cream	Doublebase gel	7.18E-02 $\pm 1.59\text{E-}02$	2.44 ± 0.57	3.07E-03* $\pm 1.45\text{E-}04$	1.43* ± 0.27	1.11E-05* $\pm 2.81\text{E-}06$
	Doublebase gel	Dermovate cream	9.30E-02 $\pm 2.90\text{E-}02$	1.99 ± 0.63	2.10E-03* $\pm 1.81\text{E-}04$	1.43* ± 0.42	9.98E-06* $\pm 3.80\text{E-}06$
30 minutes	Dermovate cream	Doublebase gel	8.17E-02 $\pm 1.48\text{E-}02$	2.11 ± 0.37	3.05E-03* $\pm 1.73\text{E-}04$	1.94* ± 0.48	1.25E-05** $\pm 2.53\text{E-}06$
	Doublebase gel	Dermovate cream	1.74E-02* $\pm 1.97\text{E-}03$	9.72* ± 1.16	1.62E-02 $\pm 6.60\text{E-}04$	1.24* ± 0.30	1.41E-05** $\pm 1.95\text{E-}06$

Table 3-7 (cont.): Estimated apparent diffusion co-efficient (D/h^2), lag time (L_t), apparent partition co-efficient (Kh), total drug permeation at 24 h (Q_{24}) and pseudo steady state drug flux (J_{ss}) obtained from the nonlinear modelling of the permeation data presented in Figure 3-11. Data are shown as mean \pm SD (n = 6). * Denotes a significant difference when D/h^2 , L_t , Kh , Q_{24} and J_{ss} values were compared to the respective permeation parameters for Dermovate cream alone. ** Denotes a significant difference when J_{ss} values were compared to the premixed formulation within the same emollient group ((Kruskal-Wallis and Mann-Whitney test; $p < 0.05$)).

Time interval	Product 1	Product 2	D/h^2 (cm)	L_t (h)	Kh (h ⁻¹)	Q_{24} (μgcm^{-2})	J_{ss} ($\mu\text{gcm}^{-2}\text{h}^{-1}$)
Premixed Dermovate cream and Diprobases ointment			7.38E-02 \pm 2.82E-02	2.86 \pm 1.92	7.23E-03* \pm 1.20E-03	1.55* \pm 0.60	1.39E-05* \pm 6.70E-06
5 minutes	Dermovate cream	Diprobases ointment	1.01E-01* \pm 1.04E-02	1.67* \pm 0.16	4.76E-03* \pm 1.03E-03	2.16* \pm 0.18	2.42E-05* \pm 5.98E-06
	Diprobases ointment	Dermovate cream	9.10E-02* \pm 1.44E-02	1.88* \pm 0.29	5.53E-03* \pm 1.91E-04	2.33* \pm 0.24	2.53E-05* \pm 4.87E-06
30 minutes	Dermovate cream	Diprobases ointment	1.94E-01* \pm 4.12E-02	0.90* \pm 0.19	2.42E-03* \pm 1.64E-04	0.93* \pm 0.17	2.33E-05* \pm 4.22E-06
	Diprobases ointment	Dermovate cream	4.46E-02* \pm 6.16E-03	3.82* \pm 0.57	2.03E-03* \pm 1.82E-04	0.13* \pm 0.04	4.52E-06* \pm 7.93E-07

When defining the parameters for the modelling of the permeation profiles, the model was fitted to the data set under the assumption that the drug concentration of Dermovate cream was unaffected when applied before or after an emollient (with a five or thirty minute interval). In contrast, when Dermovate cream was applied in a premixed system with an emollient, to reflect a simple 1:1 dilution of the TCS, the drug concentration was defined as half that of Dermovate cream. This approach enabled an evaluation of whether Dermovate cream applied before or after an emollient resulted in reduced drug flux similar to that of a diluted TCS and emollient preparation, or whether employing particular application protocols mitigated or further altered drug permeation across human skin when compared to the respective premixed TCS and emollient formulation.

The application of a premixed system incorporating Diprobase ointment or Hydromol Intensive cream into Dermovate cream resulted in a significant decrease in drug flux across human skin by 3.9 fold and 5.6 fold, respectively when compared to Dermovate cream alone ($p < 0.05$). This pattern of reduced drug flux across human skin was true for Diprobase ointment and Hydromol Intensive cream regardless of the application protocol employed when compared to the application of Dermovate cream alone and mirrored the significant decrease in total drug delivery to the skin when compared to Dermovate cream alone ($p < 0.05$; Table 3-7 and Figure 3-10).

The incorporation of Doublebase gel to Dermovate cream did not exhibit a similar trend to that observed across silicone membrane, with a significant 5.5 fold decrease in drug flux observed across human skin compared to a significant 2.2 fold increase in drug flux across silicone membrane, when compared to Dermovate cream alone ($p < 0.05$). Employing any of the application protocols for Dermovate cream and Doublebase gel also significantly decreased drug flux across skin, when compared to drug flux from Dermovate cream alone ($p < 0.05$; Table 3-7). This trend is consistent with the findings of Chapter 2 when Doublebase gel was incorporated into Elocon cream and was attributed to the solvent-membrane effects of IPM in Doublebase gel overestimating drug transport across silicone membrane. The findings were in further agreement with published reports of increased drug flux across silicone membrane from a range of permeants delivered from an IPM vehicle (Cross et al., 2001; Najib et al., 2016; Oliveira et al., 2012a; Oliveira et al., 2012b). The role

of IPM as a penetration enhancer across skin has been well investigated, with studies reporting enhanced permeation of piroxicam (Santoyo et al., 1995), nicorandil (Sato et al., 1988) and estradiol (Goldberg-Cettina et al., 1995) from formulations containing the fatty acid ester. However, IPM has also demonstrated greater enhancement effects on hydrophilic compounds compared to lipophilic compounds (Zhang et al., 2013), thus when Doublebase gel was applied with lipophilic drug clobetasol propionate ($\log P = 3.0$), the enhancement effects may have been masked by the simultaneous TCS dilution effect.

The *in vitro* silicone membrane data demonstrated a good correlation with drug flux across skin for Diprobace cream and Diprobace ointment, suggesting this model is capable of discriminating emollient effects on drug thermodynamic activity. However, limitations were observed with Doublebase gel, where it was postulated that IPM interaction with the membrane resulted in greater drug transport from the TCS across silicone compared to IPM effects on the stratum corneum, at the same concentration. Thus, it is particularly important that extrapolation of the findings should be made with support of *ex vivo* data in cases where listed excipients are known to interact with the membrane.

On analysis of the premixed formulations across emollient groups, drug flux from all the premixed TCS and emollient formulations decreased to similar extents when compared to drug flux from Dermovate cream alone ($p > 0.05$; Table 3-7 and Figure 3-12). This trend was consistent with the earlier reported significant decrease in total drug delivery to the skin to similar extents following application of the premixed formulations, with a 2.0 – 2.9 fold decrease in drug penetration observed when compared to the application of Dermovate cream alone ($p < 0.05$; Figure 3-10). Modelling of the permeation data attributed the reduced drug flux to a 2.0 – 5.6 fold significant decrease in the apparent partition co-efficient when premixed formulations were compared to Dermovate cream alone ($p < 0.05$). Contrary to the trends observed with Elocon cream where the application of a premixed system to the skin increased or decreased drug delivery to an emollient specific extent, this data set suggests comparable emollient effects on drug delivery to the skin when Dermovate cream

was applied in a premixed system with Hydromol Intensive cream, Doublebase gel and Diprobace ointment ($p > 0.05$; Figure 3-12).

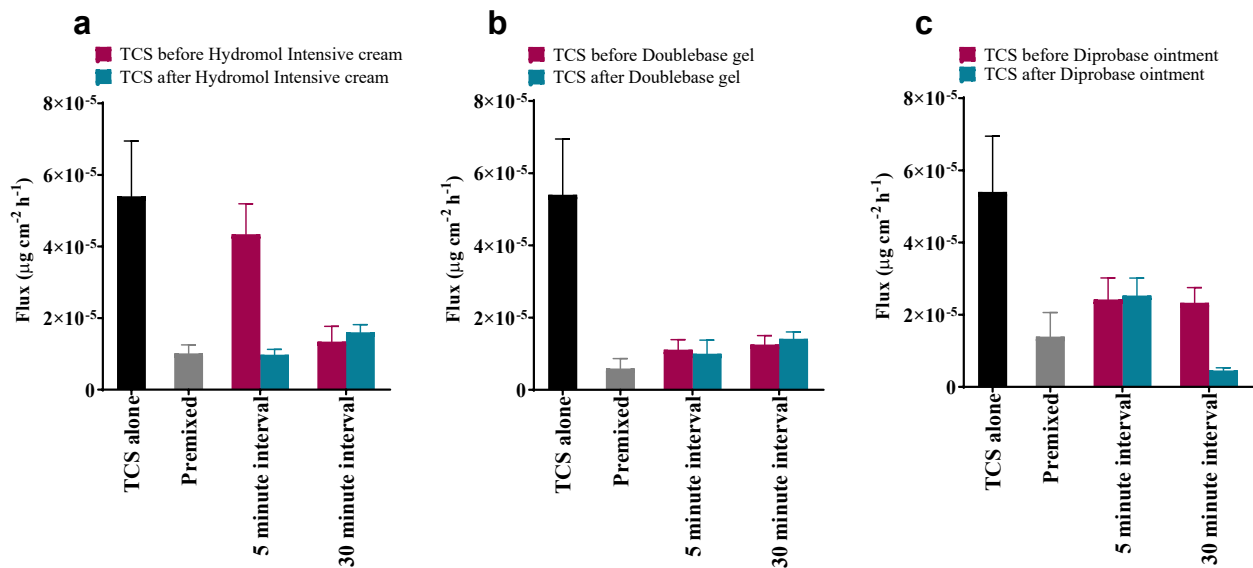


Figure 3-12: The calculated drug flux (J_{ss} from Table 3-7) from Dermovate cream when a finite dose of Dermovate cream was applied alone, in a premixed system, before an emollient or after an emollient. The emollients were (a) Hydromol Intensive cream, (b) Doublebase gel and (c) Diprobace ointment. Data are shown as the mean \pm SD ($n=6$).

A possible explanation for the observed trend is the dilution of the TCS when premixed with an emollient and consequential reduction in drug thermodynamic activity when compared to Dermovate cream alone. Incorporating Diprobace cream, Doublebase gel and Hydromol Intensive cream with Dermovate cream resulted in the inclusion of excipients with solubilising capabilities for clobetasol propionate. In the presence of drug particles held in suspension within the TCS formulation, thus at maximum thermodynamic activity, it may be expected that incorporating solubilising agents would either (a) maintain maximum thermodynamic activity however with a shorter duration of steady state flux (if drug particles are still present when the formulations are mixed), or (b) reduce thermodynamic activity (if all suspended drug particles are solubilised). However, in the absence of visible drug particles following microscopic analysis of Dermovate cream and the absence of drug particles or crystals when Dermovate cream was premixed with Diprobace cream, Doublebase gel or Hydromol Intensive cream, the data suggest that clobetasol propionate is present in the TCS formulation at a sub-saturated concentration. In further support of this theory, Harding et al. (1985)

reported a formulation composition for Dermovate cream of 47.5 % propylene glycol, 22.5 % waxes and approximately 30 % water. Considering the relatively high solubilising capacity of propylene glycol for clobetasol propionate (8.4 mg mL^{-1} ; Table 3-3), it is reasonable to suggest that the drug is present in a dissolved state in the TCS formulation. Reducing the degree of drug saturation in a formulation has been shown to proportionally decrease drug transport across silicone membrane and human skin (Davis & Hadgraft, 1991; Leveque et al., 2006). In the context of this work, incorporating excipients such as castor oil and isopropyl myristate with relatively high solubilising capabilities for clobetasol propionate is likely to reduce the degree of saturation thus thermodynamic activity of the drug in the premixed formulation to varying extents when compared to Dermovate cream alone, and consequently overall drug delivery to the skin, as observed. Conversely, inclusion of emollient excipients with poor solubilising capacity for clobetasol propionate, such as liquid paraffin or water, can act in an anti-solvent capacity and increase thermodynamic activity of the drug in the formulation. It is then important to consider that a 1:1 dilution of Dermovate cream with different emollients introduces non-identical solvents to the TCS at different ratios, resulting in variability in the degree of drug saturation in the formulation. Thus the degree to which drug thermodynamic activity in the formulation is altered is likely to be a conflated effect of the number and concentrations of (i) solubilising excipients and (ii) excipients with antisolvent effects incorporated from the emollient formulation to Dermovate cream.

The trend in significantly reduced drug delivery to the skin when Dermovate cream was applied after the emollient compared to Dermovate cream applied before the emollient was mirrored with drug flux when Dermovate cream was applied at five minute intervals with Hydromol Intensive cream and at thirty minute intervals with Diprobase ointment ($p < 0.05$). In all other cases, altering the order of application of the products did not impact on the extent to which flux was altered when compared to Dermovate cream alone. However, a general trend in significantly less total drug permeation (Q_{24}) was observed when Dermovate cream was applied thirty minutes after an emollient compared to thirty minutes before the emollient (Figure 3-11). Altering the order of application and time interval between product applications instead resulted in statistically similar flux values to the premixed

formulations in most cases ($p > 0.05$). The data sets for the altered order of, and time interval between, product applications were modelled with an assumption that the drug concentration following the application of both products was equivalent to that of Dermovate cream alone. As they exhibited similar trends to the respective premixed formulations, modelled on the assumption that the drug concentration was halved, it suggests that applying the products at similar times reduced the drug concentration on the surface of the skin, or partitioning out of the formulation in a similar manner to the respective premixed formulation. Indeed, these changes correlated with the extent to which the apparent partition co-efficient was reduced when compared to Dermovate cream alone and ranged from 2.3 fold when Dermovate cream was applied five minutes after Hydromol Intensive cream to 7.2 fold when Dermovate cream was applied thirty minutes after Diprobace ointment ($p < 0.05$). No overall trends in the apparent diffusion co-efficient were observed when comparing the application protocols within or across emollient groups with the apparent diffusion co-efficient ranging from a 2.7 fold increase when Dermovate cream was applied thirty minutes before Diprobace ointment to a 4.2 fold decrease when Dermovate cream was applied thirty minutes after Doublebase gel.

When Dermovate cream was applied five minutes before Hydromol Intensive cream, despite a significant decrease in total drug delivery to the skin when compared to Dermovate cream alone, the trend in drug flux appeared inconsistent with all other application protocols and was similar to Dermovate cream alone ($p > 0.05$; Figure 3-11). Nonlinear modelling of the permeation data attributed this to a significant 2 fold increase in the apparent diffusion parameter compared to Dermovate cream alone (D/h^2 ; Table 3-7). Though it is difficult to elucidate the exact reasoning for this, a similar trend in an increased apparent diffusion parameter was observed following the application of the premixed Dermovate cream and Hydromol Intensive cream formulation and when Dermovate cream was applied five minutes, or thirty minutes, before Diprobace ointment. It is possible that applying Dermovate cream at the same time, or shortly before, these products may result in an occlusive effect on drug permeation at the early time points. Occlusion by the emollient can enhance permeation through two means: (i) prevention of the evaporation of volatile solvents in

the formulation(s), thus maintaining the initial excipient compositions of the formulation(s) and (ii) enhanced hydration of the stratum corneum by reducing TEWL (Zhai & Maibach, 2001). The former mechanism would be expected to result in comparable, or greater, drug delivery to the skin compared to Dermovate cream alone, a trend not observed. Through the latter mechanism, increasing the stratum corneum water content has the potential to, in turn, promote diffusion of drug located close to the formulation and skin interface through the skin (Menon et al., 2003). One caveat for this theory is that skin tissue in Franz cell experiments are generally accepted to be over hydrated even without occlusion, in part as a result of exposure to larger volumes of receiver fluid than physiologically relevant (Levintova et al., 2011). However, occlusive effects on *ex vivo* human skin in Franz cells have been reported, Treffel et al. (1992) for example found that occlusion of the donor chamber increased drug permeation across human abdominal skin by 1.6 fold when compared to drug permeation from the same formulation under unoccluded conditions. Ultimately, though drug flux was unaltered when Dermovate cream was applied five minutes before Hydromol Intensive cream, drug partitioning from the formulation and total drug delivery was still significantly reduced when compared to Dermovate cream alone. It is possible that applying the products at similar times resulted in the mixing of the formulations on the surface of the skin. In this scenario, a thin residual layer of Dermovate cream is likely to have remained at the skin and formulation interface with drug permeating from this layer under occlusive conditions (thus increasing D/h^2), created by the new ‘mixed’ formulation above this layer. To feasibly interpret the data set, the Laplace transformation solution to Fick’s second law was employed assuming drug permeation across human skin was controlled by diffusion across the stratum corneum. Whilst this holds, the potential creation of a new ‘premixed’ layer on the surface of the skin is likely to introduce an additional partitioning step which consequently reduces partitioning into and diffusion across the stratum corneum in a variable manner (Figure 3-13 (A)).

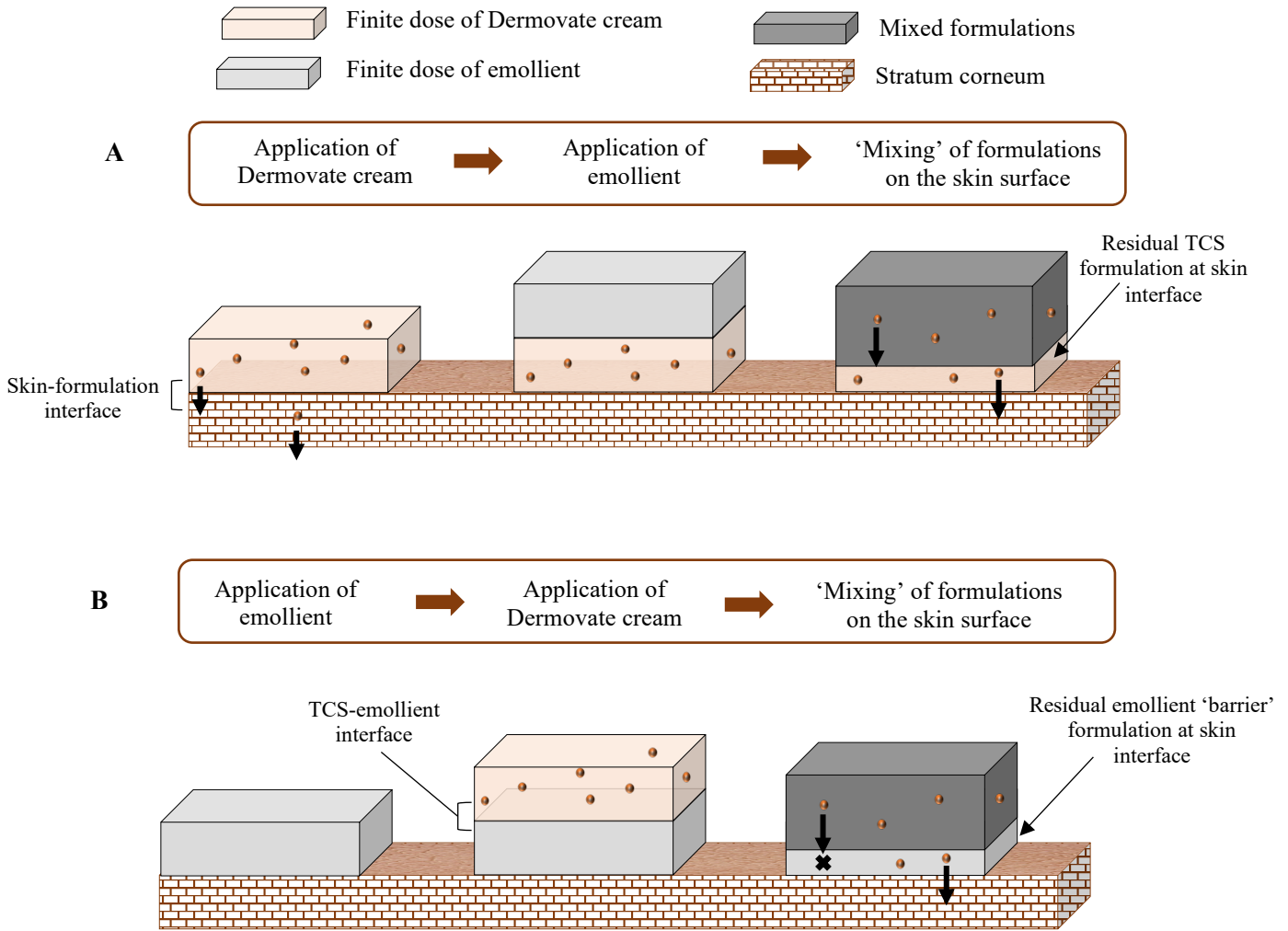


Figure 3-13: Schematic of (A) the potential 'occlusive' effect of applying an emollient to the skin surface after Dermovate cream and (B) the impact on drug diffusion towards the skin when Diprobase ointment is applied to the skin surface before Dermovate cream, effectively creating an additional barrier to drug permeation.

In the reverse scenario, applying Dermovate cream five minutes or thirty minutes after the emollient largely reduced the apparent diffusion co-efficient when compared to Dermovate cream alone ($p < 0.05$; Table 3-7). The impact of this was most clearly exemplified when Dermovate cream was applied thirty minutes after Diprobase ointment resulting in the lowest drug flux across human skin and the least amount of drug delivered to the skin in total (Figure 3-12 and Figure 3-10, respectively). As with all application protocols, it is likely that a thin film of the first product applied remained at the skin - formulation interface following application of the second product and creation of the new 'mixed' layer above this. It is possible that the emollient formulation reduced drug flux by creating

an additional barrier to drug permeation thus reducing drug partitioning and diffusion into the stratum corneum. In addition, should the residual emollient layer formed on the surface of the skin comprise of excipients into which clobetasol propionate would not favourably partition such as liquid paraffin in Diprobase ointment, this is likely to further reduce drug partitioning from the new 'mixed' layer towards the skin (Figure 3-13 B). A similar effect, though to a lesser extent, was observed on analysis of total drug delivery to the skin following the application of Dermovate cream five minutes after Diprobase ointment and it is possible that a thinner residual 'barrier' film was formed at the skin surface because of the shorter time interval between product applications.

In addition to the impact on drug partitioning out of the formulation when emollients are applied to the skin surface at similar times to the TCS, it is also valuable to consider the impact on the thermodynamic activity of penetration enhancing excipients. It has been well established that the physico-chemical properties of the drug and vehicle can influence the diffusion of a drug compound through the vehicle and partitioning out of the vehicle (Higuchi, 1960; Katz & Poulsen, 1972). For example, propylene glycol, an excipient present within Dermovate cream, is often employed as a co-solvent in formulations to alter both drug solubility in the vehicle and partitioning into the skin; the latter of which may be achieved by increasing drug solubility within the skin (Arellano et al., 1999; Schneider et al., 1996). Furthermore, a concentration dependent effect of propylene glycol on drug permeation across human skin has been established, with Diez-Sales et al. (2005) reporting an increase in the apparent partition co-efficient of acyclovir across human abdominal skin when applied in formulations of increasing propylene glycol concentration ranging from 10 % to 70 %. Altering the concentration of propylene glycol in a formulation has also been shown to have a significant effect on the permeation of ibuprofen (Herkenne et al., 2008) and diclofenac sodium (Arellano et al., 1999) across skin. Furthermore, propylene glycol, present at 47.5 % within Dermovate cream, has been shown to be crucial for achieving the expected skin blanching response for Dermovate cream (Harding et al., 1985). Harding et al. (1985) compared the percutaneous absorption of clobetasol propionate over 24 h following the applications of Dermovate cream and a modified Dermovate cream formulation, where propylene glycol content was reduced to 10 % and

replaced with dibutyl adipate. The modified Dermovate cream formulation significantly reduced the skin blanching response, plasma cortisol levels and plasma drug levels in healthy volunteers when compared to the application of the original formulation. If propylene glycol thermodynamic activity in Dermovate cream is being similarly reduced on introduction of an emollient to the skin surface at similar times, there is the potential for this to significantly reduce clobetasol propionate delivery to skin *in vivo* and consequently clinical efficacy.

3.4 Conclusion

The findings of this Chapter suggest that the application of an emollient with a very potent TCS, Dermovate cream, has the potential to significantly reduce drug delivery to the skin compared to Dermovate cream alone irrespective of the time interval between product applications or the order in which they are applied. On investigation, the application of Dermovate cream in a premixed system with Diprobace ointment, Doublebase gel or Hydromol Intensive cream significantly reduced drug permeation and penetration to human skin by up to 2.9 fold when compared to Dermovate cream alone. Applying Dermovate cream before or after the emollient, irrespective of the time interval between product application, also resulted in a significant reduction drug permeation and penetration to human skin by up to 4.4 fold when compared to Dermovate cream alone. The data strongly suggested that clobetasol propionate was present at a sub-saturated concentration in the Dermovate cream formulation and application of an emollient at similar times to the TCS potentially diluted the TCS on the surface of the skin, reducing the degree of saturation thus thermodynamic activity of the drug in the new 'premixed' formulation compared to Dermovate cream alone. Non linear modelling of the *ex vivo* permeation profiles supported this theory and largely attributed the significant decrease in drug flux to a reduction in the apparent partition co-efficient when compared to Dermovate cream alone. Across emollient groups, it was further evident that the degree to which drug thermodynamic activity in the formulations was altered was likely to be a conflated effect of (i) reduced drug thermodynamic activity, (ii) reduced propylene glycol thermodynamic activity, (iii) the number and concentrations of solubilising emollient excipients and (iv) the number and concentrations of emollient excipients with antisolvent effects.

Furthermore, clear differences emerged in the magnitude to which drug delivery was reduced when evaluating the order of product applications within emollient groups. In contrast to current clinical recommendations, applying Dermovate cream after an emollient, or leaving a time interval of 30 minutes between product applications, did not mitigate emollient effects on drug delivery to the skin. Instead applying Dermovate cream after the emollient largely decreased drug delivery to the skin to a greater extent when compared to the application of Dermovate cream before the emollient with the

same time interval. It was thought that in addition to the mixing of the formulations on the skin surface following the application of the emollient then TCS, a thin unmixed residual layer of the emollient formulation remained on the surface of the skin creating an additional barrier to drug permeation. In this scenario, drug partitioning and diffusion into the stratum corneum was likely to be further reduced compared to the application of Dermovate cream before the emollient. In support of this, significant reductions in the apparent diffusion co-efficient were largely observed when Dermovate cream was applied after an emollient compared to Dermovate cream alone, a trend not observed when Dermovate cream was applied before the emollient.

Ultimately, this work strongly suggests that (i) applying an emollient at similar times to Dermovate cream significantly reduces drug delivery to the skin, (ii) applying the TCS after an emollient can cause significantly less drug delivery to the skin compared to the reverse order of application and (iii) leaving time intervals up to thirty minutes between product applications may not be sufficient to mitigate emollient effects on TCS drug delivery to the skin. Should these findings be reflected *in vivo*, there lies the potential for the application of an emollient at similar times to Dermovate cream to impact on clinical efficacy and this evidence base will be beneficial for future clinical recommendations.

Chapter Four:

**Evaluating the impact of altering the order of, and time interval between,
the applications of Elocon cream and emollients on the percutaneous
absorption and skin retention of mometasone furoate**

4.1 Introduction

The work reported in Chapter 2 established that the application of Elocon cream (0.1 % w/w mometasone furoate) mixed with an emollient can result in significant variability in drug delivery to human skin. Notably, total drug delivery to the skin significantly increased when Elocon cream was premixed with Hydromol Intensive cream and significantly decreased when the TCS was premixed with Diprobace cream, Diprobace ointment and Doublebase gel. These findings are likely to be of clinical relevance in scenarios where topical products are applied to the skin in quick succession. However, it is also possible that products are applied to the surface of the skin with varying time intervals and in different orders (Smoker & Voegeli, 2014). In this situation mixing of the products on the skin surface may alter drug delivery to the skin to an unpredictable extent. Indeed, a clear trend emerged in Chapter 3 on evaluation of the impact of employing various application protocols on drug delivery to the skin from the very potent TCS Dermovate cream (0.05 % w/w clobetasol propionate). Mometasone furoate is a synthetic corticosteroid designed to enhance molecular potency whilst lowering the risk of local and systemic side effects (Mori et al., 1994). To achieve a clinical response, mometasone furoate must act on glucocorticoid receptors present in keratinocytes and dermal fibroblasts and dose related antiproliferative effects have been reported for both cell types (Hein et al., 1994; Wach et al., 1998). However, an increase in the delivery of potent TCSs such as Elocon cream to the skin may result in skin atrophy and delayed wound healing. Thus, to evaluate whether particular application protocols could mitigate alterations in drug delivery to the skin, the objective of this Chapter was to evaluate the impact on drug delivery to *ex vivo* human skin when the order of, and time interval between, the applications of Elocon cream and emollients were altered. To achieve this objective, drug permeation and penetration across *ex vivo* human skin was evaluated when Elocon cream was applied before an emollient (with a five minute or thirty minute interval) and after an emollient (with a five minute or thirty minute interval). The application protocols were selected to allow a comparison with the data presented in Chapter 3 to ascertain whether the findings could be extrapolated to the application of other commonly used TCS products such as Dermovate cream. The emollients selected were Diprobace

Chapter 4

cream, Diprobase ointment and Hydromol Intensive cream, based on the findings reported in Chapter 2.

4.2 Materials and methods

4.2.1 Materials

Micronised mometasone furoate was provided by MedPharm Ltd (Guildford, UK). Elocon cream (0.1 % w/w mometasone furoate), Diprobace cream, Diprobace ointment and Hydromol Intensive cream were acquired from the University of Hertfordshire Campus Pharmacy (Hertfordshire, UK). HPLC grade acetonitrile, absolute ethanol and phosphate buffered saline tablets were acquired from Fisher Scientific (Leicestershire, UK).

4.2.2 Analytical method development

The HPLC UV gradient elution method developed and implemented in Chapter 2 (Section 2.2.2) was adopted for drug quantification of mometasone furoate.

4.2.3 Formulation selection

Elocon cream, Diprobace cream, Diprobace ointment and Hydromol Intensive cream were selected for investigation based on the findings of Chapter 2. The full excipient list for the selected formulations is detailed in Table 4-1.

Table 4-1: The listed excipients for Elocon cream, Hydromol Intensive cream, Diprobase cream, Diprobase ointment. Data were obtained from the most recently published summary of product characteristics for the respective formulation.

Elocon cream	Hydromol Intensive cream	Diprobase cream	Diprobase ointment
Mometasone furoate (0.1 % w/w)	Isopropyl myristate	Phosphoric acid	White soft paraffin
Hexylene glycol	Urea (10 %)	Sodium dihydrogen phosphate	Liquid paraffin
Phosphoric acid	White soft paraffin	Sodium hydroxide	
Hydrogenated soybean lecithin	Palmitic acid	Macrogol	
White wax	Sorbitan laurate	Cetostearyl Ether	
White soft paraffin	Arlatone G	(Cetomacrogol)	
Aluminium starch	(hydrogenated castor oil)	Cetostearyl alcohol	
octenylsuccinate	Maize Starch	Liquid paraffin	
Titanium dioxide	Syncrowax HR-C	White soft paraffin	
Purified water		Chlorocresol	
		Purified water	

4.2.4 Franz cell assembly

Full thickness human scrotal skin was prepared as detailed in Section 2.2.4.1. Franz cells were assembled as detailed in Section 2.2.4.2.

4.2.5 Franz cell method development

The receiver fluid system comprising PBS and ethanol (70:30) developed in Section 2.2.5.1 (Chapter 2) was adopted for the *ex vivo* human skin Franz cell experiments. The sampling protocol and drug extraction method developed in Section 2.2.5.2 and Section 2.2.5.3 (Chapter 2) were employed for the *ex vivo* drug permeation and penetration study. No further method development was required.

4.2.6 *Ex vivo* human skin study with Elocon cream and emollients: Investigating the effect of altering the order of, and time interval between, product applications on drug delivery to the skin

4.2.6.1 Studies investigating drug permeation across *ex vivo* human skin

Franz cells were assembled with human skin as detailed in Section 2.2.4.2. The receiver chamber was filled with PBS and ethanol (70:30), informed by the findings of the receiver fluid system development studies in Chapter 2. Following the equilibration period, Franz cells were briefly removed from the water bath and skin samples were dosed with 10 μL of Elocon cream alone or 10 μL of Elocon cream followed, after a five minute interval, by 10 μL of an emollient using a calibrated positive displacement pipette. To observe the effect of altering the order of product application on mometasone furoate absorption to the skin, the reverse application regimen was employed where skin samples were dosed with 10 μL of an emollient followed, after a five minute interval, with 10 μL of Elocon cream. To investigate the impact of altering the time interval between products applications on drug absorption to the skin, experiments were conducted as detailed above but with a thirty minute time interval instead of a five minute time interval between product applications. The application protocols employed are summarised in Figure 4-1.

To ensure contact with the membrane, the product was carefully spread over the surface of the skin using the tip of a capillary piston and the Franz cell returned to the water bath to commence the experiment. The emollients selected for investigation were Diprobace cream, Diprobace ointment and Hydromol Intensive cream based on the findings presented in Chapter 2. Samples (200 μL) of the receiver fluid were taken at pre-determined intervals up to 24 h and replaced with fresh preheated receiver fluid. Drug quantification was achieved using the gradient elution analytical method summarised in Section 2.2.2.1 (Chapter 2).

Scientist[®] 3.0 (Micromath Inc, Salt Lake City, UT, USA) was used to calculate the apparent partition (Kh) and diffusion (D/h^2) parameters when the Laplace transformation solution to Fick's second law, under finite dose conditions, was fit to the experimental permeation data sets (Equation 2-4), as previously described in Section 2.2.8.1 (Chapter 2). The drug concentration in the formulation was set

to 0.1 % for the application of Elocon cream alone and all application regimes. The pseudo steady state flux (J_{ss}) and lag time (L_t) for drug permeation were estimated as previously described in Equation 2-5 and Equation 2-6 respectively.

Altered sequence and timing of product applications to <i>ex vivo</i> human skin				
	Application protocol 1	Application protocol 2	Application protocol 3	Application protocol 4
Finite application of product 1	Elocon cream	Diprobase ointment Diprobase cream Hydromol Intensive	Elocon cream	Diprobase ointment Diprobase cream Hydromol Intensive
Time interval	5 minutes		30 minutes	
Finite application of product 2	Diprobase ointment Diprobase cream Hydromol Intensive	Elocon cream	Elocon cream	Diprobase ointment Diprobase cream Hydromol Intensive

Figure 4-1: Summary of the application protocols employed to investigate the altered drug permeation and penetration to human skin in the presence of emollients.

4.2.6.2 Studies investigating drug penetration to *ex vivo* human skin

Following the drug permeation across human skin studies, Franz cells were disassembled and drug content on the skin surface (residual formulation), the epidermis and dermis determined. Removal of the residual formulation and heat separation of the epidermis and dermis was achieved by adopting the protocol described in Section 2.2.8.2. Quantification of mometasone furoate in the cotton buds, tape strips, epidermis and dermis was achieved using the extraction method developed in Section 2.2.5.3. All samples were analysed by the gradient elution analytical method summarised in Section 2.2.2.1 (Chapter 2).

4.2.7 Data treatment and statistical analysis

The concentration of mometasone furoate in the receiver fluid was corrected for previous sample removal and profiles constructed to present cumulative amount of drug permeated per unit area ($\mu\text{g cm}^{-2}$) over the exposure period. Experimental data were expressed as mean ($n = 6$) \pm standard deviation (SD), unless otherwise stated. Statistical analysis was performed using Prism 8.0 (GraphPad, USA). The Shapiro Wilk test was employed to determine the normality of all data sets. Non-parametric analysis for multiple comparisons was performed using Kruskal-Wallis and a Mann-Whitney test applied for post hoc analysis. Parametric analysis for multiple comparisons was performed using analysis of variance (ANOVA) and Tukey's post hoc test. Statistically significant differences were determined at a 95 % confidence interval ($p \leq 0.05$).

4.2.8 Kinetic evaluation of crystal formation in Elocon cream premixed with Diprobase cream

To determine the rate of drug crystal formation when Diprobase cream was introduced to Elocon cream, microscopic analysis of crystalline structures over time were conducted on premixed systems of Elocon cream and Diprobase cream (1:1). Samples of the premixed formulation were prepared and mounted on glass slides for microscopic analysis immediately after preparation and 5 minutes, 1 hour, 2 hours, 3 hours 22 hours and 24 hours after preparation. Samples were observed using a L3230 GX light microscope (GT Vision Ltd, Suffolk, UK) and images were captured using a x 50 objective lens, unless otherwise stated, using a GX CAM camera and GX Capture software (GT Vision Ltd, Suffolk, UK). Drug crystal length and diameter were measured using ImageJ (National Institute of Health, USA). Drug crystal area was calculated as a product of crystal length and diameter.

4.3 Results and discussion

To elucidate the emollient effect on TCS drug delivery to the skin when the order of application and time interval between product applications may be altered, an *ex vivo* human skin study was conducted employing some of the currently recommended application protocols. The effect of applying Elocon cream before or after an emollient on drug permeation and penetration across human skin was evaluated. Furthermore, the impact of leaving a five minute interval compared to a thirty minute interval between product applications on the extent of drug permeation and penetration across human skin was assessed.

Informed by the findings of Chapter 2, Hydromol Intensive cream and Diprobace cream were selected as emollients with the potential to enhance and reduce drug delivery to the skin, respectively. The premixed application of Diprobace ointment with a TCS demonstrated significantly reduced drug delivery to the skin when applied with Elocon cream and Dermovate cream (Chapter 2 and Chapter 3). Thus, Diprobace ointment was selected to (i) discern the effect on drug delivery to the skin when Elocon cream was mixed with a relatively simple emollient ointment base and (ii) allow the extrapolation of emollient specific trends across TCS groups.

The distribution of mometasone furoate in all matrices and the receiver fluid when applied with either Diprobace cream, Diprobace ointment or Hydromol Intensive cream using one of the four application regimens is presented in Figure 4-2.

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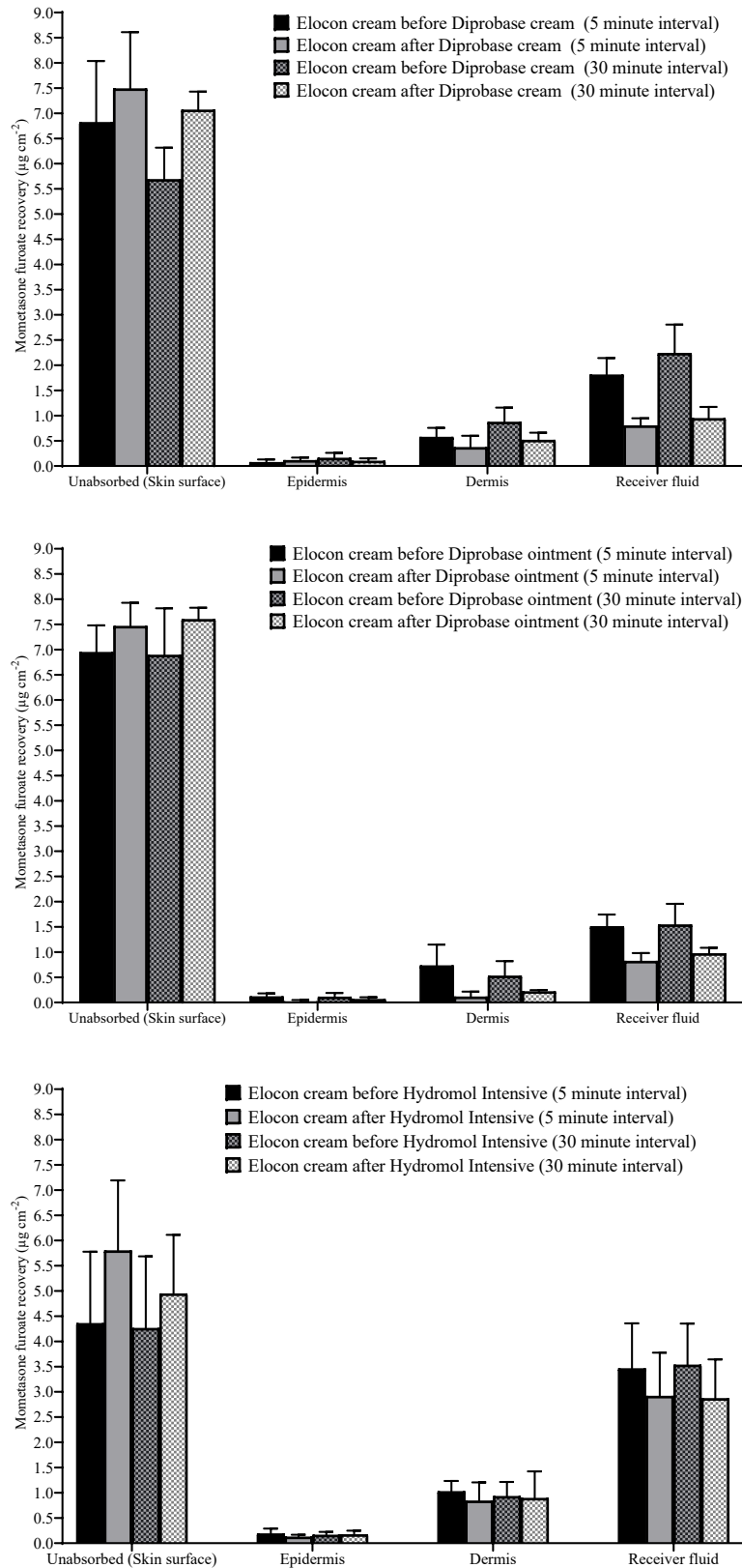


Figure 4-2: Drug distribution in the unabsorbed formulation, epidermis, dermis and receiver fluid ($\mu\text{g cm}^{-2}$) recovered after 24 h from Elocon cream following the finite application of Elocon cream applied with (a) Diprobace cream (b) Diprobace ointment or (c) Hydromol Intensive cream according to multiple application protocols. Data are shown as mean + SD (n=6).

The absolute recovery of mometasone furoate from Elocon cream ranged 89 – 108 % of the applied dose for all experiments conducted. For all application protocols employed, the trend in drug distribution followed that observed when Elocon cream was applied in a premixed system with an emollient and was evident in the following order of magnitude: unabsorbed drug > receiver fluid > dermis > epidermis. For clarity the total drug absorption (total drug content in the epidermis, dermis and receiver fluid) was used for statistical analysis as an indication of the change in absolute mometasone furoate absorption in the presence of one of the three emollients, employing one of the four application regimens (Figure 4-3).

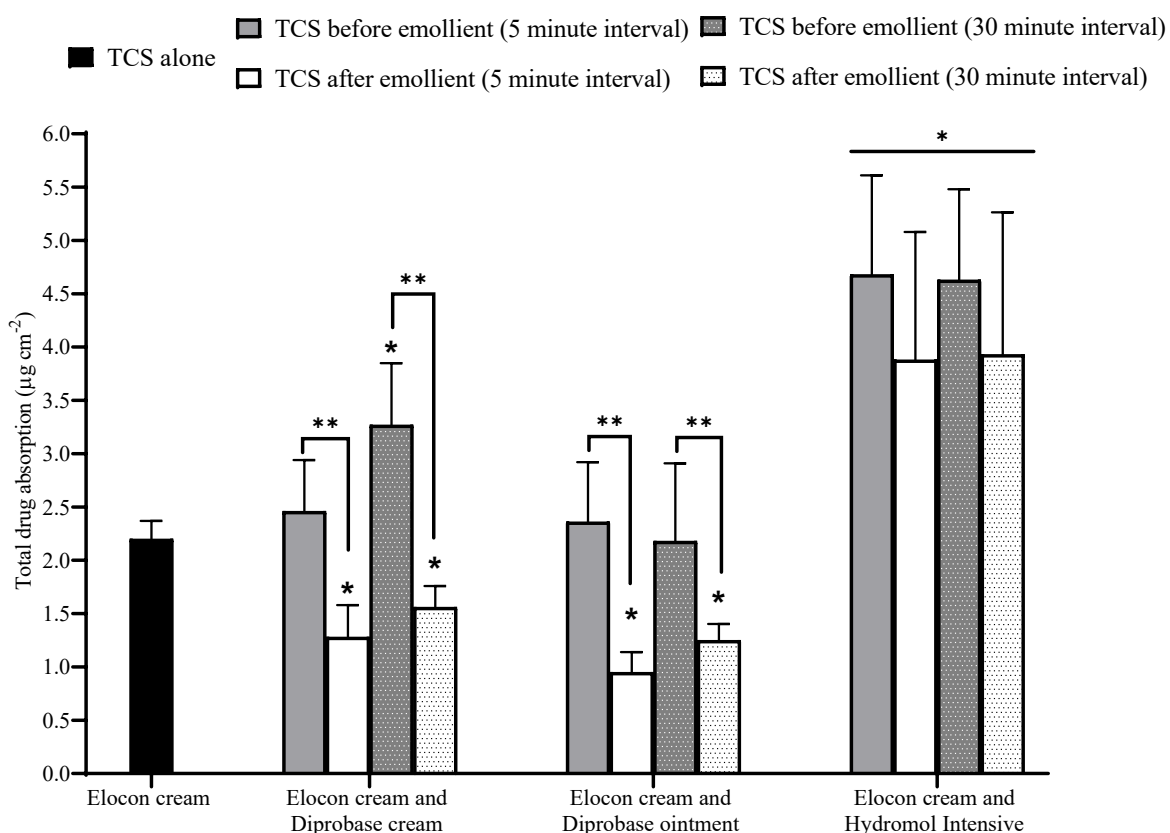


Figure 4-3: Total mometasone furoate delivered to the skin (epidermis, dermis and receiver fluid; $\mu\text{g cm}^{-2}$) following the application of a finite dose of: Elocon cream alone, Elocon cream before an emollient (with a 5 min or 30 min interval) or Elocon cream after an emollient (with a 5 min or 30 min interval). The emollients were Diprobase cream, Diprobase ointment or Hydromol Intensive cream. Data are shown as the mean + SD ($n=6$). * denotes a significant difference when compared to the total drug recovered from Elocon cream alone. ** denotes a significant difference when the application of the TCS before the emollient was compared to the application of the TCS after the emollient, for a particular time interval and emollient (Kruskal-Wallis and Mann-Whitney test; $p < 0.05$).

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The application of Elocon cream five minutes after Diprobace cream significantly decreased total drug delivery to the skin by 1.5 fold when compared to the application of Elocon cream alone ($p < 0.05$). This decrease in drug absorption when compared to Elocon cream alone was mirrored when the time interval between product applications was increased to thirty minutes (1.3 fold; $p < 0.05$). A similar 2 fold decrease in drug delivery to the skin was observed following the application of a premixed Elocon cream and Diprobace cream formulation when compared to Elocon cream alone (Chapter 2), indicative of an altered formulation on the surface of the skin to a comparable extent as the application of a premixed formulation. In contrast, applying Elocon cream thirty minutes before Diprobace cream resulted in a significant 1.7 fold increase in total drug absorption when compared to the total drug absorption from Elocon cream alone.

A similar trend was observed when Elocon cream was applied five minutes after Diprobace ointment with a significant 2 fold decrease in total drug delivery being observed when compared to Elocon cream alone ($p < 0.05$; Figure 4-3). Increasing the time interval between product applications to thirty minutes but maintaining the order of product applications still resulted in a significant decrease in total drug delivery to the skin when compared to the application of Elocon cream alone ($p < 0.05$; 1.6 fold). These trends resembled the significant 1.5 fold decrease in drug delivery to the skin following the application of a premixed Elocon cream and Diprobace ointment formulation (Chapter 2). Employing the reverse application regimen, where Elocon cream was applied before Diprobace ointment, did not significantly alter total drug delivery to the skin when compared to the application of Elocon cream alone; this was irrespective of the time interval employed between product applications (Figure 4-3).

The trends observed thus far further support the findings presented in Chapter 3, where the application of a TCS after an emollient generally decreased drug delivery to the skin when compared to the application of the TCS before the emollient. Furthermore, leaving time intervals up to thirty minutes between product applications appears not to be sufficient to mitigate emollient effects on drug delivery to the skin when the TCS is applied after the emollient.

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In addition to this established trend, an emollient specific effect was distinctly evident in the case of Hydromol Intensive cream where total drug delivery to the skin significantly increased by up to 2.4 fold, irrespective of the application protocol employed, when compared to the application of Elocon cream alone ($p < 0.05$). This finding echoed the trend observed following the premixed application of Elocon cream and Hydromol Intensive cream to skin (Chapter 2), which resulted in a 2 fold significant increase in drug delivery to the skin when compared to Elocon cream alone. It was hypothesised that emollient excipients with penetration enhancing capabilities increased drug partitioning from the saturated premixed formulation and it is possible that a similar effect was occurring when Elocon cream was applied to the skin surface at similar times to Hydromol Intensive cream.

To provide insight into the mechanistic effects impacting on drug flux when products were applied to the skin at similar times, an evaluation of the permeation of mometasone furoate across the skin was conducted. The cumulative amount of mometasone furoate permeated across human skin over 24 h following the application of Elocon cream alone and Elocon cream with an emollient according to the application protocols is presented in Figure 4-4.

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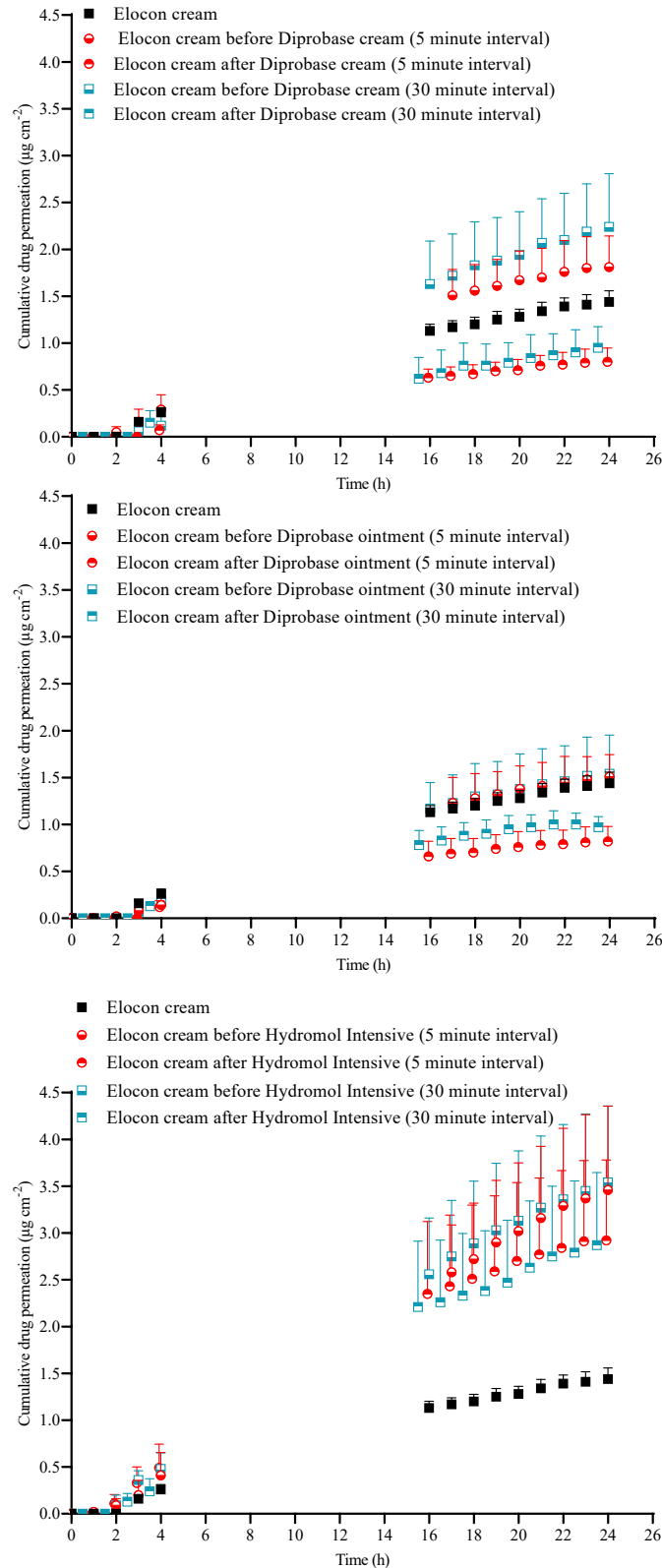


Figure 4-4: The cumulative amount of mometasone furoate ($\mu\text{g cm}^{-2}$) permeated across human scrotal skin from Elocon cream when a finite dose of Elocon cream was applied alone (■), 5 minutes before an emollient (●), 5 minutes after an emollient (●), 30 minutes before an emollient (■) or 30 minutes after an emollient (■). The emollients were (a) Diprobace cream, (b) Diprobace ointment and (c) Hydromol Intensive cream. Data are shown as mean + SD (n= 6).

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Drug permeation across human skin was evident from 3 h onwards for all application protocols. In the presence of Diprobase cream and Diprobase ointment, drug permeation largely profiled in a similar manner to mometasone furoate permeation from Elocon cream alone, with drug permeation increasing with time then plateauing at later time points. Total drug permeation (Q_{24}) ranged from 9 % of the applied dose when Elocon cream was applied five minutes after Diprobase cream to 25 % of the applied dose when Elocon cream was applied thirty minutes before Diprobase cream. Comparatively, total drug permeation from Elocon cream alone was 17 % of the applied dose. When Elocon cream was applied with Hydromol Intensive cream, drug permeation continued to increase with time over the entire experimental period for all application protocols employed. This was accompanied by greater amounts of total drug permeation (Q_{24}) compared to Elocon cream alone and ranged from 32 % of the applied dose when Elocon cream was applied thirty minutes after Hydromol Intensive cream to 40 % of the applied dose when Elocon cream was applied thirty minutes before Hydromol Intensive cream.

The permeation profiles presented in Figure 4-4 were modelled using Equation 2-4 (Chapter 2) to obtain the normalised apparent partition co-efficient and diffusion co-efficient for mometasone furoate across human skin. The model fittings were similar to the representative modelled permeation profile presented in Chapter 2 (Figure 2-12), thus are not re-presented here. The estimated permeation parameters are presented in Table 4-2. The approach established in Chapter 3 for modelling of the permeation data when the TCS and emollients were applied according to an application protocol was adopted for the permeation data presented in Figure 4-4. Thus, the model was fitted to the data set under the assumption that the drug concentration in Elocon cream on the skin surface when applied before or after an emollient (with a five or thirty minute interval) matched that of Elocon cream applied alone.

Table 4-2: Estimated apparent diffusion co-efficient (D/h^2), lag time (L_t), apparent partition co-efficient (Kh), total drug permeation at 24 h (Q_{24}) and pseudo steady state drug flux (J_{ss}) obtained from the nonlinear modelling of the permeation data presented in Figure 4-4. Data are shown as mean \pm SD (n = 6). * Denotes a significant difference when D/h^2 , L_t , Kh , Q_{24} and J_{ss} values were compared to the respective permeation parameters for Elocon cream alone (Kruskal-Wallis and Mann-Whitney test; $p < 0.05$).

Time interval	Product 1	Product 2	D/h^2 (cm)	L_t (h)	Kh (h^{-1})	Q_{24} (μgcm^{-2})	J_{ss} ($\mu\text{g cm}^{-2} \text{h}^{-1}$)
	Elocon cream		3.29E-02 \pm 2.95E-03	5.102 \pm 0.41	3.87E-03 \pm 4.77E-04	1.44 \pm 0.12	1.29E-05 \pm 2.90E-06
5 minutes	Elocon cream	Diprobace cream	5.92E-02* \pm 9.14E-03	2.89* \pm 0.48	2.13E-03* \pm 1.11E-04	1.81 \pm 0.33	1.27E-05 \pm 2.30E-06
	Diprobace cream	Elocon cream	3.00E-02 \pm 3.38E-03	5.63 \pm 0.67	2.02E-03* \pm 8.88E-05	0.80 \pm 0.15	6.07E-06* \pm 7.77E-07
30 minutes	Elocon cream	Diprobace cream	3.87E-02 \pm 5.98E-03	4.43 \pm 0.76	4.81E-03 \pm 2.56E-04	2.24 \pm 0.57	1.87E-05 \pm 3.61E-06
	Diprobace cream	Elocon cream	3.13E-02 \pm 4.20E-03	5.45 \pm 0.93	2.39E-03* \pm 1.19E-04	0.95 \pm 0.23	7.53E-06* \pm 1.29E-06
5 minutes	Elocon cream	Diprobace ointment	4.60E-02* \pm 6.40E-03	3.70* \pm 0.54	2.28E-03 \pm 1.27E-04	1.51 \pm 0.23	1.05E-05 \pm 2.02E-06
	Diprobace ointment	Elocon cream	3.10E-02 \pm 4.24E-03	5.51 \pm 0.93	2.01E-03* \pm 1.41E-04	0.82 \pm 0.16	6.23E-06* \pm 1.09E-06
30 minutes	Elocon cream	Diprobace ointment	4.66E-02* \pm 9.21E-03	3.72* \pm 0.74	2.26E-03* \pm 1.34E-04	1.54 \pm 0.41	1.0E-05 \pm 2.51E-06
	Diprobace ointment	Elocon cream	3.84E-02 \pm 4.08E-03	4.40 \pm 0.48	1.96E-03* \pm 7.61E-05	0.97 \pm 0.11	7.51E-06* \pm 7.47E-07
5 minutes	Elocon cream	Hydromol Intensive	4.16E-02* \pm 6.77E-03	4.10* \pm 0.56	7.03E-03* \pm 4.23E-04	3.46 \pm 0.90	2.97E-05* \pm 6.62E-06
	Hydromol Intensive	Elocon cream	5.10E-02* \pm 1.43E-02	3.57* \pm 1.09	4.80E-03 \pm 3.61E-04	2.92 \pm 0.86	2.44E-05* \pm 6.87E-06
30 minutes	Elocon cream	Hydromol Intensive	4.22E-02 \pm 9.01E-03	4.13 \pm 0.87	7.74E-03* \pm 6.16E-04	3.54 \pm 0.82	3.31E-05* \pm 9.43E-06
	Hydromol Intensive	Elocon cream	2.94E-02 2.39E-03	5.70 \pm 0.44	9.32E-03* \pm 9.62E-04	2.87 \pm 0.77	2.77E-05* \pm 5.13E-06

Pseudo steady state drug flux was altered to varying extents depending on the application protocol employed and the emollient applied when compared to Elocon cream alone (Figure 4-5). For Diprobace cream and Diprobace ointment, a general trend was observed where the application of Elocon cream after either emollient resulted in a significant decrease in drug flux when compared to Elocon cream alone, ranging from a 1.7 fold decrease when Elocon cream was applied thirty minutes after Diprobace cream to a 2.1 fold decrease when Elocon cream was applied five minutes after Diprobace ointment ($p < 0.05$; Table 4-2).

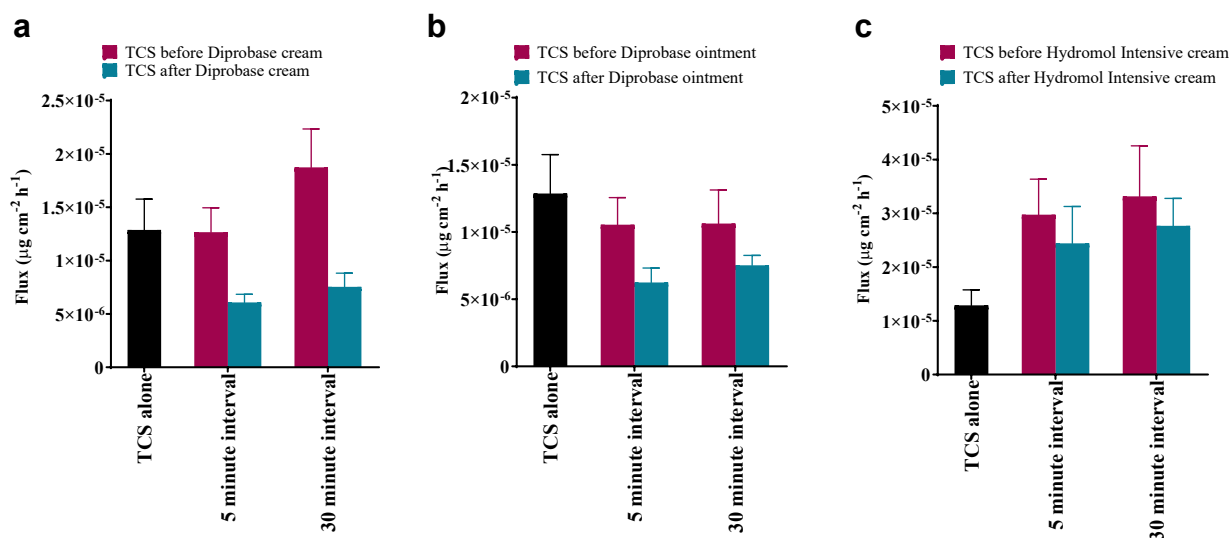


Figure 4-5: The calculated pseudo steady state drug flux (J_{ss} from Table 4-2) from Elocon cream when a finite dose of Elocon cream was applied alone, before an emollient or after an emollient. The emollients were (a) Diprobace cream, (b) Diprobace ointment and (c) Hydromol Intensive cream. Data points are shown as the mean \pm SD (n=6).

This pattern of reduced drug flux when Elocon cream was applied after Diprobace cream or Diprobace ointment, irrespective of the time interval between product applications, corresponds with the earlier reported trends in reduced total drug delivery to the skin when compared to Elocon cream alone (Figure 4-3). Analysis of the permeation parameters largely attributed the reduced drug flux to a significant decrease in the apparent partition co-efficient by up to 2 fold and an unaltered apparent diffusion co-efficient when compared to Elocon cream alone (Table 4-2). These findings appear to support the theory presented in Chapter 3 that applying a TCS after an emollient can potentially create (i) a new ‘premixed’ formulation on the surface of the skin, reducing drug partitioning towards the skin and (ii) a residual

emollient layer on the surface of the skin acting as an additional barrier to drug delivery to the skin. It was hypothesised in Chapter 2 that mometasone furoate was likely to be present in Elocon cream at saturated concentrations, thus at maximum thermodynamic activity. Similar to the application of a premixed system, the introduction of Diprobace cream or Diprobace ointment to Elocon cream on the skin surface was likely to reduce the degree of saturation of mometasone furoate in the new 'premixed' formulation through dilution, thus reduce the thermodynamic activity of the drug in the formulation(s) and drug flux across the skin. Clinically, it is also important to consider that the degree of dilution of a product cannot always predict the extent to which efficacy is altered. For example, a betamethasone-17-valerate ointment (Betnovate ointment), when diluted up to 16-fold with emulsifying ointment, did not exhibit a resultant reduction in potency as determined by the vasoconstrictor assay (Ryatt et al., 1982), findings which were further supported by the works of Gibson et al. (1982) and Gibson et al. (1983). Furthermore, Stoughton and Wullich (1989) found, on comparison branded preparations of the same TCS but with varying strengths, there to be no correlation between steroid concentration and skin blanching response in seven out of eight cases. In the final case, a positive correlation between steroid concentration and skin blanching response was observed for Synalar preparations. Therefore, it is reasonable to expect that different emollients may alter the degree of saturation of the TCS and potentially clinical efficacy of the product to varying extents.

When evaluating the effect of emollient application on TCS delivery, the role of individual excipients in the TCS and emollient formulations should not be overlooked. The prevailing approach to optimise drug delivery to the skin is through inclusion of excipients within a formulation which exhibit penetration enhancing effects (Williams & Barry, 2012). Thus, an equally important consideration is the altered thermodynamic activity of CPEs in the formulation when multiple topical products are mixed together. Elocon cream, for example is formulated with hexylene glycol; an excipient which can act to favour the partitioning of the drug out of the vehicle and into the skin (Barry, 1987; Mollgaard & Hoelgaard, 1983). The clinical effect of exploiting the actions of glycols is evident in a currently available 0.1 % w/w hydrocortisone cream (Dioderm), formulated with propylene glycol to deliver the equivalent clinical efficacy to that of the generic 1 % w/w Hydrocortisone cream BP formulation but

with one tenth of the drug strength (Whitefield & McKenzie, 1975). Therefore, reduced drug partitioning from the formulation when Elocon cream was mixed with Diprobace cream or Diprobace ointment could be a combined effect of reduced drug and hexylene glycol thermodynamic activities in the premixed formulations.

In Chapter 2 it was established that mixing Elocon cream with Diprobace cream resulted in the formation of drug crystals and this correlated with a significant reduction in drug permeation and penetration to skin from the premixed formulation. The effect of particle size on the rate of dissolution and consequently drug delivery to the skin was evidenced by Barrett et al. (1965), who reported decreased percutaneous absorption of fluocinolone acetonide from a vehicle when the drug was present in crystalline form compared to the micronised form. Furthermore, reduced drug permeation following the application of supersaturated formulations has been attributed to drug crystallisation on and in the skin (Santos et al., 2010, 2012). Similarly, the inadvertent formation of drug crystals on the skin surface when Elocon cream and Diprobace cream were mixed is likely to reduce the rate of drug dissolution, partitioning from the vehicle and consequently flux across the skin. To determine whether drug crystals formed rapidly when Diprobace cream was introduced to Elocon cream and thus whether this would be a likely mechanism occurring when the products were mixed on the surface of the skin at similar times the rate of drug crystal growth was evaluated by microscopic observations. Premixed samples of Elocon cream and Diprobace cream were observed for crystal formation at various timepoints over 24 hours and the drug crystal area was calculated. A representative image used to calculate drug crystal area is presented in Figure 4-6 and a plot of drug crystal area against time is presented in Figure 4-7.

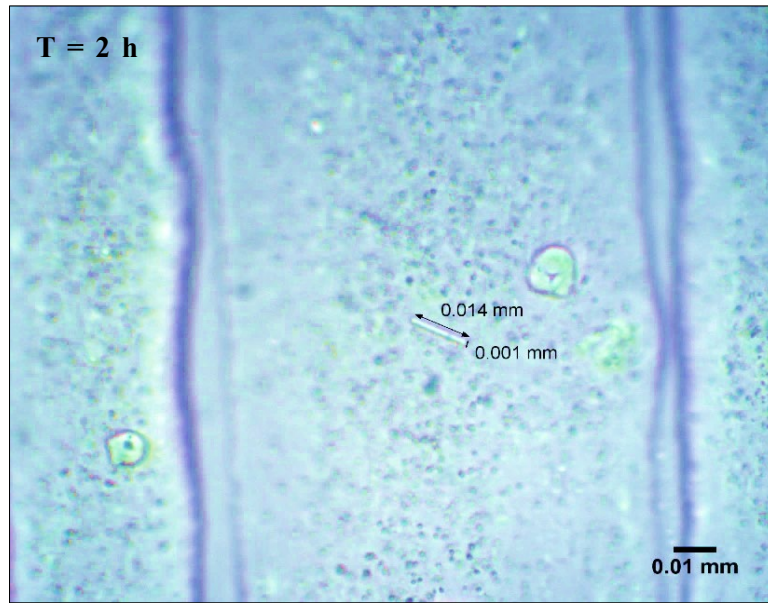


Figure 4-6: Representative image used to calculate drug crystal area taken at x 50 magnification. The image shows a drug crystal present 2 h after mixing Elocon cream and Diprobase cream (1:1).

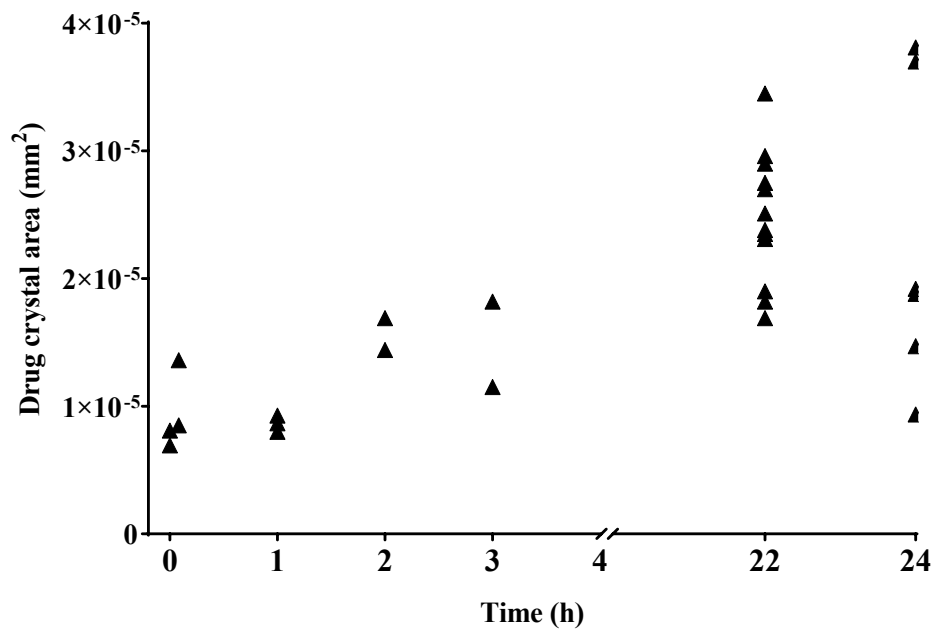


Figure 4-7: Drug crystal area (mm²) calculated from images taken at 0 min, 5 min, 1 h, 2 h, 3 h, 22 h and 24 h after the introduction of Diprobase cream to Elocon cream (1:1). Data points shows single drug crystal area measurements calculated at each timepoint.

Drug crystallisation occurs following development of nuclei of a critical size (nucleation) and subsequently crystal growth. As nucleation time decreases with increasing degrees of saturation this can offer an indication of the degree of drug saturation within a formulation (Mullin, 1972). Furthermore, it is generally accepted that slow nucleation rates are indicative of a diffusion-controlled process, thus nucleation time can offer insight into the rate of drug dissolution and diffusion from the surrounding environment towards the nucleation point (Vekilov, 2010). However, experimental observation of nuclei formation is challenging given that nuclei are typically of the order of tens of nanometres and during measurement the subsequent processes of particle agglomeration, crystal growth and secondary nucleation may distort results (Brandel & ter Horst, 2015; Raghavan et al., 2000). To allow a kinetic evaluation of crystal growth, crystallisation time was instead measured and defined as the time interval at which crystals were first observed, an approach previously employed for analysis of hydrocortisone acetate crystal formation in vehicles for topical application (Raghavan et al., 2001). Drug crystallisation was evident immediately following the introduction of Diprobace cream to Elocon cream (1:1), suggesting that crystallisation, therefore nucleation, occurred rapidly (Figure 4-7). This finding further supports the postulation of a high degree of drug saturation in Elocon cream. At early timepoints, a relatively small distribution in drug crystal area was observed, ranging from $6.9 \times 10^{-6} \text{ mm}^2$ to $1.82 \times 10^{-5} \text{ mm}^2$, suggesting that the rapidly formed crystals grew simultaneously and to similar extents. Comparatively, at later timepoints (22 – 24 h), a large size distribution and increase in the number of crystals was observed with drug crystal area ranging from $9.36 \times 10^{-6} \text{ mm}^2$ to $3.81 \times 10^{-5} \text{ mm}^2$. It was evident that smaller crystals resembling those observed at 0 – 3 h were present at 22 – 24 h suggesting that nucleation, at a slower rate, was occurring beyond 3 h. This is a likely scenario as the rapid nucleation and crystal growth observed at early timepoints potentially depleted the amount of solubilised drug available for new crystal growth, thereby hindering further nucleation until sufficient drug dissolution and diffusion from the neighbouring environment occurred. Furthermore, the presence of larger crystals (increased drug crystal area) at 22 – 24 h appears to suggest that growth of the earlier formed crystals was continuing simultaneous to nucleation at later timepoints.

Considered in the context of the application protocols employed, should Diprobace cream and Elocon cream mix on the surface of the skin, it seems reasonable to suggest that drug crystals could form rapidly. In addition to a TCS dilution effect, this may result in reduced drug partitioning from the new ‘mixed’ formulation compared to the application of Elocon cream alone and may reduce the expected drug delivery profile to the skin from once-daily products such as Elocon cream. It is, however, important to appreciate that a complex interplay may exist on the skin surface between the process of crystal formation and drug diffusion towards the skin – formulation interface, affecting drug partitioning from the formulation (Figure 4-8). Therefore, the rate of one process may govern the extent to which the other occurs.

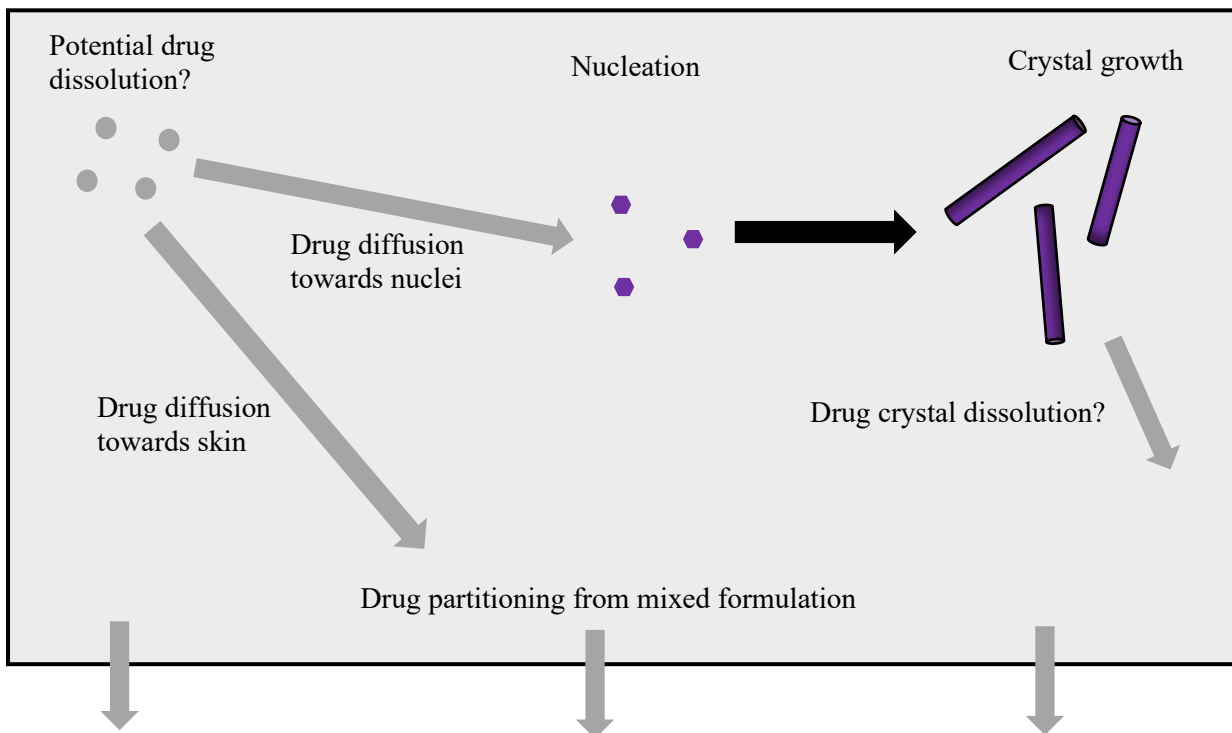


Figure 4-8: Schematic of the simultaneous processes potentially occurring in a mixed formulation of Diprobace cream and Elocon cream on the skin surface. The extent to which each process occurs may be impacted by the application protocol employed.

The earlier reported data on drug partitioning into the skin from Elocon cream with Diprobace cream showed variability with the application protocol employed (Table 4-2). As crystallisation is dependent on the degree of drug saturation amongst other factors (Mullin, 2001), employing one application protocol over another may reduce drug concentration on the skin surface to greater extents through drug partitioning, thus creating a less favourable environment for crystallisation when the products mix. This theory may contribute to the reason that applying Elocon cream thirty minutes before Diprobace cream did not match the trend of a significantly reduced apparent partition co-efficient observed with all other application protocols for the emollient, when compared to Elocon cream alone ($p < 0.05$; Table 4-2). Instead the apparent partition co-efficient, and drug flux, from Elocon cream applied thirty minutes before Diprobace cream was unaltered and total drug delivery was significantly increased when compared to Elocon cream alone (Table 4-2 and Figure 4-3). This correlated with the lowest amount of drug recovered on the skin surface (unabsorbed formulation) when comparing all application protocols for Diprobace cream with Elocon cream (Figure 4-2).

An opposing concern is whether drug crystallisation from the application of Elocon cream and Diprobace cream has the potential to occur in the stratum corneum, thus creating a TCS reservoir within the skin. Vickers (1963) first reported the presence of a drug reservoir in the SC, finding repeated occlusion of skin sites treated with a single application of 1 % w/w fluocinolone acetonide to induce skin blanching responses for up to 11 days following application. A substantial body of evidence has since been reported supporting the presence of a stratum corneum reservoir for a range of drugs (Stoughton, 1965, 1966; Vickers, 1969). More recently, the formation of drug reservoirs in the skin, and consequently a reduction in drug permeation, has been attributed to drug crystallisation in the skin (Goh et al., 2017; Hadgraft & Lane, 2016). In the *ex vivo* Franz cell study, a potential sign of drug crystallisation in the stratum corneum may be a greater fraction of the dose delivered to the skin (sum of epidermal, dermal and receiver fluid drug recovery) found in the epidermis when Elocon cream was applied before or after Diprobace cream, compared to the application of Elocon cream alone. The percent distribution of dose delivered to the epidermis, dermis and receiver fluid is presented in Table 4-3.

Table 4-3: The percent distribution of dose delivered to the epidermis, dermis and receiver fluid following the application of Elocon cream alone or Elocon cream with Diprobace cream to human skin. Data are presented as mean \pm SD (n=6). * denotes a significant difference when the percent of the delivered dose in the epidermis, dermis and receiver fluid were compared to the respective values for Elocon cream alone (Kruskal-Wallis and Mann-Whitney test; $p < 0.05$).

Application protocol	Distribution of the dose delivered (%)		
	Epidermis	Dermis	Receiver fluid
Elocon cream	3.43 \pm 1.59	21.39 \pm 2.00	75.18 \pm 2.77
Elocon cream before Diprobace cream (5 min)	2.85 \pm 2.15	23.04 \pm 4.23	74.11 \pm 5.76
Elocon cream after Diprobace cream (5 min)	9.83 \pm 3.46*	27.05 \pm 11.15	63.12 \pm 11.21
Elocon cream before Diprobace cream (30 min)	4.99 \pm 3.39	27.03 \pm 8.38	67.98 \pm 11.58
Elocon cream after Diprobace cream (30 min)	6.79 \pm 3.23*	32.63 \pm 10.23	60.58 \pm 12.55*

On analysis, applying Elocon cream after Diprobace cream resulted in a significantly greater proportion of the delivered dose in the epidermis, when compared to epidermal drug delivery from Elocon cream alone ($p < 0.05$). With a thirty minute interval, this order of application was coupled to a significantly lower proportion of the delivered dose in the receiver fluid when compared to Elocon cream alone ($p < 0.05$). Though preliminary, these findings are of interest as they appear to hint at the formation of a drug reservoir in the epidermis when particular application protocols were employed for Elocon cream and Diprobace cream. However, a distinction has yet to be made between drug present in the epidermis in solubilised or crystalline form. Early reports of a steroid reservoir in the stratum corneum were confirmed through skin blanching assays which provided useful insight to the bioavailability of drug remaining in the stratum corneum (Stoughton & Fritsch, 1964). Recently, however, attempts have focused on employing spectroscopic approaches to image drug delivery to the stratum corneum, and these have led to reservoir formation being attributed specifically to drug crystallisation (Belsey et al., 2014; Goh et al., 2017; Saar et al., 2011). Employing a combination of these techniques for future investigation will enable the confirmation of drug crystals in the stratum corneum when Elocon cream is applied with Diprobace cream and offer an indication of the impact on drug bioavailability compared to the application of Elocon cream alone.

When Hydromol Intensive cream was applied at similar times to Elocon cream, drug flux significantly increased irrespective of the application protocol employed, compared to the application of Elocon cream alone ($p < 0.05$; Table 4-2). The increase in drug flux ranged from 1.9 fold when Elocon cream was applied five minutes after Hydromol Intensive cream to 2.6 fold when Elocon cream was applied thirty minutes before Hydromol Intensive cream. This was accompanied by significant increases in total drug delivery to the skin, similar to the 2 fold increase observed following the application of a premixed Elocon cream and Hydromol Intensive cream formulation when compared to Elocon cream alone ($p < 0.05$; Chapter 2). Of notable interest in the excipient list of Hydromol Intensive cream are isopropyl myristate (IPM), an aliphatic ester, and urea, a commonly used hydrating agent; both of which have the potential to enhance skin penetration of the TCS (Beastall et al., 1986; Feldmann, 1974). Should incorporation of emollient excipients into the TCS formulation on the skin surface inadvertently enhance skin penetration of the drug, total drug delivery to the skin is likely to be increased variably, depending on the effective concentration of the excipients and potential synergistic activity with the TCS excipients such as hexylene glycol.

To discern the likely effects of IPM and urea on drug delivery to the skin at concentrations relevant to a premixed Elocon cream and emollient formulation, an *ex vivo* human skin Franz cell study was conducted where Elocon cream was spiked with relevant concentrations of IPM or urea. Hydromol Intensive cream is listed to contain 10 % urea (British National Formulary, 2020b), thus Elocon cream was spiked with 5 % w/w urea as an equivalent concentration in a 1:1 mix of Elocon cream and Hydromol Intensive cream. The concentration of IPM in Hydromol Intensive cream is not currently listed, however IPM is typically present at 1 – 10 % in topical formulations (Brinkmann & Muller-Goymann, 2003; Reid et al., 2013; Sheskey et al., 2019). Doublebase gel, an earlier investigated emollient, contains IPM at 15 % w/w (British National Formulary, 2020b), thus a concentration of 7.5 % w/w was selected to offer an indication of the potential enhancing effects at this concentration. To elucidate the potential occlusive effects of emollients when applied with Elocon cream on drug delivery to the skin, an additional investigation was conducted where Elocon cream was applied under occlusion.

The drug distribution in the epidermis, dermis and receiver fluid alongside total drug delivery to the skin is presented in Figure 4-9.

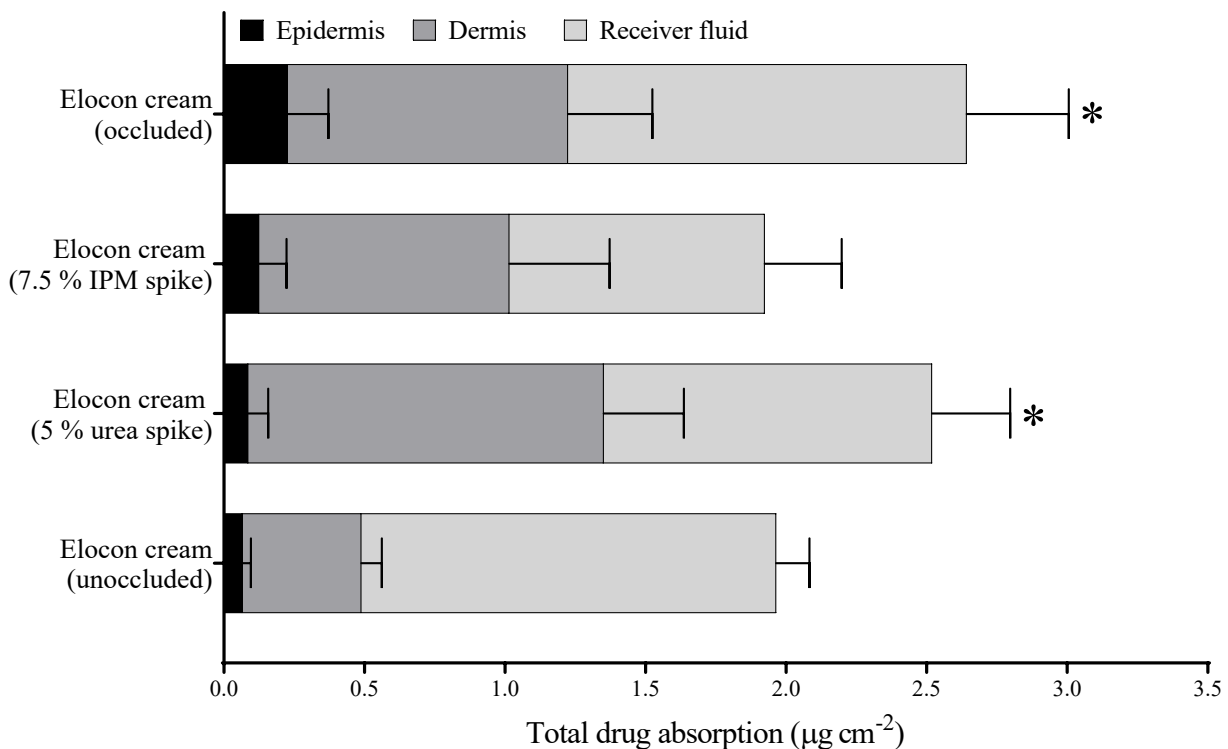


Figure 4-9: Distribution of mometasone furoate in the epidermis, dermis and receiver fluid following the finite application of Elocon cream (unoccluded), Elocon cream spiked with 5% w/w urea, Elocon cream spiked with 7.5 % w/w IPM and Elocon cream (occluded). Data are shown as mean + SD (n= 6). * Denotes a significant difference when the total drug delivery (epidermis, dermis and receiver fluid) for each formulation was compared to Elocon cream (unoccluded) (Kruskal-Wallis and Mann-Whitney test; $p < 0.05$).

The hygroscopic nature of urea lends to an increase in the water holding capacity in the stratum corneum and when present at 10 % in a cream (as with Hydromol Intensive cream), it can double the water holding capacity of the stratum corneum (Brown & Williams, 2019). At a 5 % w/w concentration, urea significantly enhanced total drug delivery to the skin from Elocon cream by 1.28 fold, when compared to the application of Elocon cream alone ($p < 0.05$). It appeared that the significant increase in total drug delivery to the skin, by up to 2.4 fold, when Elocon cream was applied at similar times to Hydromol Intensive cream may be partially attributable to the actions of urea. In contrast, at 7.5 % w/w, IPM demonstrated a negligible effect on enhancing drug delivery to the skin compared to Elocon cream alone ($p > 0.05$). The mechanism through which IPM is thought to achieve permeant enhancement is

through altering the lipid bilayer (Brinkmann & Müller-Goymann, 2005) or promoting drug solubility within the skin (Santos et al., 2012). However, successful enhancement also requires consideration of the solvent thermodynamic activity in the formulation. Refai and Müller-Goymann (2002) investigated the influence of diluting a TCS containing CPEs on percutaneous absorption. It was reported that a three fold dilution of a saturated Soventol cream (1 % w/w hydrocortisone) containing IPM with a hydrophilic base resulted in a five fold reduction in drug flux across human skin, a finding attributed to the reduced thermodynamic activity of IPM in the formulation. The negligible enhancement in drug delivery to the skin observed when 7.5 % w/w IPM was incorporated into Elocon cream, compared to Elocon cream alone, may be attributable to low solvent thermodynamic activity in the formulation. Thus, if IPM is present at similar concentrations in Hydromol Intensive cream this is unlikely to be a contributing factor to the enhanced skin permeation observed.

Occlusion of the skin surface can enhance drug permeation through increased hydration of the stratum corneum (Elias et al., 2002). On analysis, occlusion of the donor chamber following the application of Elocon cream significantly enhanced drug delivery to the skin by 1.34 fold compared to non-occluded conditions ($p < 0.05$), an interesting observation as skin tissue in Franz cell experiments are generally thought to be over hydrated (Levintova et al., 2011). A similar 1.6 fold increase in drug permeation has been reported following occlusion of the donor chamber in an *ex vivo* human skin study (Treffel et al., 1992). Though occlusion of the donor chamber may not entirely replicate the occlusive effects of an emollient applied after a TCS, the occlusive nature of some semi solid topical formulations has been reported. Barry and Woodford (1975), for example, demonstrated that occlusion, and re-occlusion, of a range of TCS ointment formulations induced skin blanching no more than the non-occluded application of the same products, attributing this to the inherent occlusive nature of the formulation base. It is possible that Hydromol Intensive cream may have enhanced permeation through occlusion, in addition to the actions of urea. Equally, if other emollient products are applied after a TCS, they may exhibit similar occlusive effects and enhance drug delivery to the skin, a potential concern for the safety profiles of potent and very potent TCSs.

4.4 Conclusion

The work conducted in this Chapter was with an aim to elucidate the emollient effects on TCS drug delivery to *ex vivo* skin when the order of application and time interval between the applications of Elocon cream and emollients were altered. On analysis, drug delivery to the skin was altered to varying extents depending on the application protocol employed and the emollient applied when compared to Elocon cream alone.

In the case of Diprobace cream and Diprobace ointment, a general trend was observed where the application of Elocon cream after either emollient resulted in a significant decrease in total drug delivery to the skin and pseudo steady state drug flux when compared to Elocon cream alone. Analysis of the permeation parameters largely attributed the reduced drug flux to a significant decrease in the apparent partition co-efficient and an unaltered apparent diffusion co-efficient when compared to Elocon cream alone. Expanding on the findings of Chapter 2, it was hypothesised that applying Elocon cream after Diprobace cream or Diprobace ointment potentially created (i) a new 'premixed' formulation on the surface of the skin which reduced drug partitioning towards the skin and (ii) a residual emollient layer on the surface of the skin acting as an additional barrier to drug delivery to the skin. It was thought this was partially attributable to a dilution effect, where the introduction of Diprobace cream or Diprobace ointment to Elocon cream on the skin surface was likely to reduce the degree of saturation of mometasone furoate in the new 'premixed' layer, thus reduce the thermodynamic activity of the drug in the formulation(s) and drug flux across the skin. With regards to Diprobace cream, it was established that the new 'premixed' layer on the surface of the skin was also likely to result in rapid formation of drug crystals on introduction of the emollient to Elocon cream, contributing to the reduced drug partitioning from the formulation towards the skin. This finding further supported the hypothesis of a high degree of drug saturation in Elocon cream as reported in Chapter 2.

In addition to the impact of application protocols on drug delivery to the skin, an emollient specific effect was distinctly evident when Hydromol Intensive cream was applied with Elocon cream, with total drug delivery to the skin significantly increasing when compared to Elocon cream alone,

irrespective of the application protocol employed. The data indicated that introduction of urea to Elocon cream on the skin surface could potentially enhanced percutaneous absorption, a finding which is of particular concern for treatments involving potent or very potent TCSs.

Should these findings translate *in vivo*, healthcare professionals and patients will need to be aware of the impact of varying application protocols on drug delivery to the skin and prescribers may need to select TCS and emollient combination therapies with an appreciation for particular emollient effects on TCS performance.

Chapter Five:

An evaluation of the Aron regimen: Drug stability, percutaneous absorption and skin retention of betamethasone dipropionate and fusidic acid from the Aron mix

5.1 Introduction

The work presented in Chapter 2, Chapter 3 and Chapter 4 investigated the impact of emollient products on TCS delivery to the skin. Significant changes in the percutaneous absorption and skin retention of two TCSs (mometasone furoate and clobetasol propionate) were found when the products were applied to the skin surface at similar times. It was clear, for some product combinations, that mixing TCSs and emollients resulted in complex formulation changes which altered the delivery profile of the TCSs to extents not explained by simple dilution alone. In some clinical cases, a third topical product, topical antibiotics, may be prescribed alongside TCS and emollient therapy to treat areas of infected eczema. The impact of another drug (topical antibiotic) in a further complex system (TCS, topical antibiotic and emollient) on the delivery of both agents to the skin has yet to be evaluated and hence this became the focus of the work presented in this Chapter.

Alongside the prescribing of topical antibiotics for the restricted application to affected sites only, in severe cases of atopic eczema unresponsive to treatment with topical corticosteroids, calcineurin inhibitors, systemic anti-inflammatory treatment or phototherapy patients may seek alternative, unconventional treatments in an attempt to manage the skin condition. Once such emerging treatment is the Aron Regimen, pioneered by Dr Richard Aron, a therapy with a focus on decolonising the skin of *S. aureus* (Aron, 2019). The causative link between *S. aureus* skin colonisation and atopic dermatitis has been reported, with evidence that δ - toxin, a peptide toxin secreted by *S. aureus* cells, promotes mast cell degranulation and may thus play a critical role in the pathogenesis of atopic dermatitis (Nakamura et al., 2013; Schlievert et al., 2008). Indeed, it is thought that *S. aureus* is extensively present in areas of affected and unaffected skin in up to 90 % of atopic dermatitis patients (Wollenberg et al., 2018b). Building on this premise, the Aron regimen employs a three-component system of commonly prescribed topical products in the treatment of skin conditions: an emollient, a topical corticosteroid and a topical antibiotic. However, these products are compounded into one tailored formulation (the Aron mix). Unlike the conventional application of these products, the Aron regimen entails frequent application of the Aron mix to all affected, and unaffected, areas of the body up to six times a day for one to two weeks, after which the frequency of application is gradually

tapered down according to response to treatment. Dr Aron's rationale behind heavily diluting the TCS and topical antibiotic is to allow uninterrupted therapy (and more frequent applications) thereby preventing the risk of 'steroid rebound' or recolonisation of the skin by *S. aureus*. However, the findings of the work presented in Chapter 2 and Chapter 3 indicate that dilution of a topical product does not result in a proportional, nor predictable, decrease in drug delivery to the skin, and an assumption that it does fails to consider the complex formulation changes that may be occurring when diluting topical medicinal products with an emollient dissimilar to the product base. Thus, it should not be readily assumed that dilution of a TCS or topical antibiotic proportionally reduces drug thermodynamic activity and the potency of the product, thus associated side effects. Consistent with these findings, a poor correlation between TCS dilution and potency is largely reported in literature (Gibson et al., 1982; Refai & Müller-Goymann, 1999; Stoughton & Wullich, 1989).

Whilst the success of the Aron regimen has been reported by patients, carers and Dr Aron (Aron, 2019; The Daily Telegraph, 2014; The Guardian, 2018), the body of evidence is largely anecdotal. To date, a single case series has attempted to evaluate the efficacy of the Aron regimen (Lakhani et al., 2017). However, this study was unblinded, lacked a control group and was conducted retrospectively; thus, variables such as adherence to the Aron regimen or the simultaneous use of additional therapy were not controlled, or accounted for. Furthermore, a double blinded randomised controlled trial evaluating the benefits of TCS and antibiotic therapy over TCS therapy alone in the treatment of *S. aureus* infected atopic eczema and dermatitis found both treatments to provide equivalent therapeutic effects (Gong et al., 2006). However, whilst the use of topical antibiotics should be limited where eczema flares do not show signs of a severe infection, cases of severely infected eczema are likely to benefit from topical (or oral) antibiotic treatment (Francis et al., 2016; National Institute for Health and Care Excellence, 2018b). Previous investigations into the effect of TCS dilution with emollients on drug delivery to the skin revealed that emollient excipients with penetration enhancing capabilities can significantly enhanced drug delivery to the skin, despite a two fold dilution of the saturated formulation (Chapter 2). Similarly, substantial dilution of the topical antibiotic with an emollient base and TCS formulation may introduce excipients with penetration enhancing capacity to the antibiotic formulation resulting in

greater antibiotic delivery to affected and unaffected skin sites than anticipated. This raises the question of whether it is suitable (or indeed beneficial) for patients to apply the Aron regimen to all affected, and unaffected, skin areas as frequently as recommended by Dr Aron.

The wide spread application of topical antibiotic for extended periods opposes current recommendations that the treatment is limited to two weeks (Eichenfield et al., 2014; National Institute for Health and Care Excellence, 2018b; Wollenberg et al., 2018a) and the role of this therapy in increasing antibiotic resistance in the community warrants exploration given that high levels of fusidic acid resistant *S. aureus* have been reported in community settings (Shah & Mohanraj, 2003). Additionally, the long term impact of frequently applying heavily diluted, though not necessarily lower potency, TCSs on large areas of the skin for extended periods has not been established. From the formulation considerations raised in previous Chapters, mixing topical products can induce complex formulation changes in the final product resulting in an altered drug delivery profile of the medicinal products. It was postulated that these changes were as a result of altered drug and solvent thermodynamic activities in the mixed formulations relative to the individual marketed products. These considerations are equally applicable to the extemporaneous mixing of a TCS, antibiotic and emollient according to the Aron regime. Fucidin cream (2 % fusidic acid), for example, is formulated with a proportion of glycerol which can enhance the water holding capacity of the stratum corneum and increase drug permeation (Batt et al., 1988). Dilution of this excipient on introduction of the TCS and emollient to the topical antibiotic may reduce the solvent thermodynamic activity and penetration enhancing capabilities across skin, simultaneous to potential decreases in TCS and topical antibiotic thermodynamic activities in the mixed formulation. Diprosone cream (0.05 % betamethasone dipropionate) and Fucidin cream also contain proportions of antimicrobial preservatives, antioxidants and buffering agents to maintain the chemical and physical stability of the marketed preparations. The extemporaneous dilution of these products may lead to chemical and physical instabilities in the mixed formulation, further altering the stability, expected shelf life and performance of the extemporaneously prepared Aron mix to an unpredictable extent.

Chapter 5

Thus, the objectives of the work described in this Chapter were to evaluate the Aron mix for drug stability and *ex vivo* formulation performance compared to the applications of a TCS alone and topical antibiotic alone. To achieve these objectives the chemical and physical stability of the Aron mix was assessed over the recommended period of use (two months). The percutaneous absorption and skin distribution of fusidic acid from Fucidin cream, betamethasone dipropionate from Diprosone cream and both drugs from the Aron mix were investigated. To complement these data, drug transport studies were performed on silicone membrane to evaluate the effect varying the ratios of the TCS, topical antibiotic and emollient in the Aron mix on drug transport, particularly important as tailoring the Aron regimen to patient age, weight and severity of the condition is one of the cornerstones of Dr Aron's treatment.

5.2 Materials and methods

5.2.1 Materials

Fusidic acid (Ph Eur) and betamethasone dipropionate (Ph Eur) were acquired from Carbosynth Ltd (Compton, UK). Diprosone cream and Diprobase cream were acquired from the University of Hertfordshire campus pharmacy (Hertfordshire, UK). Fucidin cream was acquired from Bushey Pharmacy (Bushey, UK). Raman grade calcium fluoride slides were acquired from Crystran Ltd (Dorset, UK). Phosphate buffered saline (PBS) tablets, acetonitrile (HPLC grade) and absolute ethanol (99 + %) were acquired from Fisher Scientific (Leicestershire, UK). Sodium chloride (Ph Eur) was acquired from Sigma Aldrich (Dorset, UK).

5.2.2 Analytical method development

5.2.2.1 Development of the HPLC method for the quantification of betamethasone dipropionate and fusidic acid

The HPLC system was comprised of an Agilent 1260 Infinity quaternary pump and high performance autosampler coupled to an Agilent 1260 multi wavelength UV/Vis detector set to 210 nm for fusidic acid detection and 240 nm for betamethasone dipropionate detection (Agilent Technologies, UK). Chromatographic analysis was performed using a reverse phase Kinetex™ C₁₈ column (5 μ particle size, 250 mm x 4.6 mm; Phenomenex, UK) in conjunction with a SecurityGuard™ guard cartridge system packed with a C₁₈ cartridge (4 mm x 3 mm; Phenomenex, UK), a sample injection volume of 40 μL and a constant flow rate of 1 mL min⁻¹. The final mobile phase for gradient elution of fusidic acid and betamethasone dipropionate is summarised in Table 5-1. The Agilent ChemStation software (Agilent Technologies, UK) was used for data acquisition. Under these conditions, betamethasone dipropionate eluted at 13.8 min and fusidic acid eluted at 14.4 min.

Table 5-1: The HPLC – UV gradient profile employed for the detection and quantification of fusidic acid and betamethasone dipropionate in standards and samples.

Time (min)	Mobile phase composition (%)		
	Mobile phase A: Sodium phosphate buffer, pH 3.1	Mobile phase B: HPLC grade Acetonitrile	Mobile phase C: Water (18.2 MΩ cm ⁻¹)
0	65	35	0
5	65	35	0
15	30	70	0
16	0	95	5
20	0	35	65
22	0	35	65
23	65	35	0
24	65	35	0

5.2.2.2 Preparation of mobile phase

The mobile phase comprised a 30 mM sodium phosphate buffer solution (pH 3.1), HPLC grade acetonitrile and deionised water (18.2 MΩ MilliQ). To prepare 1 L of the buffer solution, 2.94 g of phosphoric acid was weighed into a 1 L volumetric flask with 7.594 g of sodium chloride. The volumetric flask was made up to approximately 900 mL with water (18.2 MΩ cm⁻¹, Milli-Q), a stirrer bar was introduced to the flask and the solution was stirred for 30 minutes to ensure dissolution of the powders. The pH of the buffer solution was then adjusted to pH 3.1 using a 1 M sodium hydroxide solution then made up to volume with water (18.2 MΩ cm⁻¹, Milli-Q). The final buffer solution was stirred thoroughly, filtered through a 0.2 μm nylon filter and stored for up to 1 week at 2 – 8 °C. All mobile phase solutions were degassed prior to use.

5.2.2.3 Preparation of calibration standards

A 100 μg mL⁻¹ combined stock solution of fusidic acid and betamethasone dipropionate was prepared by weighing 10 mg of each drug into a 100 mL volumetric flask and making up to volume with the diluent, acetonitrile. Calibration standards, over the nominal concentration range 0.01 μg mL⁻¹ – 100

$\mu\text{g mL}^{-1}$ were prepared by appropriate dilution of the stock solution with the diluent. Drug quantification was achieved using HPLC method summarised in Section 5.2.2.1.

5.2.2.4 Forced degradation of betamethasone dipropionate and fusidic acid

To ensure that potential drug degradation products were sufficiently resolved from the drug peaks of interest, the potential for betamethasone dipropionate or fusidic acid to degrade under acidic, basic or oxidative conditions was investigated and analysed by HPLC. Stock solutions (1 mg mL^{-1}) of betamethasone dipropionate and fusidic acid were prepared by weighing 100 mg of drug into a 100 mL volumetric flask and making up to volume with acetonitrile. Following this, 5 mL of 1 M sodium hydroxide (NaOH), 1M hydrochloric acid (HCl) or hydrogen peroxide (H_2O_2 ; 3 % w/w) were added to vials containing 5 mL of the stock solution and the vials were stored at $60\text{ }^\circ\text{C}$ for 24 h. After this period, aliquots of the samples were filtered, adjusted to pH 3 using NaOH or HCl as required and diluted appropriately with acetonitrile: water (50:50) for analysis using the HPLC method presented in Section 5.2.2.1.

5.2.2.5 Determination of the fitness for purpose of the analytical method

The HPLC method was validated for linearity, precision and accuracy in accordance with the International Conference on Harmonization (ICH) guidelines (ICH, 2005). Linearity was determined by the correlation coefficient (R^2) for concentrations ranging from $0.05\text{ }\mu\text{g mL}^{-1}$ to $100\text{ }\mu\text{g mL}^{-1}$. The standard error for the predicted y value for all x values in the regression (STEYX) was calculated and used, as previously described, in Equation 2-1 and Equation 2-2 to calculate the limits of detection (LOD) and limits of quantification (LOQ), respectively, for betamethasone dipropionate and fusidic acid (Section 2.2.2.3).

Determination of the precision of the analytical method was achieved by intra-day and inter-day analysis. Intra-day precision was measured by 6 replicate injections of $5\text{ }\mu\text{g mL}^{-1}$, $50\text{ }\mu\text{g mL}^{-1}$ and $100\text{ }\mu\text{g mL}^{-1}$ samples of standards of betamethasone dipropionate and fusidic acid prepared on the same day. Inter-day precision was assessed through the analysis of 6 replicate injections of $5\text{ }\mu\text{g mL}^{-1}$, $50\text{ }\mu\text{g mL}^{-1}$ and $100\text{ }\mu\text{g mL}^{-1}$ samples prepared in triplicate on 3 separate days.

The accuracy of the analytical method was evaluated by preparing triplicate samples of betamethasone dipropionate and fusidic acid in the diluent at three concentrations (low, medium and high) and quantifying using the gradient HPLC UV method. Accuracy was determined as previously described using Equation 2-3 (Section 2.2.2.3).

5.2.3 Formulation selection

The products used in the Aron mix were Diprosone cream, Fucidin cream and Diprobase cream. The listed excipients for the formulations are presented in Table 5-2.

Table 5-2: The listed excipients for Diprosone cream, Fucidin cream and Diprobase cream. Data were obtained from the most recently published summary of product characteristics for the respective formulation.

Diprosone cream	Fucidin cream	Diprobase cream
Betamethasone dipropionate 0.064% w/w	Fusidic acid 2 % w/w	Chlorocresol
Sodium dihydrogen phosphate dihydrate	Butylhydroxyanisole (E320)	Macrogol Cetostearyl Ether (Cetomacrogol)
Phosphoric acid	Cetanol	Cetostearyl alcohol
White soft paraffin	Glycerol	Liquid paraffin
Liquid paraffin	Liquid paraffin	White soft paraffin
Cetomacrogol 1000	Potassium sorbate	Phosphoric acid
Cetostearyl alcohol	Polysorbate 60	Sodium dihydrogen phosphate
Sodium hydroxide	White soft paraffin	Sodium hydroxide
Purified water	All- <i>rac</i> - α -tocopherol	Purified water
Sodium dihydrogen phosphate dihydrate	Hydrochloric acid	
Chlorocresol	Purified water	

5.2.4 Stability testing of the Aron formulation

Three batches of the Aron mix were prepared by weighing appropriate amounts of Fucidin cream, Diprosone cream and Diprobase cream in a 1:2:20 ratio into a glass dish. The products were mixed for approximately 1 min to ensure homogeneity and 10 g samples of the Aron mix were transferred to clear borosilicate glass vials for storage. Sample vials were sealed with parafilm to prevent evaporation during the experimental period. Additional samples were stored in clear plastic screw cap containers to investigate the suitability of the primary packaging material to prevent weight change during storage.

Sufficient samples were prepared to enable full investigation over the experimental period (one sample vial per batch, per timepoint) and the weight of each sample vial was recorded prior to commencing the stability investigation. Representative samples from each batch were then stored at 25 °C, 2-8 °C, 30 °C and 40 °C until analysis. At specified time points over 2 months, samples were removed from storage to analyse the visual appearance, weight loss of product, change in apparent pH and drug content. The timepoints were 0, 1 day, 3 days, 5 days, 7 days, 14 days, 21 days, 28 days and 56 days.

5.2.4.1 Physical stability of the Aron mix: Visual appearance, weight loss and apparent pH

At the selected time point, samples were removed from storage at 25 °C, 2-8 °C, 30 °C and 40 °C, the vial was dried and reweighed to calculate weight loss. The visual appearance of the formulation was assessed by noting the formulation colour, clarity and physical state. An analysis of the apparent pH of the formulation was conducted using a calibrated pH meter (Hanna Instruments, UK). The pH electrode tip was fully submerged in the formulation and gently swirled to ensure contact of the formulation with the electrode. All pH readings were recorded to ± 0.01 pH unit.

5.2.4.2 Chemical stability of the Aron mix: Development of an extraction method for determination of betamethasone dipropionate and fusidic acid in the Aron mix.

To develop an extraction method to suitably recover and quantify betamethasone dipropionate and fusidic acid in the Aron mix, the suitability of acetonitrile as an extraction solvent was investigated. Acetonitrile was selected based on the findings of the drug stability investigations (discussed in Section 5.2.8.5). A 1 g sample of Fucidin cream was weighed into a 100 mL volumetric flask to achieve a fusidic acid target concentration of 200 $\mu\text{g mL}^{-1}$. A 2 g sample of Diprosone cream was weighed into a 100 mL volumetric flask to achieve a betamethasone dipropionate target concentration of 10 $\mu\text{g mL}^{-1}$. All samples were prepared in triplicate. The volumetric flasks were filled to volume with acetonitrile, a stirrer bar was introduced to each flask and samples were stirred for 24 h at room temperature. At 24 h, aliquots of each sample were filtered through 0.45 μm PTFE filters, appropriately diluted in acetonitrile if required and analysed using the HPLC method summarised in Section 5.2.2.1.

5.2.5 Chemical stability of the Aron mix: Determining the content of betamethasone dipropionate and fusidic acid in the Aron mix

At the selected time points, a 1 g sample of the Aron mix was weighed into a 100 mL volumetric flask. The flask was filled to volume with the extraction diluent (acetonitrile), a stirrer bar was introduced to the flask and the samples were stirred for 24 h at room temperature. At 24 h, aliquots of each sample were filtered through 0.45 µm PTFE filters, appropriately diluted in acetonitrile and analysed on the HPLC method summarised in Section 5.2.2.1.

5.2.6 Raman microscopy of Diprosone cream, Fucidin cream and Aron mix 9

Raman microscopy of crystalline structures observed in Diprosone cream, Fucidin cream and Aron mix 9 was performed using a Renishaw inVia Raman microscope (Renishaw Plc, UK), calibrated for peak position and intensity using a silicon reference block. Aron mix 9 was prepared 1 h in advance of analysis by weighing appropriate amounts of Fucidin cream, Diprosone cream and Diprobase cream in a 1:2:10 ratio into a glass dish and mixing thoroughly to ensure homogeneity. Samples of Diprosone cream alone, Fucidin cream alone and Aron mix 9 were mounted on Raman grade calcium fluoride slides for spectral analysis. Raman spectra were obtained using the x 100 long working distance magnification lens, a laser excitation wavelength of 785 nm, five accumulations per sample and an acquisition time of 10 s. Three replicate areas were scanned for each analysis and the single, most representative spectrum selected for presentation.

5.2.7 Franz cell assembly

Full thickness human scrotal skin was prepared as detailed in Section 2.2.4.1. Franz cells were assembled as detailed in Section 2.2.4.2.

5.2.8 Franz cell method development

5.2.8.1 Selection of the receiver fluid system for *ex vivo* Franz cell experiments

To ensure adequate solubility of fusidic acid and betamethasone dipropionate in the receiver fluid, the solubility of fusidic acid and betamethasone dipropionate in PBS alone (pH 7.4) and PBS with ethanol (10 %, 20 % or 30 %) was determined. Saturated solutions of betamethasone dipropionate or fusidic acid were prepared independently as follows: adequate amounts of each drug were added to the range of solutions until the formation of a suspension (determined by the presence of visible particles in solution) and then stirred for 24 h at room temperature. Aliquots of samples were filtered through Millex Millipore 0.22 µm syringe filters, appropriately diluted in acetonitrile and drug content was quantified using the HPLC method summarised in Section 5.2.2.1.

5.2.8.2 Determining drug – filter binding

Membrane binding studies were conducted to determine whether fusidic acid or betamethasone dipropionate had the potential to bind to PTFE filters during the drug extraction process. Saturated solutions of fusidic acid and betamethasone dipropionate in acetonitrile were prepared as detailed in Section 5.2.2.2 and filtered through a 0.22 µm PTFE filter. Aliquots of filtered and unfiltered solutions were diluted prior to quantification, achieved using the HPLC method summarised in Section 5.2.2.1.

5.2.8.3 Selecting the appropriate receiver fluid sampling time points for *in vitro* and *ex vivo* Franz cell experiments

A study was conducted to establish a sampling protocol to adequately profile betamethasone dipropionate and fusidic acid transport across silicone membrane whilst maintaining sink conditions for the duration of the experiment. Franz cells (n=3) were assembled with silicone membrane and the receiver chamber filled with the receiver fluid system developed in Section 5.2.8.1 (PBS pH 7.4 and ethanol; 70:30 or 80:20). The membrane was dosed with 500 mg of Fucidin cream or Diprosone cream by weight. Samples (200 µl) of the receiver fluid were taken periodically up to 26 h and replaced with fresh preheated receiver fluid. A sampling protocol was also required to investigate the permeation of both drugs across human skin following the finite dosing of Diprosone cream and Fucidin cream. To

achieve this, skin samples were mounted in Franz cells (n=3) and the receiver chamber filled with the receiver fluid system developed in Section 5.2.8.1 (PBS pH 7.4 and ethanol; 70:30 or 80:20). Skin samples were dosed with 10 μL of Diprosone cream or Fucidin cream and samples (200 μL) of the receiver fluid were taken periodically up to 24 h and replaced with fresh preheated receiver fluid. The drug concentration at each time point was determined using the HPLC method summarised in Section 5.2.2.1.

5.2.8.4 Development of drug extraction method for betamethasone dipropionate and fusidic acid from skin matrices

The suitability of acetonitrile as an extraction solvent for fusidic acid and betamethasone dipropionate from all matrices (skin surface and donor chamber, epidermal membrane, dermal membrane), following 24 h drug skin permeation experiments, was investigated. Using a positive displacement pipette, 10 μL of a 1 mg mL^{-1} solution of fusidic acid or betamethasone dipropionate in acetonitrile, prepared as detailed in Section 5.2.2.3, were added to vials containing: cotton buds, tape strips, epidermal membranes, dermal membranes and an empty vial serving as the control. All vials were left at 37 °C for 24 h. Following this period, 1 mL of acetonitrile was added to each vial, the vials sonicated for 10 minutes and placed on a roller shaker for 18 h. Extraction solvents were removed entirely from the vials, filtered through 0.22 μm PTFE filters and quantified using the HPLC method summarised in Section 5.2.2.1. Following data analysis, it was deemed necessary to conduct a second extraction to ensure full recovery ($100 \pm 10\%$ of dose applied) of fusidic acid and betamethasone dipropionate from all matrices.

5.2.8.5 Stability of betamethasone dipropionate and fusidic acid in the extraction solvent and receiver fluid systems

To ascertain the potential for betamethasone dipropionate or fusidic acid to degrade in the selected receiver fluid system over the experimental period, drug stability in PBS pH 7.4 and ethanol (80:20 and 70:30) was determined when samples were stored at 37 °C for 24 h. To ascertain the potential for betamethasone dipropionate or fusidic acid to degrade in the selected receiver fluid systems (PBS pH 7.4 and ethanol; 70:30 or 80:20) or extraction solvent (acetonitrile) during storage, drug stability in each solvent system was determined when samples were stored at 25 °C and 2-8 °C for two weeks. Stock

solutions of 100 $\mu\text{g mL}^{-1}$ of betamethasone dipropionate and fusidic acid in the solvent systems were prepared as detailed in Section 5.2.2.3 and aliquots stored in the respective conditions. Samples were analysed at 0 days, 1 day, 5 days, 7 days and 14 days following storage, using the HPLC method summarised in Section 5.2.2.1. To indicate stability, the concentrations of fusidic acid or betamethasone dipropionate at each time point were compared to the respective drug concentration in freshly prepared samples.

5.2.9 *In vitro* silicone membrane drug transport studies

5.2.9.1 Varying the ratios of Diprosone cream to Diprobase cream in the Aron mix

To investigate the effect of varying the ratios of Diprosone cream to Diprobase cream on betamethasone dipropionate transport across a synthetic membrane, an *in vitro* drug transport study was conducted using variations of the Aron mix with a fixed ratio of Fucidin cream. The ratios of Fucidin cream to Diprosone cream to Diprobase cream were: Aron mix 1 (1:2:20), Aron mix 2 (1:4:18), Aron mix 3 (1:6:16) and Aron mix 4 (1:10:12). The formulations were prepared 1 h prior to dosing using the method detailed in Section 5.2.4. Franz cells were assembled with silicone membrane as detailed in Section 2.2.4.2. Informed by the findings of the receiver fluid system development studies, the receiver chamber was filled with PBS pH 7.4 and ethanol (70:30) and Franz cells were equilibrated in a water bath set to 37 °C. Following the equilibration period, Franz cells were briefly removed from the water bath and the membrane was dosed with 1 g of Diprosone cream alone, Fucidin cream alone, Aron mix 1, Aron mix 2, Aron mix 3 or Aron mix 4 applied to the donor chambers by weight. The formulations were carefully spread over the membrane surface using a spatula to ensure contact with the membrane. Six replicate Franz cells were assembled for each formulation investigated. Samples (200 μl) of the receiver fluid were taken periodically up to 26 h and replaced with fresh preheated receiver fluid. Drug quantification in samples was achieved using the HPLC method summarised in Section 5.2.2.1.

5.2.9.2 Varying the ratios of Fucidin cream to Diprobase cream in the Aron mix

To investigate the effect of varying the ratios of Fucidin cream to Diprobase cream on fusidic acid transport across silicone membrane, an *in vitro* drug transport study was conducted using variations of

the Aron mix with a fixed ratio of Diprosone cream. The experimental design was similar to that described in Section 5.2.9.1 but the ratios of Fucidin cream to Diprosone cream to Diprobase cream were: Aron mix 5 (0.5:2:20.5), Aron mix 6 (3:2:18), Aron mix 7 (5:2:16) and Aron mix 8 (7:2:14). The receiver fluid system, dosing quantity, sampling timepoints and method for drug quantification remained as detailed in Section 5.2.9.1.

5.2.10 *Ex vivo* human skin Franz cell study: Aron mix 9

5.2.10.1 Studies investigating drug permeation across *ex vivo* human skin

Franz cells were assembled with human skin as detailed in Section 2.2.4.2. The receiver chamber was filled with a PBS pH 7.4 and ethanol mixture (80:20), informed by the findings of the receiver fluid system development studies (Section 5.2.8.1 and Section 5.2.8.5). Following the equilibration period, Franz cells were briefly removed from the water bath then skin samples were dosed with 10 μL of Diprosone cream, Fucidin cream or Aron mix 9 using a positive displacement pipette. Aron mix 9 was prepared one hour in advance of dosing in the ratios of 1:2:10 of Fucidin cream, Diprosone cream and Diprobase cream, respectively using the method detailed in Section 5.2.4. To ensure contact with the membrane, the product was carefully spread over the surface of the skin using the tip of a capillary piston and the Franz cell returned to the water bath to commence the experiment. Samples (200 μL) of the receiver fluid were taken at pre-determined intervals up to 24 h and replaced with fresh preheated receiver fluid. Drug quantification was achieved using the HPLC method summarised in Section 5.2.2.1.

5.2.10.2 Studies investigating drug penetration in *ex vivo* human skin

Following the drug permeation across human skin studies, Franz cells were disassembled and the drug content on the skin surface (residual formulation), the epidermis and dermis were determined as previously described in Section 2.2.8.2 (Chapter 2). Quantification of betamethasone dipropionate and fusidic acid in the cotton buds, tape strips, epidermis and dermis was achieved using the extraction method developed in Section 5.2.8.4. All samples were analysed by the gradient elution analytical method summarised in Section 5.2.2.

A summary of the experimental design employed for all Franz cell studies is presented in Figure 5-1. The compositions of all Aron mix formulations investigated are presented in Table 5-3.

5.2.11 Data treatment and statistical analysis

The concentration of betamethasone dipropionate and fusidic acid in the receiver fluid was corrected for previous sample removal and profiles constructed to present cumulative amount of drug permeated per unit area ($\mu\text{g cm}^{-2}$) over the exposure period. Linear regression was performed on infinite dose data sets to determine mean drug flux.

Experimental data were expressed as mean ($n = 6$) \pm standard deviation (SD), unless otherwise stated. Statistical analysis was performed using Prism 8.0 (GraphPad, USA). The Shapiro Wilk test was employed to determine the normality of all data sets. Non-parametric analysis for multiple comparisons was performed using Kruskal-Wallis and a Mann–Whitney test applied for post hoc analysis. Parametric analysis for multiple comparisons was performed using analysis of variance (ANOVA) and Tukey's post hoc test. Statistically significant differences were determined at a 95 % confidence interval ($p \leq 0.05$).

Experimental design for investigated formulations		
	<i>In vitro</i> Franz cell experiments	<i>Ex vivo</i> Franz cell experiments
Membrane	Silicone	Human skin
Dosing model	Infinite	Finite
Application protocol	Diprosone cream alone Fucidin cream alone Aron mix 1 Aron mix 2 Aron mix 3 Aron mix 4 Aron mix 5 Aron mix 6 Aron mix 7 Aron mix 8	Diprosone cream alone Fucidin cream alone Aron mix 9

Figure 5-1: Summary of the experimental design employed for *in vitro* (Aron mixes 1-8) and *ex vivo* (Aron mix 9) Franz cell experiments.

Table 5-3: The percent compositions of Fucidin cream, Diprosone cream and Diprobace cream in the Aron formulations investigated.

	Percent composition of products in the Aron mix		
	Fucidin cream	Diprosone cream	Diprobace cream
Aron mix 1 (1:2:20)	4.35	8.70	86.96
Aron mix 2 (1:4:18)	4.35	17.39	78.26
Aron mix 3 (1:6:16)	4.35	26.09	69.57
Aron mix 4 (1:10:12)	4.35	43.48	52.17
Aron mix 5 (0.5:2:20.5)	2.17	8.70	89.13
Aron mix 6 (3:2:18)	13.04	8.70	78.26
Aron mix 7 (5:2:16)	21.74	8.70	69.57
Aron mix 8 (7:2:14)	30.43	8.70	60.87
Aron mix 9 (1:2:10)	7.69	15.38	76.92

5.3 Results and discussion

5.3.1 Analytical methods

A gradient elution HPLC method with dual wavelength detection was developed to enable the simultaneous quantification of betamethasone dipropionate and fusidic acid in samples following *in vitro* and *ex vivo* Franz cell experiments. Calibration standards were prepared over a nominal concentration range and analysed by the HPLC method detailed in Section 5.2.2.1. The elution time of excipients from Fucidin cream, Diprosone cream and Diprobase cream in addition to extracts from skin endogenous compounds, cotton buds and scotch tape were determined to ensure no interference with the peaks of interest. The resulting HPLC gradient method enabled drug elution with suitable retention times and sufficient resolution from all other peaks. Sample chromatograms of betamethasone dipropionate and fusidic acid detected at 240 nm and 210 nm are presented in Figure 5-2(a) and Figure 5-2 (b), respectively.

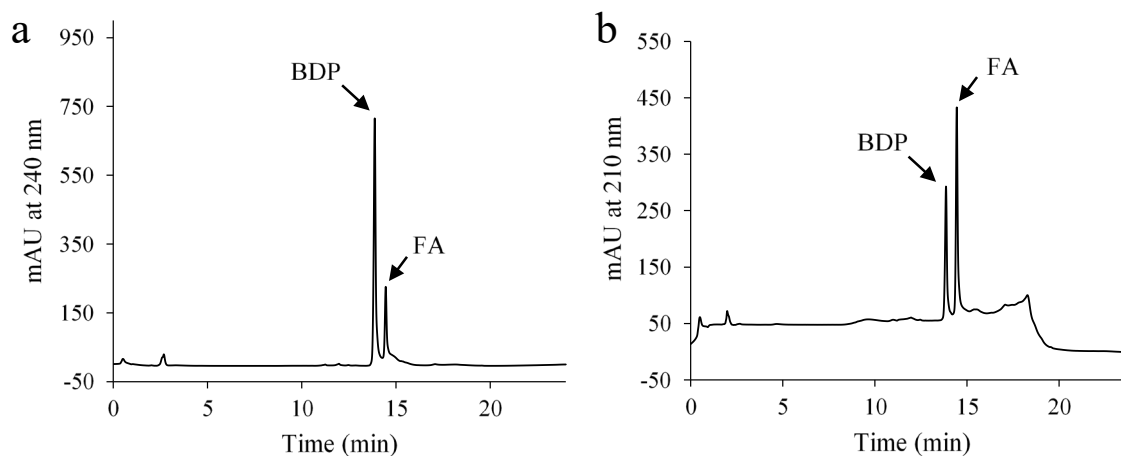


Figure 5-2: Sample chromatograms obtained following the analysis of a combined stock solution of betamethasone dipropionate (BDP) and fusidic acid (FA) at $100 \mu\text{g mL}^{-1}$, detected at 240 nm for BDP quantification and 210 nm for FA quantification. The drug elution time was 13.8 min for BDP and 14.4 min for FA.

To ensure that the degradation products of betamethasone dipropionate and fusidic acid did not interfere with elution of either drug peak, betamethasone dipropionate and fusidic acid were exposed to three stress conditions (acidic, basic and oxidative) for 24 h and the HPLC method was evaluated for specificity at both wavelengths (210 nm and 240 nm). The percent recovery of betamethasone dipropionate and fusidic acid and the retention times of additional peaks present at 210 nm and 240 nm are presented in Table 5-4.

Table 5-4: The percent recovery of betamethasone dipropionate (BDP) and fusidic acid (FA) and retention times of degradation peaks when exposed to acidic (HCl), basic (NaOH) and oxidative (H₂O₂) conditions. Assay specificity was conducted at two wavelengths: 240 nm for quantification of betamethasone dipropionate and 210 nm for quantification of fusidic acid. Percent recovery is presented as the mean of three replicates.

Stress condition	Recovery (%)	Retention times of additional peaks at 240 nm (min)	Retention times of additional peaks at 210 nm (min)
None - FA control	99.85	-	-
FA in 1 M NaOH, 60 °C, 24 h	1.85	2.15, 4.53, 6.92, 7.52, 11.32, 14.76, 16.24	2.35, 3.96, 6.92, 7.52, 11.32, 12.72, 13.78, 13.94, 14.76, 15.86, 16.24
FA in 1 M HCl, 60 °C, 24 h	3.36	4.53, 6.92, 7.52, 11.32, 11.83, 14.76	2.35, 3.96, 6.92, 7.52, 11.32, 11.85, 12.72, 13.78, 14.76, 15.86
FA in 3 % v/v H ₂ O ₂ , 60 °C, 24 h	0.20	2.15, 4.53, 6.92, 7.52, 11.32, 12.08, 12.12	2.35, 3.96, 6.92, 7.52, 11.32, 12.08, 12.12, 12.72
None – BDP control	99.90	-	-
BDP in 1 M NaOH, 60 °C, 24 h	0.16	2.15, 6.92, 7.52, 10.54, 11.32, 12.3, 14.76	2.35, 6.92, 7.52, 11.32, 12.72, 13.76, 14.76, 15.86
BDP in 1 M HCl, 60 °C, 24 h	8.35	6.92, 7.52, 11.32, 12.00, 14.78	2.35, 3.96, 6.92, 7.52, 11.32, 12.08, 12.72, 13.68, 14.78, 15.85
BDP in 3 % v/v H ₂ O ₂ , 60 °C, 24 h	6.91	4.53, 6.92, 7.52, 11.32, 14.78	2.72, 4.52, 6.92, 7.52, 11.32, 15.39

Betamethasone dipropionate and fusidic acid degraded to varying extents under the stress conditions investigated. The percent recovery was lowest for betamethasone dipropionate when exposed to basic conditions over 24 h (0.16 % drug recovery; Table 5-4). This is consistent with literature reports that betamethasone dipropionate is more stable under acidic conditions compared to basic conditions, with maximum stability of the drug achieved when pH values are between pH 4-6 (Simonsen et al., 2004). In contrast, fusidic acid demonstrated greatest susceptibility to degradation under oxidative conditions (0.20 % drug recovery; Table 5-4), a finding also reported by Sharma et al. (2019).

In addition to the retention times of the degradation peaks listed in Table 5-4, sample chromatograms of the degradation peaks are presented in Figure 5-3. Under basic conditions, a degradation product of fusidic acid was detected at 210 nm with a retention time of 13.78 min, co-eluting with betamethasone dipropionate at 13.8 min. However, this product was not detected at 240 nm, the wavelength at which betamethasone dipropionate quantification was achieved, thus would not interfere with analysis. In summary, all degradation peaks were sufficiently resolved from betamethasone dipropionate at 240 nm and fusidic acid at 210 nm to enable drug quantification at the respective wavelengths.

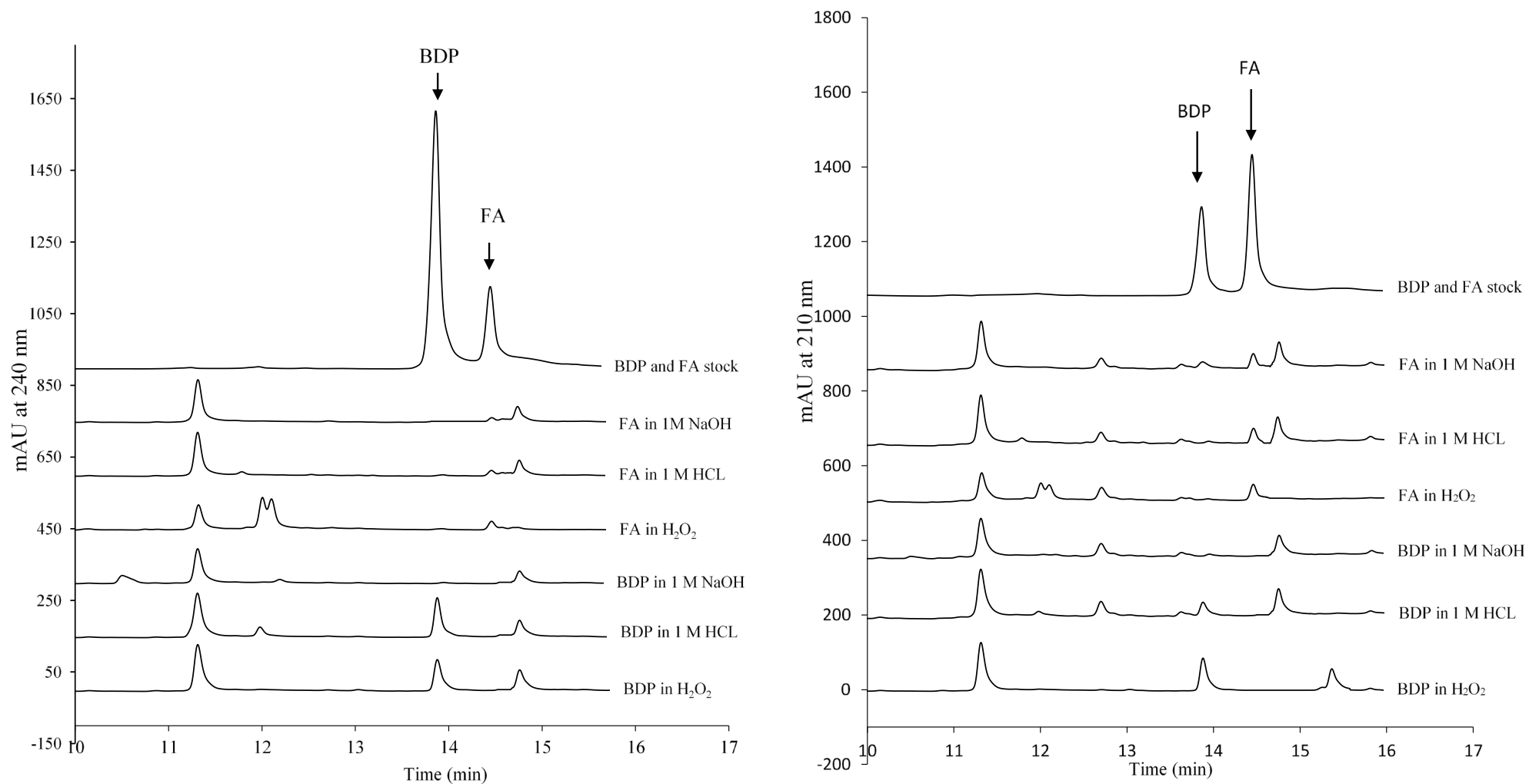


Figure 5-3: Sample chromatograms show the degradation peaks observed for betamethasone dipropionate (BDP) and fusidic acid (FA) under acidic (HCL), basic (NaOH) and oxidative (H₂O₂) conditions when detected at (a) 240 nm for quantification of BDP and (b) 210 nm for quantification of FA.

Calibration curves were constructed to enable the quantification of betamethasone dipropionate and fusidic acid in samples following formulation stability investigations, *in vitro* and *ex vivo* Franz cell studies. The calibration curves for betamethasone dipropionate and fusidic acid are presented in Figure 5-4(a) and Figure 5-4(b), respectively.

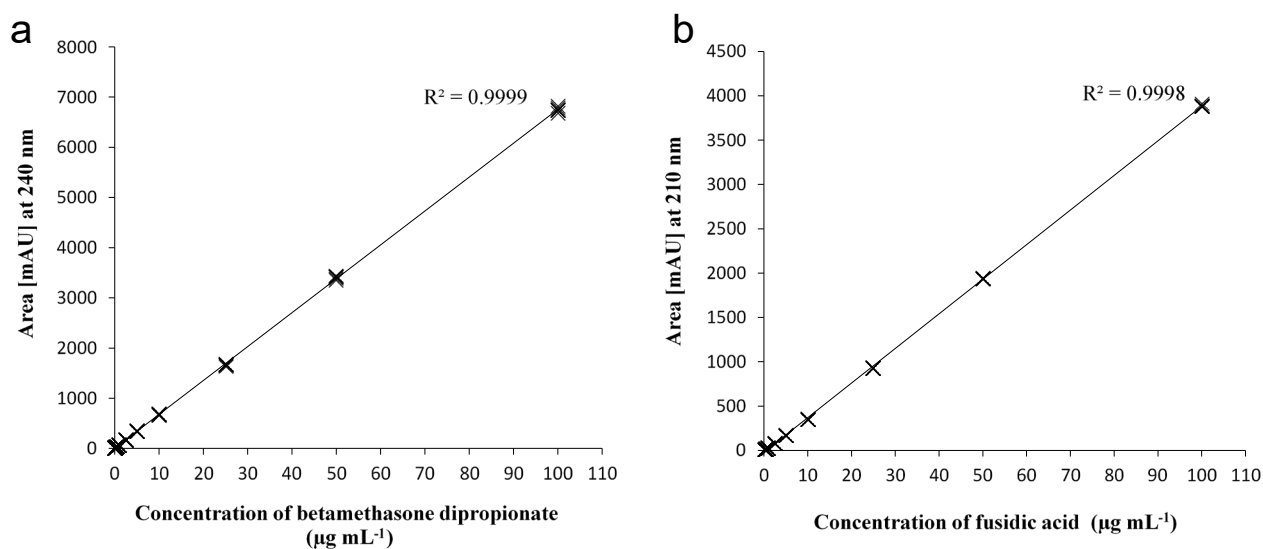


Figure 5-4: Calibration curve for (a) betamethasone dipropionate and (b) fusidic acid standards obtained following analysis with the gradient elution HPLC UV method over the concentration range of 0.05 µg mL⁻¹ to 100 µg mL⁻¹. The peak area of each injection is plotted against each concentration (six individual data points per concentration).

The analytical methods were evaluated for linearity, precision and accuracy in accordance with the International Conference on Harmonization (ICH) guidelines (ICH, 2005). The linearity, accuracy and system suitability parameters for betamethasone dipropionate and fusidic acid are summarised in Table 5-5. The analytical method implemented passed all specification criteria as defined by the ICH guidelines (ICH, 2005) with suitable LOD and LOQ levels for the quantification of small amounts of betamethasone dipropionate and fusidic acid in samples.

Table 5-5: Summary of the parameters determined for the ‘fitness for purpose’ of the HPLC method developed for quantification of betamethasone dipropionate and fusidic acid in samples.

Parameter	Betamethasone dipropionate	Fusidic acid	Limits as per ICH guidelines
Linearity (R^2)	0.9999	0.9998	> 0.999
Intra-day precision (% RSD) 5 $\mu\text{g mL}^{-1}$, 50 $\mu\text{g mL}^{-1}$ and 100 $\mu\text{g mL}^{-1}$	0.25, 0.33, 0.39	0.37, 0.29, 0.44	$\text{RSD} \leq 2\%$
Inter-day precision (% RSD) 5 $\mu\text{g mL}^{-1}$, 50 $\mu\text{g mL}^{-1}$ and 100 $\mu\text{g mL}^{-1}$	0.34, 0.52, 0.47	0.53, 0.52, 0.46	$\text{RSD} \leq 2\%$
Accuracy (%) 5 $\mu\text{g mL}^{-1}$, 50 $\mu\text{g mL}^{-1}$ and 100 $\mu\text{g mL}^{-1}$	98.85, 99.42, 101.87	98.78, 98.64, 100.41	$100 \pm 2\%$
Limit of detection ($\mu\text{g mL}^{-1}$)	0.20	0.31	Report result
Limit of quantification ($\mu\text{g mL}^{-1}$)	0.61	0.95	Report result
Tailing factor	1.2	1.5	< 2
Resolution	5.11	5.06	> 2

5.3.2 Stability testing of the Aron formulation

During pharmaceutical formulation development critical quality attributes of a topical product, such as the physical and chemical characteristics of the formulation, are evaluated to ensure they fall within appropriate limits and will maintain the product quality (ICH, 2009). The Aron mix, in comparison, is extemporaneously prepared thus is not subject to the regulatory safeguards of licensed medicinal products with no stability data currently available for the formulation. To evaluate the physical and chemical stability of the Aron mix, a series of stability investigations were conducted investigating changes in the physical appearance of the product, weight loss or gain, pH alterations and drug content over two months.

The weight change of a product can indicate whether excipient formulations are evaporating from the product (weight loss) or moisture is being absorbed into the formulation (weight gain) over the period of storage or use thus can offer an indication of the suitability of the storage container to preserve the quality of the product. When extemporaneously dispensed by pharmacies, the Aron mix is often prepared and dispensed in plastic containers (Patient Blog 1, 2014; Patient Blog 2, 2015; Patient Blog 3, 2015); thus, to replicate in use conditions, samples of Aron mix 1 were stored in plastic, screw cap containers for the weight change investigation. Weight change of the Aron mix formulation was negligible at all investigated storage conditions over two months ($p > 0.05$; Figure 5-5). An evaluation of the physical appearance of the formulations under the same conditions revealed no change in homogeneity, colour or texture over 2 months compared to the appearance of the Aron mix on commencing the experiment (data not shown).

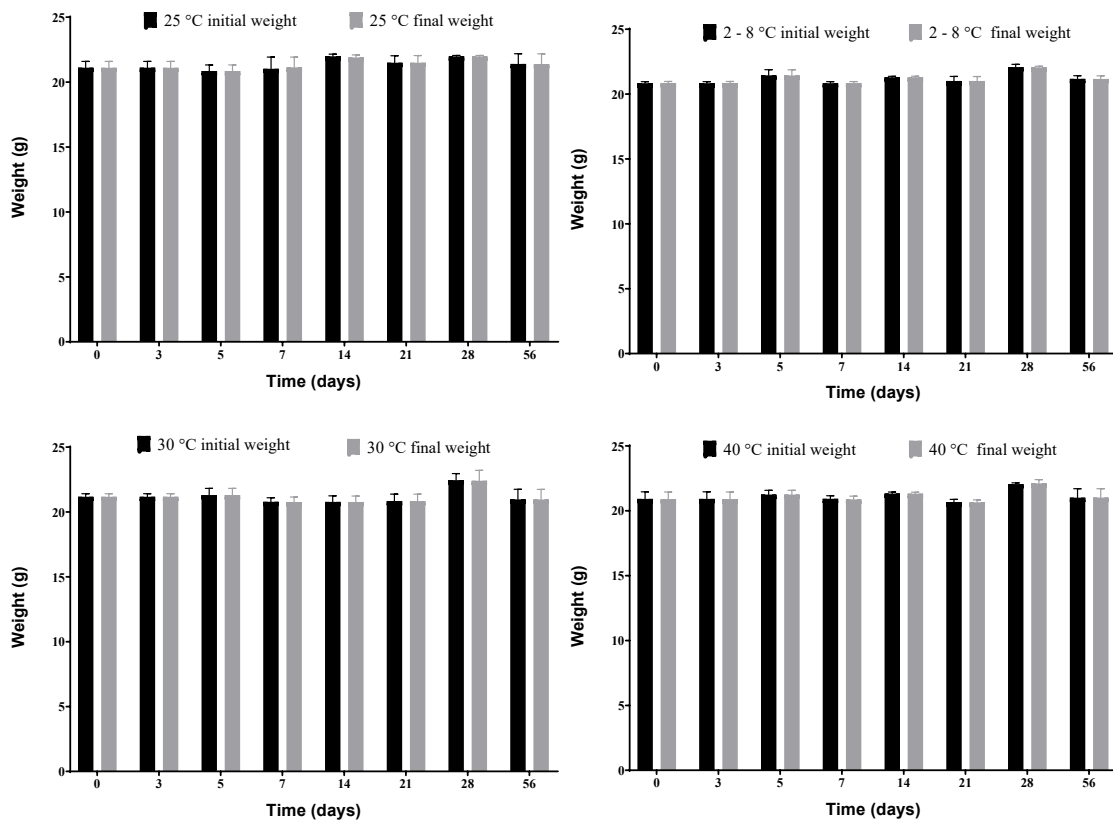


Figure 5-5: Weight change of Aron mix 1 (1:2:20) when stored in plastic screw-cap containers at 25 °C, 2-8 °C, 30 °C and 40 °C over two months. Data bars shows the mean weight of three batches (+SD) at each time point.

A change in pH of a formulation can alter the solubility and stability of the drug in the formulation, thus the apparent pH of Aron mix 1 was monitored over two months at all investigated storage conditions. For comparison, the apparent pH of Diprosone cream, Fucidin cream and Diprobase cream alone were measured at the start of the study and are reported in Table 5-6. Diprosone cream, Fucidin cream and Diprobase cream were formulated at similar pH levels (5.48, 5.40 and 5.09, respectively). The average pH readings over two months for three batches of the Aron mix are reported in Table 5-7. On analysis, small variations in pH were observed in Aron mix 1 over the investigated time, with pH readings ranging from pH 4.84 – pH 5.26. However, this did not amount to a significant change in the pH of the Aron mix ($p > 0.05$) or a large change from the pH of the original formulations, thus if the chemical stability of betamethasone dipropionate or fusidic acid is compromised when stored at these conditions for two months, it is unlikely to be attributed to the pH of the formulation.

Table 5-6: The apparent pH of Diprosone cream, Fucidin cream and Diprobase cream stored at 25 °C. Data are shown as the mean pH readings of three samples and the range is denoted in brackets.

Formulation	pH reading
Diprosone cream	5.48 (0.12)
Fucidin cream	5.40 (0.13)
Diprobase cream	5.09 (0.04)

Table 5-7: The apparent pH of the Aron formulation (1:2:20) over two months when stored at 2-8 °C, 25 °C, 30 °C and 40 °C. Data are shown as the mean pH readings of three batches and the range is denoted in brackets.

Time (days)	2-8 °C	25 °C	30 °C	40 °C
1	5.07 (0.30)	4.97 (0.20)	4.97 (0.20)	5.08 (0.10)
3	4.90 (0.21)	4.96 (0.23)	4.84 (0.05)	4.87 (0.15)
5	5.25 (0.04)	5.26 (0.02)	5.19 (0.20)	5.05 (0.17)
7	5.11 (0.12)	5.02 (0.12)	5.02 (0.06)	5.01 (0.04)
14	5.07 (0.17)	5.09 (0.38)	5.11 (0.20)	5.00 (0.03)
21	4.98 (0.01)	5.14 (0.22)	5.10 (0.29)	4.97 (0.02)
28	4.91 (0.12)	5.09 (0.18)	5.07 (0.14)	5.08 (0.14)
56	4.97 (0.03)	4.99 (0.10)	5.12 (0.09)	5.10 (0.08)

Stability data for betamethasone dipropionate and fusidic acid in the marketed formulations will have been investigated during formulation development. However, no data exist on the stability of these drugs in the compound Aron formulations. To evaluate betamethasone dipropionate and fusidic acid stability in Aron mix 1 (1:2:20), the drug content in the formulation was determined following storage at 2-8 °C, 25 °C, 30 °C and 40 °C over two months. The storage temperatures were selected in accordance with the ICH guidance on the stability testing of new drug substances and products (ICH, 2003). The findings are presented in Figure 5-6.

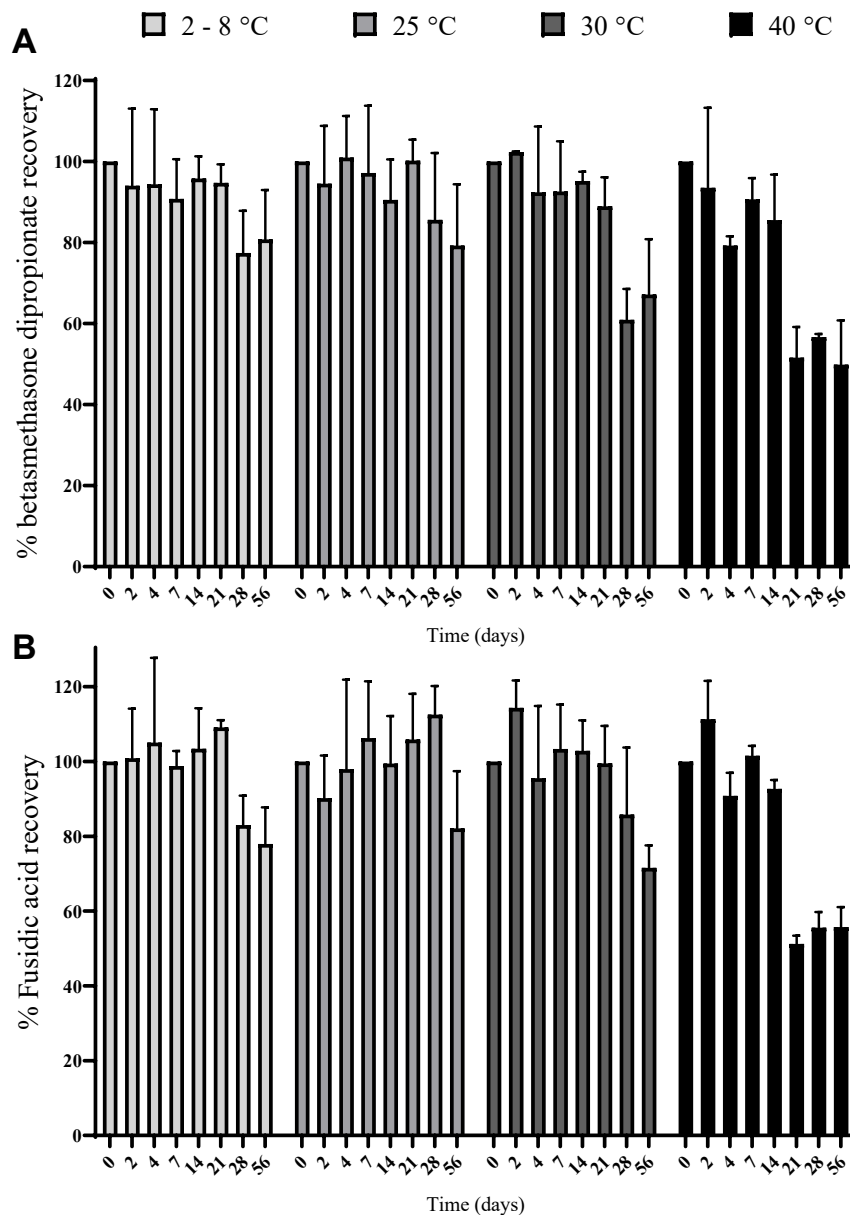


Figure 5-6: The percent recovery of (A) betamethasone dipropionate and (B) fusidic acid in the Aron formulation (1:2:20) over two months when stored at 2-8 °C, 25 °C, 30 °C and 40 °C. Data bars show the mean recovery from three batches and error bars denote the range of data points.

At some timepoints, considerable batch-to-batch variability in drug content was observed (Figure 5-6). As weight change from the formulation was negligible and samples were sealed to prevent evaporation, it is unlikely that the variability observed is attributable to loss of volatile components thus altered drug concentration in Aron mix 1. Instead, the variability between batches may reflect a lack of content uniformity in the product arising from poor drug and excipient homogeneity in the formulation, one of the risks associated with the extemporaneous preparation of products (Paediatric Formulary Committee, 2019). Dr Aron recommends that patients or carers identify a pharmacy to prepare the unlicensed preparation for use; however, if unsuccessful patients or carers are advised that they may mix the creams themselves (Aron, 2019), a particular concern given the absence of stability data or lack of a recommended shelf life for the product. Thus, the intention of this investigation was to ascertain the stability of the extemporaneous formulation as prepared in current practice with limited quality assurance processes and the variability in the data set provides insight to the formulation effects of extemporaneous preparation on batch-to-batch drug content.

The stability of betamethasone dipropionate in the Aron mix at 2-8 °C, 25 °C and 30 °C was greater than 80 % of the initial drug content after 14 days (Figure 5-6). Between 21 days and 56 days, drug recovery from Aron mix 1 stored at 2-8 °C, 25 °C, 30 °C decreased to 80 %, 79 % and 67 % of initial drug content, respectively. An accelerated rate of loss of betamethasone dipropionate content was observed when the formulation was stored at 40 °C with drug content ranging from 93 % of initial drug content after 2 days to 50 % of initial drug content after 56 days. Comparatively, fusidic acid demonstrated greater stability in Aron mix 1 at 2-8 °C, 25 °C and 30 °C with average drug content above 80 % of initial drug content for up to 28 days. Between 28 days and 56 days, fusidic acid recovery from Aron mix 1 stored at 2-8 °C, 25 °C, 30 °C decreased to 77 %, 82 % and 71 % of initial fusidic acid content, respectively. As observed with betamethasone dipropionate, an accelerated rate of loss of fusidic acid content was observed when the formulation was stored at 40 °C with drug content ranging from 111 % of initial drug content after 2 days to 55 % of initial drug content after 56 days. The ICH guidelines define a 'significant change' in the stability profile of a drug product as a 5 % change in assay from the initial value (ICH, 2003). Based on this criterion, betamethasone dipropionate and

fusidic acid in Aron mix 1 failed to meet the specified criterion over the two month period when stored at the four investigated storage conditions.

The decrease in drug stabilities observed may be attributable to the incorporation of diluents dissimilar to the TCS and topical antibiotic base. The importance of diluent selection on the stability of a topical formulation was exemplified by Ryatt et al. (1982) who found that a 1 in 4 dilution of Betnovate ointment (betamethasone-17-valerate) with emulsifying ointment resulted in over 60 % drug degradation in the formulation within 6 h. However the equivalent dilution with Unguentem M maintained drug chemical stability in the diluted preparation for 5 months (Ryatt et al., 1983). Byrne et al. (2017) later reported a positive correlation between the rate of betamethasone-17-valerate isomerisation and the concentration of emulsifier in a formulation, postulating that inclusion of the emulsifier increased drug solubility and rate of isomerisation in the aqueous phase of the formulation.

As the SPCs for Diprosone cream and Diprobace cream recommend that the products are not stored above 25 °C (Bayer, 2019; MSD, 2019), it is likely the same recommendation is made for storage of the Aron mix. Based on the criterion specified in the ICH guidelines, an extemporaneous preparation of Aron mix 1 stored at 25 °C exhibited a significant change in drug content after 7 days (< 95 % of initial drug contents). However, pharmacies typically recommend an expiry date of two weeks to one month for recently prepared extemporaneous preparations (Paediatric Formulary Committee, 2019) and Dr Aron has previously recommended that the preparation is suitable for use beyond the recommended expiry date, a statement not supported by the drug stability data presented in this Chapter.

An additional concern is the impact on preservative content and efficacy when Diprosone cream and Fucidin cream are compounded in the Aron mix. Diprosone cream contains a proportion of chlorocresol, an antimicrobial preservative commonly used in topical formulations (MSD, 2019; Sheskey et al., 2019). Fucidin cream contains butylhydroxyanisole (E320) and all-*rac*- α -tocopherol (Vitamin E) to prevent oxidative degradation of fusidic acid (Leo Laboratories Ltd, 2015; Sheskey et al., 2019). The substantial dilution of these excipients when compounded in the Aron mix may impact on antioxidant and antimicrobial preservative content and effectiveness over the period of use of the product. To

ascertain the effectiveness of the preservative concentration in the Aron mix, further microbial investigations should be conducted.

5.3.3 Raman microscopy of Diprosone cream, Fucidin cream and Aron mix 9

Samples of Diprosone cream and Fucidin cream were analysed by Raman microscopy to provide an indication of whether drug was present in the formulations at saturated or sub saturated levels. No potential drug particles were observed for Diprosone cream; thus it is likely that betamethasone dipropionate was present at subsaturated concentrations thus at submaximal drug thermodynamic activity in the formulation. On analysis of Fucidin cream, fusidic acid particles were present in two distinct solid forms: rod shaped and square, planar shaped crystals. The crystal image is presented in Figure 5-7 (A) and the corresponding Raman spectra are presented in Figure 5-8. This finding strongly suggested that Fucidin cream was formulated with a high degree of drug saturation, thus the drug was likely to be at maximum thermodynamic activity in Fucidin cream. To ascertain whether fusidic acid or betamethasone dipropionate drug crystals were present in Aron mix 9, the formulation employed for the *ex vivo* skin permeation and penetration study, samples of Aron mix 9 (1:2:10) were analysed by light microscopy and a representative image is presented in Figure 5-7 (B). Fusidic acid drug crystals, confirmed by Raman microscopy, were evident in Aron mix 9 (Figure 5-7 B and Figure 5-8) following a 13 fold dilution of Fucidin cream in Diprosone cream and Diprobase cream, however appeared to be less numerous than observed in Fucidin cream alone.

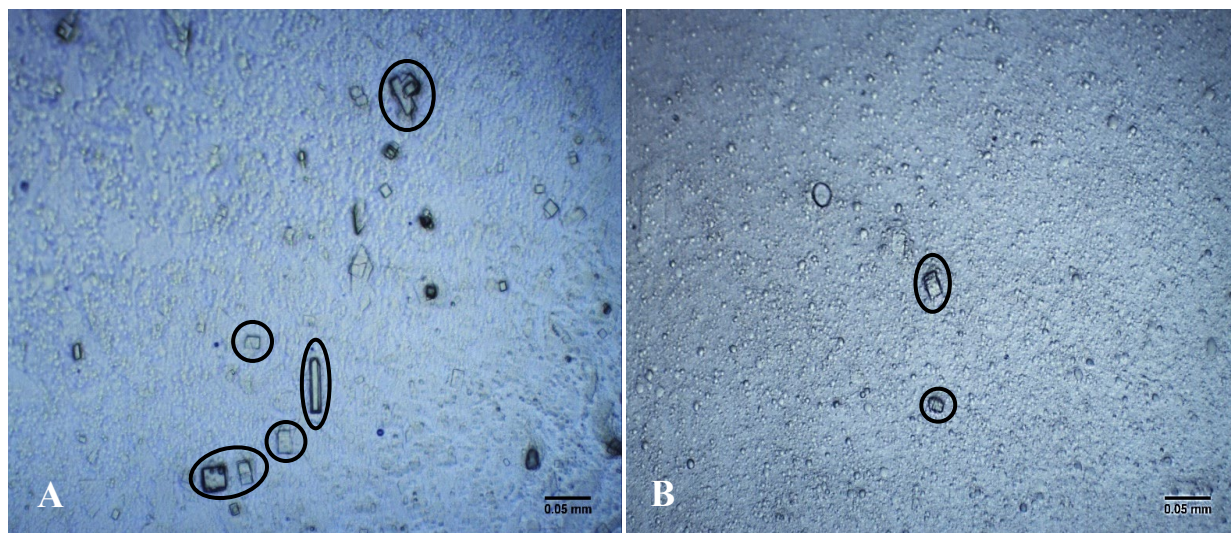


Figure 5-7: Representative light microscope images (x 20 magnification) of (A) Fucidin cream and (B) Aron mix 9. Aron mix 9 contained Fucidin cream, Diprosone cream and Diprobase cream in a 1:2:10 ratio, prepared 1 h before analysis. Crystalline structures, circled in the above images, were evident in formulation A and B and attributed to fusidic acid.

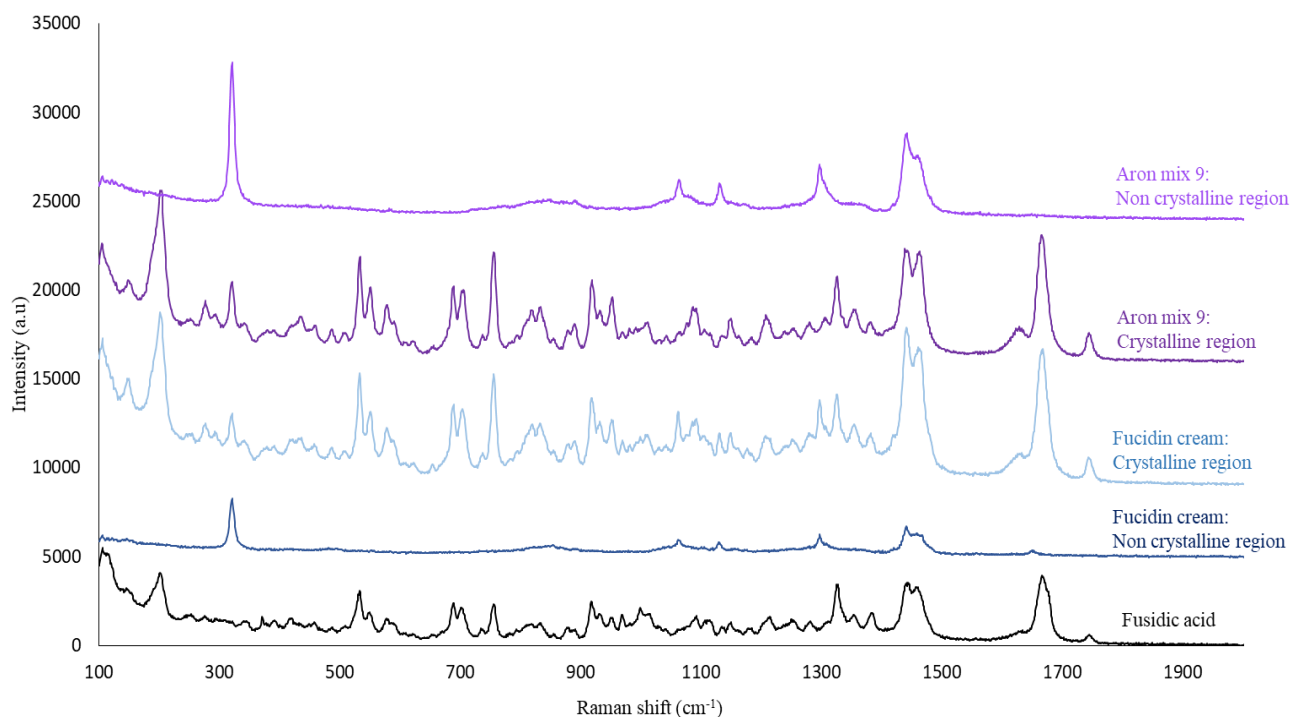


Figure 5-8: Raman spectra obtained from fusidic acid, crystalline and non-crystalline regions of Fucidin cream and Aron mix 9. Aron mix 9 contained Fucidin cream, Diprosone cream and Diprobase cream in a 1:2:10 ratio, prepared 1 h before analysis. Spectra were obtained at x100 magnification, a laser excitation wavelength of 785 nm, five accumulations per sample and an acquisition time of 10 s.

5.3.4 Franz cell method development

The saturated solubility of betamethasone dipropionate and fusidic acid in a range of receiver fluid systems was determined to ascertain whether sink conditions were likely to be maintained for the duration of the experimental period. Four receiver fluid systems of PBS (pH 7.4) and varying concentrations of ethanol were investigated. The findings are presented in Table 5-8.

Table 5-8: The solubility of betamethasone dipropionate and fusidic acid in various solvent systems. Data are presented as the mean of three replicates. The range is denoted in brackets.

Solvent	Solubility of betamethasone dipropionate ($\mu\text{g mL}^{-1}$)	Solubility of fusidic acid ($\mu\text{g mL}^{-1}$)
PBS	0.18 (0.04)	240.92 (5.68)
PBS + 10 % ethanol	3.31 (0.11)	429.70 (3.91)
PBS + 20 % ethanol	12.82 (0.76)	645.32 (2.98)
PBS + 30 % ethanol	97.92 (2.56)	831.62 (7.59)

Betamethasone dipropionate demonstrated limited solubility in PBS alone and greatest solubility in a system comprising PBS and ethanol (70:30). Comparatively, fusidic acid demonstrated greater solubility in PBS alone, and the solubilising capabilities of the solvent system increased with increasing ethanol content. The Aron mix contains unmatched concentrations of betamethasone dipropionate (0.064% w/w strength formulation) and fusidic acid (2 % w/w strength formulation) mixed in varying ratios, thus the dose delivered to the receiver fluid was expected to vary considerably between the two drugs. To ensure adequate solubility of both drugs in the receiver fluid system whilst maintaining sink conditions, two receiver fluids were selected for further investigation: PBS and ethanol (70:30) and PBS and ethanol (80:20). A pilot *in vitro* and *ex vivo* Franz cell study was conducted to determine the suitability of the selected receiver fluid systems and inform the sampling protocols for the full scale *in vitro* and *ex vivo* experiments.

Following the application of infinite doses of Diprosone cream and Fucidin cream to silicone membrane the receiver fluid was sampled at time points up to 26 h. Cumulative drug transport was calculated over this period and the resultant permeation profiles are presented in Figure 5-9.

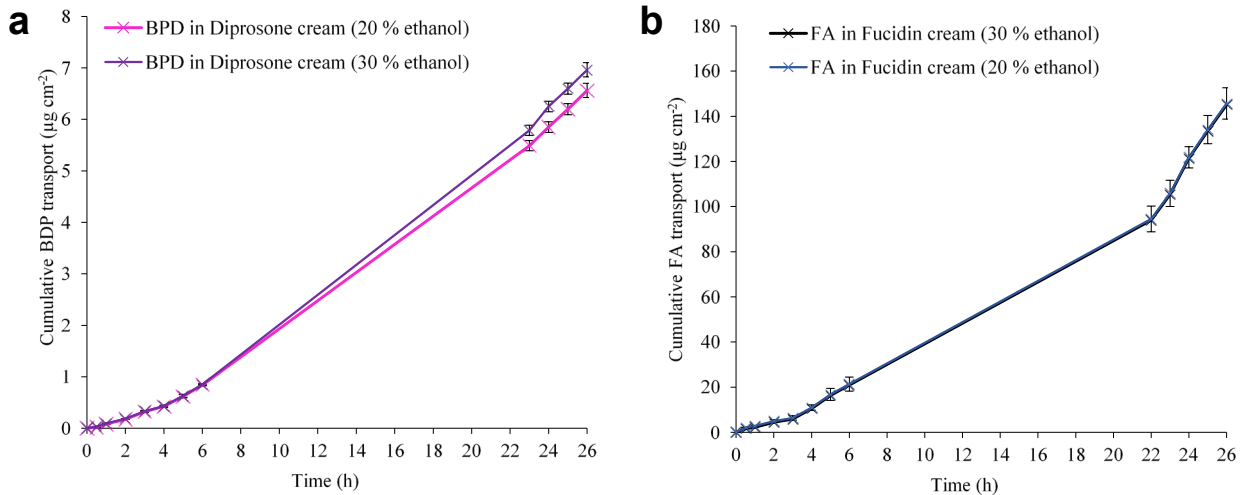


Figure 5-9: Drug transport profile shows the cumulative amounts of (a) betamethasone dipropionate (BDP) and (b) fusidic acid (FA) transport across silicone membrane following the applications of infinite doses of Fucidin cream and Diprosone cream, respectively. Two receiver fluid systems were investigated: PBS and ethanol (70:30) and PBS and ethanol (80:20). Data are shown as the mean of three replicates; error bars denote the range of data points.

On analysis of the drug transport profile where silicone membrane was dosed with Diprosone cream, betamethasone dipropionate was detected in both receiver fluid systems from 1 h onwards (Figure 5-9 a). The maximum concentration of betamethasone dipropionate in the receiver fluid was 13 % of the saturated solubility in PBS and ethanol (80:20) and 1.7 % of the saturated solubility in PBS and ethanol (70:30). Following the finite dosing of Fucidin cream to silicone membrane, fusidic acid was detected in both receiver fluid systems from 1 h onwards (Figure 5-9 b). Sink conditions were maintained for the duration of the experimental period, with fusidic acid concentrations not exceeding 5 % of the saturated solubility in PBS and ethanol (80:20) or 4 % of the saturated solubility in PBS and ethanol (70:30). As sink conditions are accepted to be maintained when the concentration of each drug in the receiver fluid system does not exceed 10 % of the drug saturated solubilities in the receiver fluid system (Higuchi, 1960), a system comprising PBS and ethanol (80:20) would not be suitable, given the potential for betamethasone dipropionate concentrations to violate sink conditions. Thus, PBS and ethanol (70:30) was selected as the receiver fluid system for the *in vitro* drug transport experiments. The spread and number of sampling time points were appropriate to enable profiling of both drugs in the selected receiver fluid system, thus were adopted for the full scale *in vitro* drug transport experiments.

The *ex vivo* Franz cell experiments across human skin employed a finite dose model to closely replicate the clinical use of the products. It was anticipated that a decrease in the dose applied would consequently decrease the dose delivered to the receiver fluid, and therefore the concentrations of the drugs in the receiver fluid system from the *ex vivo* studies compared to those obtained from the *in vitro* experiments. To select the most appropriate receiver fluid system for the finite dose *ex vivo* experiments, the suitability of PBS and ethanol (70:30) or PBS and ethanol (80:20) as a receiver fluid system was investigated. Following the application of finite doses of Diprosone cream and Fucidin cream to human skin the receiver fluid was sampled at time points up to 24 h. Cumulative drug permeation was calculated over this period and the resultant permeation profiles are presented in Figure 5-10.

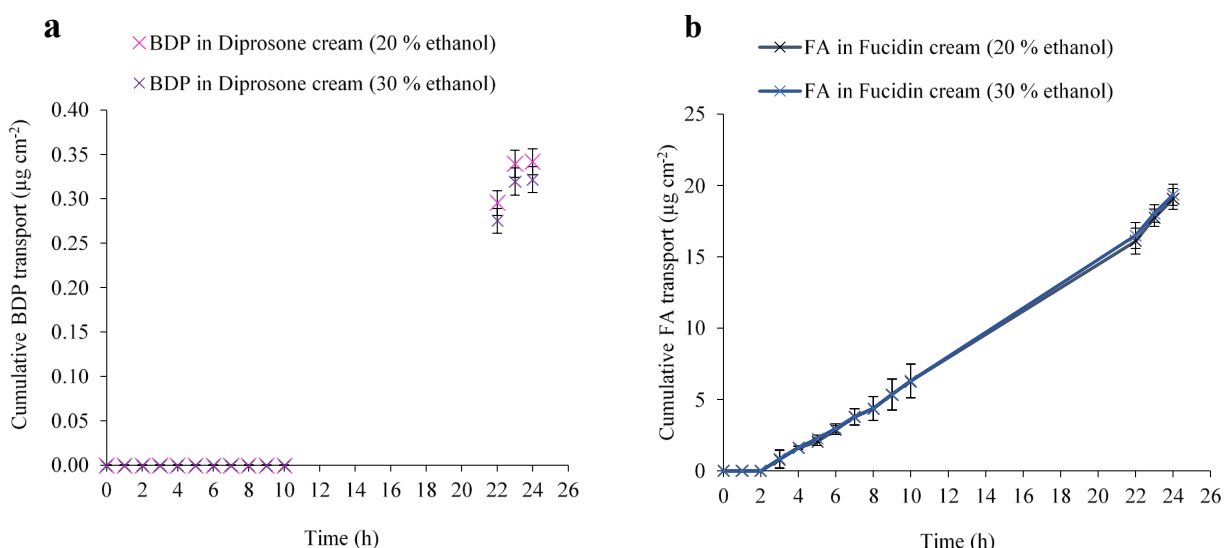


Figure 5-10: Permeation profile shows the cumulative amounts of (a) betamethasone dipropionate (BDP) and (b) fusidic acid (FA) permeation across human skin following the applications of finite doses of Fucidin cream and Diprosone cream, respectively. Two receiver fluid systems were investigated: PBS and ethanol (70:30) and PBS and ethanol (80:20). Data are shown as the mean of three replicates; error bars denote the range of data points.

Drug permeation was consistently low (not detected) over 0-10 h, following the application of Diprosone cream to human skin. Over 22-24 h, small amounts of betamethasone dipropionate were detected in the receiver fluid and maximum drug concentrations did not exceed 0.7 % of the saturated solubility in PBS and ethanol (80:20) receiver fluid system and 0.09 % of the saturated solubility in the PBS and ethanol (70:30) receiver fluid system, thus sink conditions were maintained in both systems.

Following the application of Fucidin cream, drug permeation across human skin was evident from 2 h onwards (Figure 5-10 b). As with betamethasone dipropionate, sink conditions were maintained in both receiver fluid systems with the concentration of fusidic acid not exceeding 0.8 % and 0.6 % of the saturated solubility in PBS and ethanol (80:20) and PBS and ethanol (70:30), respectively. Given the data from the solubility experiment and findings of the pilot *ex vivo* experiment, a receiver fluid system with of PBS and ethanol (80:20) was selected for the full scale *ex vivo* Franz cell experiments.

During the *ex vivo* drug penetration study, the distribution of betamethasone dipropionate and fusidic acid in the layers of the skin was investigated. To achieve this, the suitability of acetonitrile as an extraction solvent was evaluated. Drug recovery following the dosing of a spiked formulation of betamethasone dipropionate or fusidic acid to cotton buds, tape strips, epidermis and dermis was assessed and is presented in Figure 5-11. On analysis, two sequential extractions were sufficient to recover 94–99% of betamethasone dipropionate and 95–100 % of fusidic acid from the samples thus acetonitrile was an appropriate extraction solvent to employ.

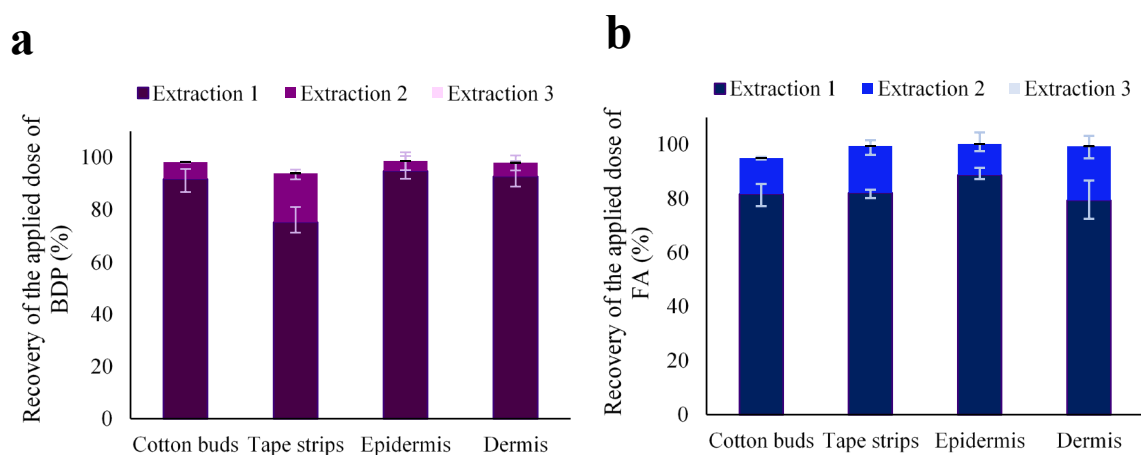


Figure 5-11: The percent recovery after 24 h of (a) betamethasone dipropionate (BDP) and (b) fusidic acid (FA) from matrices following a 10 μL dose of a standard solution of 1 mg mL^{-1} of the drug. The extraction solvent investigated was acetonitrile. Data are presented as the mean of three replicates for each extraction. Error bars denote the range of values.

To determine whether betamethasone dipropionate and fusidic acid were stable in the receiver fluid during the experimental period, the stability of both drugs in the receiver fluid systems (PBS and ethanol; 70:30 and 80:20) at 37 °C was determined over 24 hours. To ascertain the potential for betamethasone dipropionate and fusidic acid to degrade in the selected receiver fluid systems or extraction solvent (acetonitrile) during storage, the stability of both drugs when stored at 2–8 °C and 25 °C was evaluated. The findings are presented in Table 5-9.

Table 5-9: Stability of betamethasone dipropionate (BDP) and fusidic acid (FA) in the receiver fluid systems and extraction solvent at 37 °C, 25 °C and 2-8 °C. Data are presented as the percent of drug concentration compared to freshly prepared standards for three replicates. The range is denoted in brackets.

Storage condition	Time (days)	Solvent system					
		PBS and ethanol (70:30)		PBS and ethanol (80:20)		Acetonitrile	
		BDP	FA	BDP	FA	BDP	FA
-	0	100	100	100	100	100	100
37 °C	1	99.77 (0.18)	99.98 (0.45)	99.57 (0.33)	99.85 (0.32)	-	-
	1	85.39 (0.96)	99.88 (0.12)	83.87 (0.56)	99.76 (0.68)	99.79 (0.41)	99.81 (0.35)
25 °C	7	64.18 (1.97)	99.95 (1.08)	66.48 (0.53)	99.94 (1.28)	99.82 (0.58)	99.13 (1.11)
	12	58.53 (2.15)	99.79 (1.02)	52.11 (1.68)	99.12 (0.52)	99.48 (0.12)	99.56 (0.44)
	14	59.96 (2.37)	99.84 (0.03)	50.97 (3.92)	99.38 (1.66)	98.96 (0.39)	96.28 (1.74)
	1	99.34 (1.19)	99.16 (0.19)	98.67 (0.23)	99.42 (0.24)	99.85 (0.24)	100.11 (0.98)
2 – 8 °C	7	96.89 (0.20)	99.20 (0.41)	96.42 (0.76)	99.78 (0.55)	100.26 (0.52)	99.88 (1.07)
	12	68.35 (0.77)	99.31 (0.54)	60.08 (0.85)	99.28 (1.40)	98.62 (0.63)	99.42 (0.71)
	14	42.40 (0.85)	99.91 (1.70)	48.12 (4.91)	99.85 (0.09)	100.58 (0.54)	98.27 (0.53)

Betamethasone dipropionate and fusidic acid were stable (> 95 %) in both receiver fluid systems at the experimental temperature (37 °C) over the experimental period (24 h). Betamethasone dipropionate demonstrated poor stability in both receiver fluid systems when stored at 25 °C with drug recovery ranging from 99.34 % to 50.97 % over two weeks. However, betamethasone dipropionate was stable in both receiver fluid systems for seven days when stored at 2–8 °C, with drug recovery exceeding 95 % compared to freshly prepared samples. Fusidic acid was stable in both receiver fluid systems for 14

days when stored at 25 °C and 2–8 °C. Thus, all drug samples in PBS and ethanol (70:30 or 80:20) were analysed within one week of sample collection to ensure suitable analysis of the data. On analysis, betamethasone dipropionate and fusidic acid were stable in the extraction solvent (acetonitrile) when stored at 25 °C and 2-8 °C for two weeks, with percent drug concentrations within ± 2 % of freshly prepared standards at the same concentration (Table 5-9).

5.3.5 *In vitro* silicone membrane drug transport studies: Varying the ratios of products in the Aron mix

The Aron formulations are tailored to suit the patient's age, weight and the severity of the condition, thus varying ratios of Fucidin cream and Diprosone cream diluted in a Diprobase cream base are frequently extemporaneously prepared and dispensed. To investigate the impact on betamethasone dipropionate and fusidic acid transport across silicone membrane when Diprosone cream was diluted to varying extents, a series of formulations were prepared with a fixed concentration of Fucidin cream (4.35 % w/w) and varying concentrations of Diprosone cream (8.70–43.48 % w/w) in a Diprobase cream base (Aron mix 1-4). To then investigate the impact of diluting Fucidin cream to varying extents on betamethasone dipropionate and fusidic acid transport, a series of formulations were prepared with a fixed concentration of Diprosone cream (8.70 % w/w) and varying concentrations of Fucidin cream (2.17–30.43 % w/w) in a Diprobase cream base (Aron mix 5-8). Full details of the formulation compositions are presented in Table 5-3. *In vitro* drug transport experiments across silicone membrane were conducted and the drug transport profiles for Diprosone cream, Fucidin cream and Aron mix 1-4 are presented in Figure 5-12.

A

Formulation	Q ₂₆ for BDP ($\mu\text{g cm}^{-2}$)	Rank (Q ₂₆)	J _{4-24h} for BDP ($\mu\text{g cm}^{-2}\text{h}^{-1}$)	Rank (J _{4-24h})
Diprosone cream	6.48 ± 0.37	1	2.70E-01 ± 1.48E-02	1
Fucidin cream	0.00 ± 0.00*	-	0.00 ± 0.00*	-
Aron mix 1 (1:2:20)	0.72 ± 0.03*	5	3.09E-02 ± 1.54E-03*	5
Aron mix 2 (1:4:18)	1.33 ± 0.05*	4	5.85E-02 ± 2.91E-03*	4
Aron mix 3 (1:6:16)	1.81 ± 0.10*	3	7.94E-02 ± 4.57E-03*	3
Aron mix 4 (1:10:12)	2.44 ± 0.14*	2	1.04E-01 ± 5.95E-03*	2

B

Formulation	Q ₂₆ for FA ($\mu\text{g cm}^{-2}$)	Rank (Q ₂₆)	J _{4-24h} for FA ($\mu\text{g cm}^{-2}\text{h}^{-1}$)	Rank (J _{4-24h})
Diprosone cream	0.00 ± 0.00*	-	0.00 ± 0.00*	-
Fucidin cream	82.18 ± 5.39	1	2.87 ± 1.75E-01	1
Aron mix 1 (1:2:20)	7.02 ± 0.44*	4	2.50E-01 ± 1.72E-02*	3
Aron mix 2 (1:4:18)	7.02 ± 0.41*	3	2.49E-01 ± 1.58E-02*	4
Aron mix 3 (1:6:16)	7.12 ± 0.48*	2	2.53E-01 ± 1.84E-02*	2
Aron mix 4 (1:10:12)	6.95 ± 0.42*	5	2.47E-01 ± 1.52E-02*	5

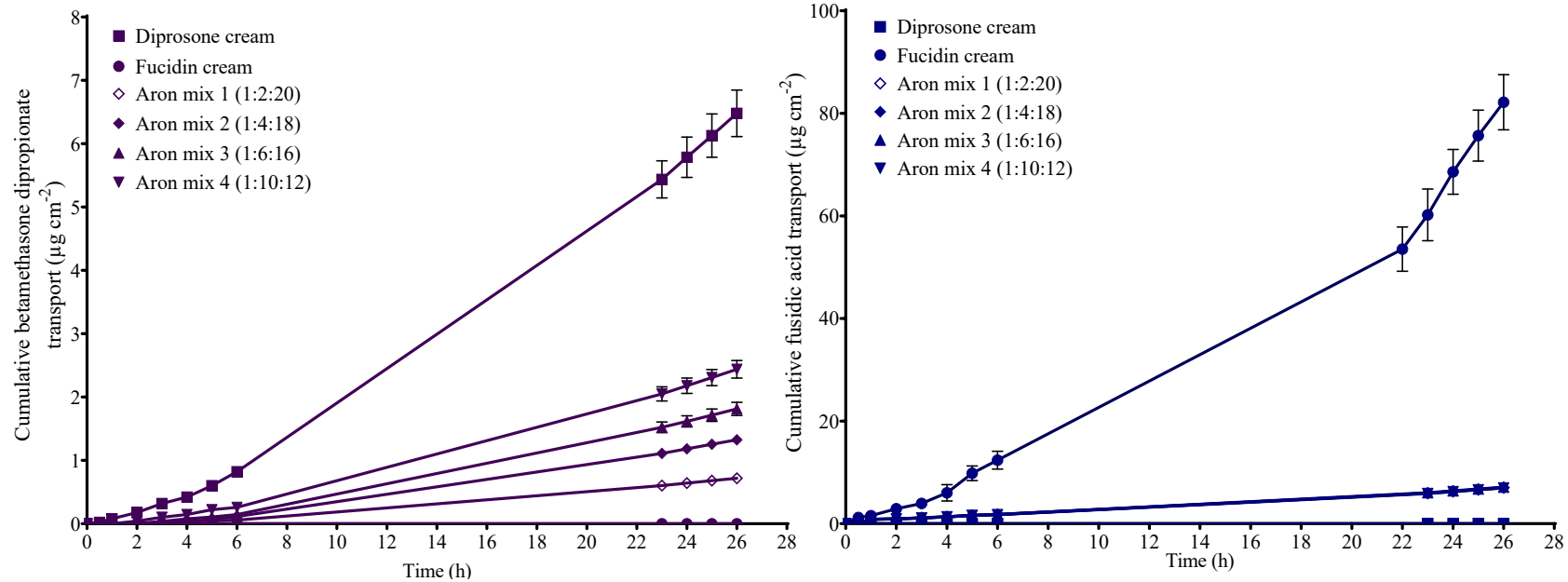


Figure 5-12: Cumulative (A) betamethasone dipropionate (BDP) transport and (B) fusidic acid (FA) transport across silicone membrane following the application of an infinite dose of Diprosone cream alone (■), Fucidin cream alone (●) or mixes of Fucidin cream, Diprosone cream and Diprosone cream in the following ratios: Aron mix 1 (1:2:20; ◇), Aron mix 2 (1:4:18; ◆), Aron mix 3 (1:6:16; ▲) or Aron mix 4 (1:10:12; ▼). Data are shown as the mean of six replicates (\pm SD). * Denotes a significant difference when Q₂₆ or J_{4-24 h} for the Aron mixes were compared to the respective parameters for (A) Diprosone cream and (B) Fucidin cream; one way ANOVA and Tukey's post hoc test ($p < 0.05$).

Following the application of Diprosone cream to silicone membrane, betamethasone dipropionate transport was evident from 0.5 h onwards and increased with time up to 26 h (Figure 5-12 a). This was accompanied by the greatest amounts of total betamethasone dipropionate transport at 26 h compared to Aron mix 1-4 (Q_{26} in Figure 5-12 a). Betamethasone dipropionate transport from Aron mix 1-4 was evident from 3 h onwards, thereafter increasing with time up to 26 h. A general trend of decreasing Q_{26} with decreasing concentrations of Diprosone cream in the Aron mix was observed for betamethasone dipropionate. Fusidic acid transport from Aron mix 1-4, which were matched for the concentration of Fucidin cream, was evident from 1 h onwards and profiled in similar manners with similar Q_{26} values (Figure 5-12 b). As the transport of betamethasone dipropionate and fusidic acid was linear between 4 h and 24 h for all investigated formulations in Figure 5-12, this range was selected to calculate average flux from all formulations.

Betamethasone dipropionate flux from Aron mix 1-4 was significantly reduced when compared to flux from Diprosone alone ($p < 0.05$; Figure 5-12 a). The reduction in drug flux ranged from a 2.6 fold decrease when Diprosone cream was diluted to 43.48 % w/w in Aron mix 4, to an 8.8 fold decrease when Diprosone cream was diluted to 8.7 % w/w in the Aron mix 1, when compared to Diprosone cream alone. As observed with Q_{26} , a trend of decreasing betamethasone dipropionate flux with decreasing concentrations of Diprosone cream in the Aron mix was evident.

The work reported in Chapters 2, 3 and 4 raised concerns about the compatibility of TCS products and emollients when mixed on the skin surface, with Diprosone cream inducing drug crystallisation when mixed with Elocon cream, for example. The summary of product characteristics (SPC) for Diprosone cream lists Diprosone cream as the base vehicle for the TCS and suggests that control of the dosage regimen can be achieved by diluting Diprosone cream with Diprosone cream (MSD, 2019). Following Raman microscopy of Diprosone cream, drug particles were not evident in the formulation suggesting that betamethasone dipropionate was present in Diprosone cream at a subsaturated concentration. In this scenario, it is therefore expected that employing Diprosone cream as a diluent would have resulted in a decrease in drug thermodynamic activity proportional to the degree of dilution in the Aron mix.

Indeed, a linear relationship was observed between the concentration of Diprosone cream in the Aron mix and betamethasone flux across silicone membrane ($R^2 = 0.9906$; Figure 5-13).

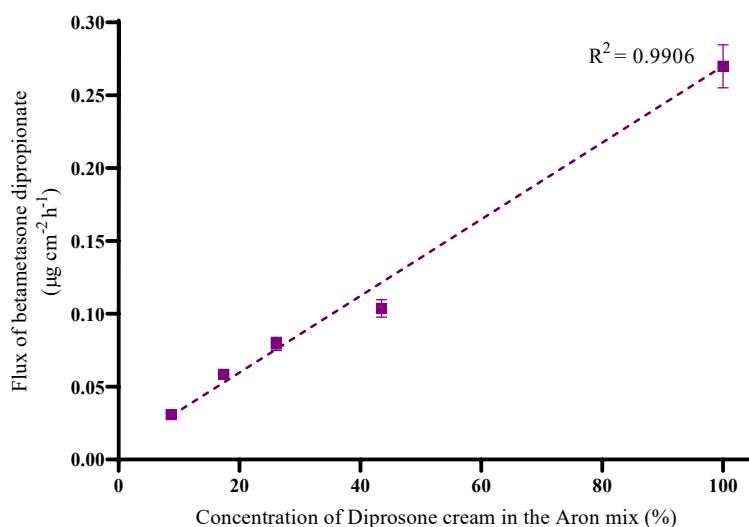


Figure 5-13: The correlation between the concentration of Diprosone cream in the Aron mix and betamethasone dipropionate flux across silicone membrane. Data points show the mean of six replicates (\pm SD).

It is, however, important to appreciate that the Aron mix entails a complex dilution of Diprosone cream, by Fucidin cream and Diprobace cream, with the potential dilution effect of Fucidin cream on Diprosone cream being unreported, to date. Thus, the thermodynamic activity of betamethasone dipropionate is not altered by the diluent alone, but also impacted by the excipients and API present in Fucidin cream. On evaluation, a proportional relationship was observed between the degree of dilution of Diprosone cream in Aron mix 1-4 and the decrease in betamethasone dipropionate flux across silicone membrane with a 2.3–11.5 fold dilution of Diprosone cream resulting in a 2.6–8.7 fold decrease in betamethasone dipropionate flux, compared to Diprosone cream alone. As Diprobace cream is listed as a compatible base in which to dilute Diprosone cream, the margin of difference observed is likely to be attributable to the formulation effects of Fucidin cream where excipients such as glycerol or Polysorbate-60 (Tween 60) or indeed fusidic acid may alter the solubility of betamethasone dipropionate in the Aron mix, thus decrease drug thermodynamic activity and flux to unpredictable extents. An opposing concern is whether dilution of the same excipients in Fucidin cream reduces the excipient thermodynamic activity in the formulation and in turn, fusidic acid thermodynamic activity when formulated in the Aron mix.

Aron mix 1–4 contained matched concentrations of Fucidin cream (4.35 %) diluted by 23 fold in varying proportions of Diprosone cream and Diprobase cream. Overall, fusidic acid flux from Aron mix 1-4 was significantly reduced by up to 11.5 fold compared to Fucidin cream alone ($p < 0.05$), thus the reduction in drug flux and decrease in drug thermodynamic activity was not proportional to the degree of dilution of the product. A potential explanation for this is that fusidic acid is formulated as a suspension in Fucidin cream and thus when the cream is diluted with a mix of Diprosone cream and Diprobase cream, the degree of drug saturation and hence drug thermodynamic activity might be expected to be maintained, compared to the dilution of a subsaturated formulation. Indeed, this trend appeared to be consistent with the findings of the Raman microscopy investigation, where fusidic acid particles were observed in Fucidin cream. Additionally, use of a diluent (Diprobase cream) dissimilar to the base of Fucidin cream may have contributed to a change in drug thermodynamic activity to an unpredictable extent and this serves as one of the reasons that the extemporaneous dilution of topical products is not recommended (British National Formulary, 2020a). The change in proportions of Diprobase cream to Diprosone cream appeared to have a negligible effect on fusidic acid flux across silicone membrane with fusidic acid flux decreasing by 11.4–11.5 fold following the applications of Aron mix 1–4, compared to Fucidin cream alone. This observation was somewhat unsurprising given the similarity in the excipient lists of Diprosone cream and Diprobase cream (Table 5-2).

The effect of diluting Fucidin cream to varying degrees in the Aron mix on fusidic acid and betamethasone dipropionate flux across silicone membrane was then evaluated, with matched concentrations of Diprosone cream. The drug transport profiles for fusidic acid and betamethasone dipropionate following the application of Aron mix 5-8 are presented in Figure 5-14 a and Figure 5-14 b, respectively.

A

Formulation	Q ₂₆ for FA ($\mu\text{g cm}^{-2}$)	Rank (Q ₂₆)	J _{2-7h} for FA ($\mu\text{g cm}^{-2} \text{h}^{-1}$)	Rank (J _{2-7h})
Fucidin cream	82.18 ± 5.39	1	2.87 ± 1.75E-01	1
Diprosone cream	0.00 ± 0.00*	-	0.00 ± 0.00*	-
Aron mix 5 (0.5:2:20.5)	3.34 ± 0.26*	6	1.23E-01 ± 2.79E-02*	6
Aron mix 1 (1:2:20)	5.56 ± 0.24*	5	2.45E-01 ± 3.38E-02*	5
Aron mix 6 (3:2:18)	8.89 ± 0.85*	4	3.04E-01 ± 7.13E-02*	4
Aron mix 7 (5:2:16)	21.51 ± 1.47*	3	6.59E-01 ± 3.01E-02*	3
Aron mix 8 (7:2:14)	41.15 ± 3.03*	2	1.11 ± 7.09E-02*	2

B

Formulation	Q ₂₆ for BDP ($\mu\text{g cm}^{-2}$)	Rank (Q ₂₆)	J _{2-7h} for BDP ($\mu\text{g cm}^{-2} \text{h}^{-1}$)	Rank (J _{2-7h})
Fucidin cream	0.00 ± 0.00	-	00.00 ± 0.00	-
Diprosone cream	6.48 ± 0.37	1	2.70E-01 ± 1.48E-02	1
Aron mix 5 (0.5:2:20.5)	0.56 ± 0.03*	6	5.64E-02 ± 3.11E-03*	5
Aron mix 1 (1:2:20)	0.65 ± 0.22*	5	5.99E-02 ± 8.18E-03*	3
Aron mix 6 (3:2:18)	0.69 ± 0.15*	4	6.22E-02 ± 5.51E-03*	2
Aron mix 7 (5:2:16)	0.75 ± 0.11*	3	5.21E-02 ± 4.07E-03*	6
Aron mix 8 (7:2:14)	0.91 ± 0.21*	2	5.96E-02 ± 4.08E-03*	4

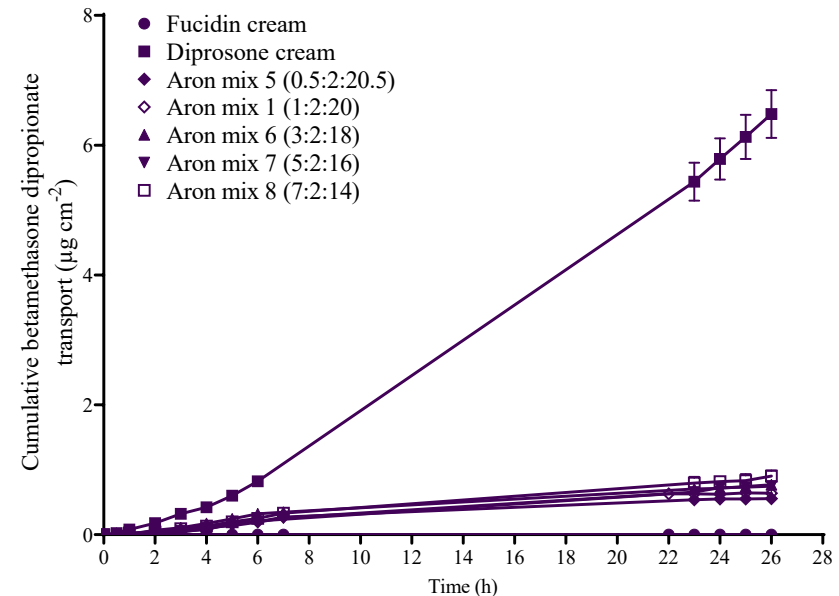
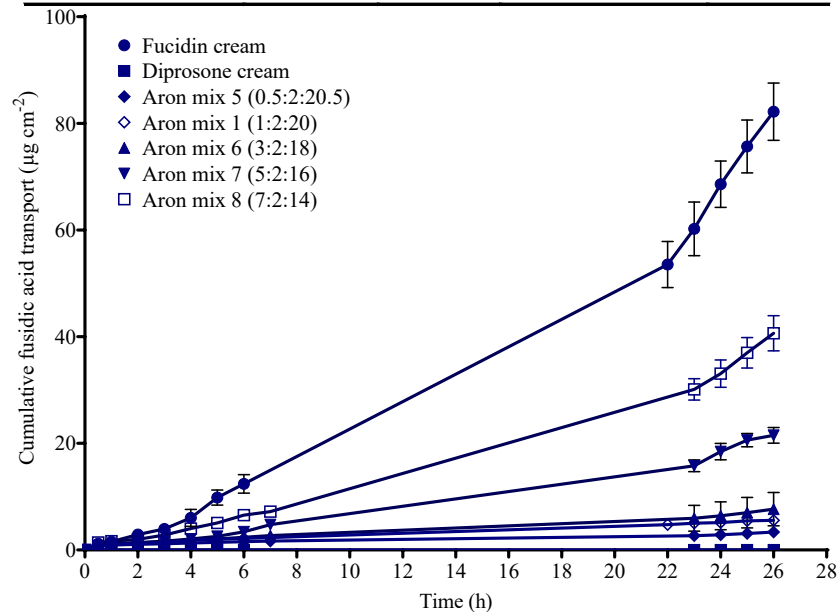


Figure 5-14: Cumulative (A) fusic acid transport (FA) and (B) betamethasone dipropionate (BDP) transport across silicone membrane following the application of an infinite dose of Fucidin cream alone (●), Diprosone cream alone (■) or mixes of Fucidin cream, Diprosone cream and Diprosone cream in the following ratios: Aron mix 5 (0.5:2:20.5; ◇), Aron mix 1 (1:2:20; ◆), Aron mix 6 (3:2:18; ▲), Aron mix 7 (5:2:16; ▼) or Aron mix 8 (7:2:14; □). Data are shown as the mean of six replicates (\pm SD). * Denotes a significant difference when Q₂₆ or J_{2-7h} for the Aron mixes were compared to the respective parameters for (A) Fucidin cream and (B) Diprosone cream; one way ANOVA and Tukey's post hoc test ($p < 0.05$).

Following the application of Fucidin cream to silicone membrane, fusidic acid transport was evident from 0.5 h onwards and increased with time up to 26 h (Figure 5-14 a). This was accompanied by the greatest amounts of total fusidic acid transport at 26 h compared to Aron mix 1 and 5-8 (Q_{26} in Figure 5-14a). Fusidic acid transport from Aron mix 1 and 5-8 was evident from 0.5 h onwards and increased with time up to 26 h (Figure 5-14a). A general trend of decreasing Q_{26} with decreasing concentrations of Fucidin cream in the Aron formulation was observed for fusidic acid. Betamethasone dipropionate transport from Aron mix 1 and 5-8 was evident from 1 h onwards and increased with time up to 7 h, after which the rate of transport slowed, with all formulations profiling in a similar manner (Figure 5-14 b). A slight trend of decreasing Q_{26} for betamethasone dipropionate with decreasing concentrations of Fucidin cream was observed (and increasing concentrations of Diprosone cream), though the concentration of Diprosone cream remained the same. This was likely to be attributable to the increasing proportion of Diprosone cream in the Aron mix, the base vehicle of Diprosone cream, as the proportion of Fucidin cream decreased, thereby creating a less complex base for dilution. The trend was exemplified by Aron mix 5, the formulation with the highest proportion of Diprosone cream (89.13 %), where the 11.5 fold dilution of Diprosone cream resulted in a proportionate 11.6 fold decrease in Q_{26} ($p < 0.05$). Comparatively, the same degree of dilution of Diprosone cream, but with a more complex base in Aron mix 8 (60.87 % Diprosone cream) resulted in a less proportionate 7.1 fold decrease in Q_{26} compared to Diprosone cream alone ($p < 0.05$).

As the transport of betamethasone dipropionate and fusidic acid was linear between 2 h and 7 h for all investigated formulations in Figure 5-14, this range was selected to calculate average flux from all formulations. Fusidic acid flux from Aron mix 1 and 5-8 was significantly reduced when compared to flux from Fucidin cream alone ($p < 0.05$; Figure 5-14a). The reduction in fusidic acid flux ranged from a 2.6 fold decrease when Fucidin cream was diluted to 30.43 % w/w in Aron mix 8, to a 23 fold decrease when Fucidin cream was diluted to 2.17 % w/w in Aron mix 5, when compared to Fucidin cream alone. As observed with Q_{26} , a trend of decreasing fusidic acid flux with decreasing concentrations of Fucidin cream in the Aron mix was evident. A linear correlation of $R^2 = 0.9783$ was obtained between the concentration of Fucidin cream in the Aron mix and drug flux across silicone membrane (Figure 5-15).

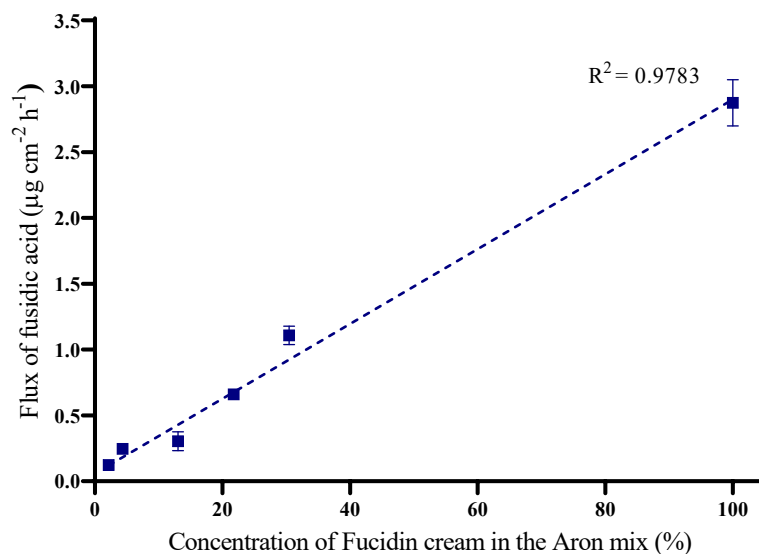


Figure 5-15: The correlation between the concentration of Fucidin cream in the Aron mix and fusidic acid flux across silicone membrane. Data points show the mean of six replicates (\pm SD).

Though a linear relationship was observed, the degree of dilution of Fucidin cream did not result in a directly proportional reduction in fusidic acid flux across silicone membrane. Instead the 3.3–46.1 fold dilution of Fucidin cream resulted in a 2.6–23.3 fold decrease in fusidic acid flux (Figure 5-14a). Thus, unlike the trend observed with betamethasone dipropionate, the thermodynamic activity of fusidic acid in Aron mix 1 and 5–8 was reduced by a mechanism not fully explained by simple dilution of the formulation and may be attributable to the high degree of drug saturation in Fucidin cream. Additionally, dilution of the topical antibiotic in complex bases dissimilar to the product (varying ratios of Diprobace cream and Diprosone cream) may have introduced excipients with solubilising or antisolvent effects into the topical antibiotic formulation, altering the solubility of fusidic acid in Aron mix 1 and 5-8 to differing extents. These findings are consistent with the work presented in Chapter 2 where a two fold dilution of a saturated formulation (Elocon cream) with Diprobace cream resulted in a 1.6 fold decrease in drug transport across silicone membrane. To investigate whether the change in drug thermodynamic activity in the Aron mixes resulted in a material difference in drug delivery to the skin, the percutaneous absorption and skin distribution of both drugs in the Aron mix was evaluated.

5.3.6 *Ex vivo* human skin Franz cell study: Aron mix 9

An *ex vivo* human skin Franz cell study was conducted to evaluate the delivery of betamethasone dipropionate and fusidic acid in and across human skin following a finite application of Diprosone cream, Fucidin cream and Aron mix 9. The Aron mix was formulated to contain a higher proportion of Diprosone cream and Fucidin cream in Diprobase cream (1: 2: 10) than typically prescribed by Dr Aron, to enable detection of both drugs in the skin matrices and receiver fluid. The resulting skin penetration profile of betamethasone dipropionate and fusidic acid following the application of Diprosone cream alone, Fucidin cream alone and the Aron mix 9 is illustrated in Figure 5-16.

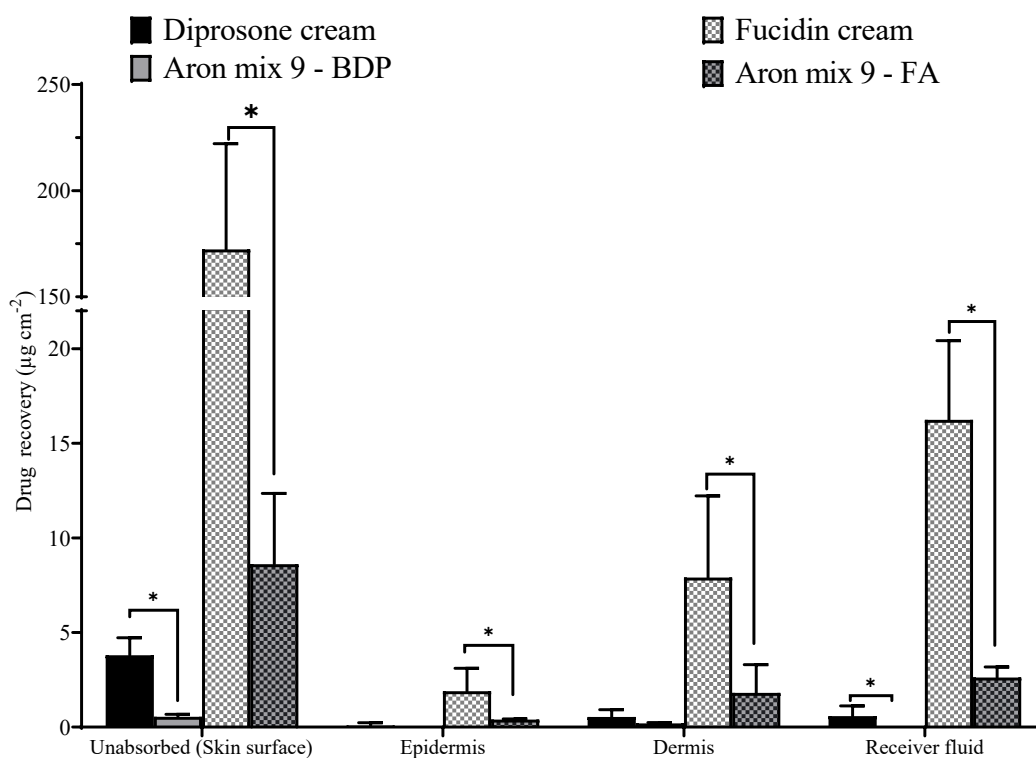


Figure 5-16: Betamethasone dipropionate (BDP) and fusidic acid (FA) distribution in the unabsorbed formulation, epidermis, dermis and receiver fluid ($\mu\text{g cm}^{-2}$) recovered after 24 h following the finite applications of from Diprosone cream alone, Fucidin cream alone and Aron mix 9. Aron mix 9 contained Fucidin cream, Diprosone cream and Diprobase cream mixed in a 1:2:10 ratio. Data are shown as mean + SD ($n = 6$). * denotes a significant difference when BDP or FA from Aron mix 9 was compared respectively to drug recovery from Diprosone cream or Fucidin cream in each compartment (Mann-Whitney test; $p < 0.05$).

The absolute recovery of betamethasone dipropionate and fusidic acid ranged 94–108 % of the applied dose for all experiments conducted, falling within the OECD defined acceptable criteria (OECD, 2019). After 24 h, the majority of betamethasone dipropionate and fusidic acid remained unabsorbed on the skin surface. The distribution of both drugs was largely evident in the following order of magnitude: unabsorbed formulation > receiver fluid > dermis > epidermis. However, betamethasone dipropionate was not detected in the receiver fluid following the application of Aron mix 9 and very low levels of the drug were recovered from the dermis (Table 5-10). On analysis the application of Aron mix 9 resulted in significantly less drug delivery to all compartments when compared to the application of Diprosone cream alone or Fucidin cream alone ($p < 0.05$; Figure 5-16). For clarity the total drug absorption (total drug content in the epidermis, dermis and receiver fluid) was used for statistical analysis as an indication of the change in absolute betamethasone dipropionate and fusidic acid absorption from Aron mix 9 when compared to Diprosone cream or Fucidin cream.

Table 5-10: The distribution of betamethasone dipropionate (BDP) and fusidic acid (FA) in epidermis, dermis, receiver fluid and total absorbed (sum of epidermis, dermis and receiver fluid) following the application of Diprosone cream alone, Fucidin cream alone or Aron mix 9 (1:2:10). Aron mix 9 contained Fucidin cream, Diprosone cream and Diprobace cream mixed in a 1:2:10 ratio. Data are shown as mean \pm SD (n = 6). * denotes a significant difference when total BDP or FA delivery from Aron mix 9 was compared to total drug delivery from Diprosone cream or Fucidin cream, respectively (Mann-Whitney test; $p < 0.05$).

	Epidermis ($\mu\text{g cm}^{-2}$)	Dermis ($\mu\text{g cm}^{-2}$)	Receiver fluid (Q_{24} ; $\mu\text{g cm}^{-2}$)	Total absorbed ($\mu\text{g cm}^{-2}$)
Diprosone cream - BDP	0.08 \pm 0.15	0.53 \pm 0.39	0.58 \pm 0.34	1.19 \pm 0.83
Aron mix 9 (1:2:10) – BDP	0.00 \pm 0.00	0.19 \pm 0.04	0.00 \pm 0.00	0.20 \pm 0.04*
Fucidin cream - FA	1.90 \pm 1.22	7.91 \pm 4.31	16.24 \pm 4.18	26.05 \pm 6.94
Aron mix 9 (1:2:10) - FA	0.40 \pm 0.03	1.81 \pm 1.49	2.62 \pm 0.56	4.83 \pm 1.84*

Total betamethasone dipropionate delivery to the skin significantly decreased by 6 fold following the application of Aron mix 9 when compared to Diprosone cream alone ($p < 0.05$; Table 5-10). This decrease was roughly proportional to the 6.5 fold dilution of Diprosone cream in the Aron mix. Comparatively, total fusidic acid delivery to the skin from Aron mix 9 significantly decreased by 5.4 fold compared to the application of Fucidin cream alone ($p < 0.05$), disproportionate to the 13 fold dilution of Fucidin cream in the Aron mix. This trend was consistent with the findings of the *in vitro* drug transport experiments and suggests that whilst Diprosone cream was a suitable diluent for Diprosone cream, the dilution effect on fusidic acid permeation was less predictable.

An evaluation of the impact on the permeation of betamethasone dipropionate and fusidic acid across the skin when Diprosone cream and Fucidin cream were applied alone or in the Aron mix was also conducted. The cumulative amounts of betamethasone dipropionate and fusidic acid permeated across human skin following the application of Diprosone cream alone, Fucidin cream alone and Aron mix 9 is presented in Figure 5-17.

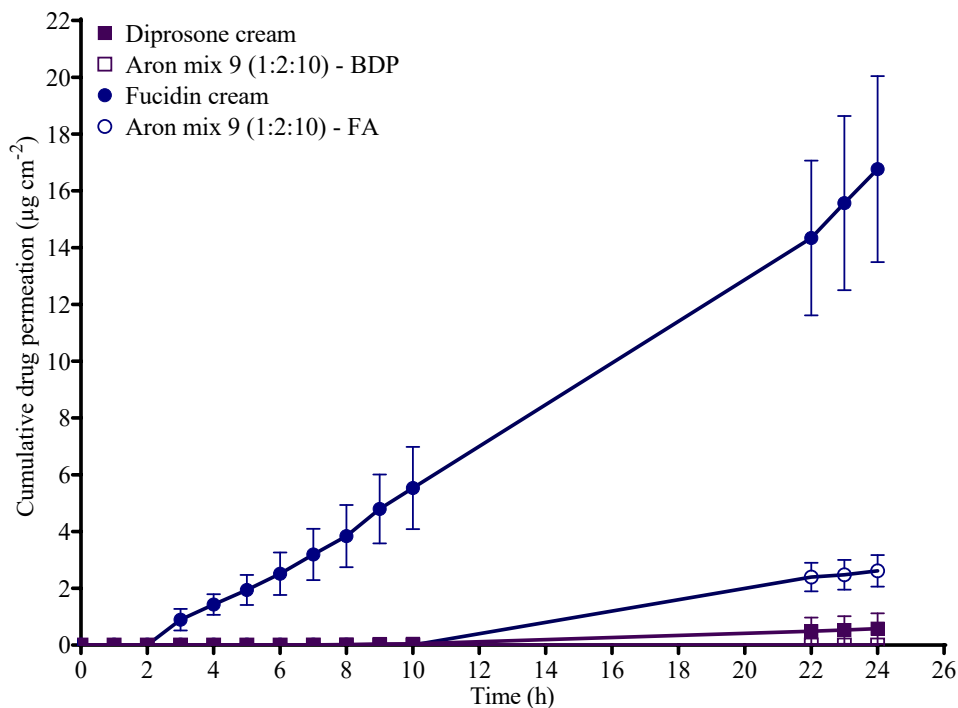


Figure 5-17: The cumulative amount of betamethasone dipropionate (BDP) and fusidic acid (FA) permeated across human skin from Diprosone cream alone (■; BDP), Fucidin cream alone (●; FA) and Aron mix 9 (□ denotes BDP and ○ denotes FA). Aron mix 9 contained Fucidin cream, Diprosone cream and Diprosone cream mixed in a 1:2:10 ratio. Data are shown as mean \pm SD (n = 6).

Consistent with the findings of the pilot *ex vivo* Franz cell experiment, betamethasone dipropionate permeation was consistently low (not detected) over 0-10 h, following the application of Diprosone cream to human skin. At 24 h the cumulative amount of drug permeating the skin was $0.58 \mu\text{g cm}^{-2}$. Following the application of Aron mix 9, betamethasone dipropionate was not detected in the receiver fluid over the entire experimental period. Fusidic acid permeation was evident from 3 h onward following the application of Fucidin cream and fusidic acid concentration increased at a constant rate with time. At 24 h, total drug permeation from Fucidin cream was $16.24 \mu\text{g cm}^{-2}$. Furthermore, it appeared that pseudo steady state flux, thus drug thermodynamic activity, was maintained up to 24 h following the application of Fucidin cream, further indicative of a high degree of drug saturation in Fucidin cream. Following the application of Aron mix 9, fusidic acid permeation was consistently low (not detected) over 0–10 h and total drug permeation at 24 h was $2.62 \mu\text{g cm}^{-2}$.

The low drug permeation rates from Aron mix 9 were consistent with the observed decrease in drug delivery to the epidermis and dermis (Table 5-10). The premise for heavily diluting the topical corticosteroid and antibiotic in the Aron mix is to allow an increase in the frequency of product application even to unaffected areas, up to six times daily (Aron, 2019). Thus, it could be argued that the significant decreases in drug delivery to the skin observed, compared to the applications of Diprosone cream or Fucidin cream alone, may alleviate the concerns associated with overuse of the products. However, dilution (reduced drug concentration) of a topical formulation does not always correlate with the extent of drug delivery to the skin, as observed from the findings presented herein. In the case of Diprosone cream, the overall decrease in drug delivery was proportional to the degree of dilution of the formulation. For Fucidin cream, this correlation was less proportional, where the substantial dilution of the saturated formulation did not reduce drug delivery to the skin to the same extent. Raman microscopy of Aron mix 9 confirmed the presence of fusidic acid particles in the formulation, however these appeared to be far less numerous than observed in the original Fucidin cream formulation. Thus, it is possible that the driving force for drug permeation from Aron mix 9 was greater than would be expected compared to simple dilution of a subsaturated formulation, however less than the driving force in Fucidin cream alone. Likewise, it is also important to consider the change

in thermodynamic activity of potential penetration enhancers, as this has the potential to impact on drug permeation and penetration. Whilst Diprosone cream does not contain any notable potential penetration enhancers, Fucidin cream is formulated with a proportion of glycerol, an hygroscopic excipient which can increase the water holding capacity of the stratum corneum (Batt et al., 1988). Furthermore, under conditions of low humidity, such as that created in stratum corneum affected by dry skin conditions, glycerol has been shown to interact with lipid bilayers to maintain the physical properties of hydrated lipid bilayer systems (Björklund et al., 2013; Nowacka et al., 2012). In order to realise its penetration enhancing potential, glycerol must partition out of the formulation and into the stratum corneum. However, simultaneous to the decrease in fusidic acid thermodynamic activity in Aron mix 9, mixing Fucidin cream with Diprobase cream and Diprosone cream is likely to have resulted in a decrease in the concentration, thus thermodynamic activity, of glycerol compared to Fucidin cream alone. In this case, the extent to which total fusidic acid delivery to the skin was altered is likely to have been influenced by (i) the decrease in drug thermodynamic activity and (ii) the decrease in thermodynamic activity of potential penetration enhancers in Aron mix 9.

Binary drug systems have been shown to exhibit eutectic properties where depression of the drug melting points, when formulated at a certain ratio, can enhance percutaneous absorption of both compounds. Most notably, this approach has been commercially exploited for the dual delivery of a lidocaine and prilocaine from a topical formulation (EMLA; Eutectic Mixture of Local Anaesthetics) (Juhlin et al., 1979, 1980) and extensive research has been conducted in an attempt to elucidate the mechanisms through which enhanced permeation is achieved (Fiala et al., 2010; Fiala et al., 2016; Nyqvist-Mayer et al., 1986). The influence of betamethasone dipropionate on fusidic acid delivery to the skin has yet to be investigated, however it may be possible that fusidic acid thermodynamic activity was also affected by the presence of, and ratio with, betamethasone dipropionate in the formulation. Future work to ascertain the drug solubilities in the mixed formulation are required to fully investigate this theory.

S. aureus infections are typically localised to the skin surface and stratum corneum (Arikawa et al., 2002). However, skin colonisation of *S. aureus* has been found to extend beyond the epidermal barrier and into the dermis in lesional sites of atopic dermatitis patients (Nakatsuji et al., 2016). Thus, successful treatment requires delivery of fusidic acid in sufficient concentrations to the stratum corneum, epidermal and dermal target sites to decolonise the affected sites and prevent recurrent skin infections. The summary of product characteristics for Fucidin cream advises that fusidic acid concentrations of 0.03-0.12 $\mu\text{g mL}^{-1}$ (equivalent to 58–232 nM) are sufficient to inhibit nearly all strains of *S. aureus* (Leo Laboratories Ltd, 2015). Furthermore, a similar minimum inhibitory concentration of 0.13 $\mu\text{g mL}^{-1}$ for fusidic acid against *S. aureus* has been reported in the literature (Alsterholm et al., 2010). The recovered drug levels in the epidermis and dermis are presented in Table 5-11 for comparison with the reported MIC for fusidic acid.

Table 5-11: Fusidic acid (nM) recovered from human epidermal and dermal skin following the application of Fucidin cream alone and Aron mix 9. Aron mix 9 contained Fucidin cream, Diprosone cream and Diprobace cream mixed in a 1:2:10 ratio. Data are shown as the mean of 6 replicates.

	Drug recovery (nM)	
	Epidermis	Dermis
Fucidin cream	781528	283576
Ratio compared to MIC^a	3365.194	528.6072
Aron mix 9	122763	54927.94
Ratio compared to MIC^a	1221.055	236.5151

^a Ratio was calculated as mean drug recovery/ MIC of 232 nM (equivalent to 0.12 $\mu\text{g mL}^{-1}$)

On analysis, drug concentration in the epidermis was 3,365 fold and 1,221 fold greater than the MIC for fusidic acid, following the application of Fucidin cream alone and Aron mix 9 respectively. In a similar trend, drug concentration in the dermis was 528 fold and 236 fold greater than the MIC of fusidic acid, following the application of Fucidin cream alone and Aron mix 9 respectively (Table 5-11). It should be noted that a direct extrapolation of *ex vivo* findings to the *in vivo* scenario should be made with caution, as the over hydrated state of *ex vivo* skin in a water bath, presence of ethanol in the receiver fluid system and reduced barrier to drug permeation presented by scrotal skin may overestimate the

extent of drug delivery to the skin compared to healthy *in vivo* skin. However, skin barrier integrity is known to be compromised in inflammatory skin conditions (Cork et al., 2006; Ogawa & Yoshiike, 1992) and it has been reported that scrotal skin possess a thinner stratum corneum and a weaker barrier to the delivery of salicylic acid compared to abdominal skin (Smith et al., 1961). Thus, employing a scrotal skin model could provide a better indication of the drug permeability of disease – state skin compared to other anatomical sites. It may be suggested that drug was delivered to the epidermal and dermal tissue in sufficient concentrations, following a single application of Aron mix 9, for activity against *S. aureus*. It is important to consider this finding in the context of the Aron regimen, which involves repeat applications of the product to affected and unaffected areas of the skin up to six times a day during the initial phase of treatment. The application of fusidic acid to large areas of clinically uninfected skin is counterintuitive from an antimicrobial stewardship perspective, given that an increase in fusidic acid resistant *S. aureus* has occurred parallel to an increase in prescribing of topical fusidic acid products in the community (Brown & Thomas, 2002; Dobie & Gray, 2004; Ellington et al., 2015). Furthermore, treatment with the topical antibiotic in the Aron mix continues for a minimum of two weeks, an approach employed to prevent *S. aureus* recolonisation in the skin. Whilst anecdotal evidence has been reported by Dr Aron for the success of this regimen, clinical recommendations widely caution against the extended use of topical antibiotics in the treatment of clinically infected atopic dermatitis (Bath-Hextall et al., 2010; Eichenfield et al., 2014; Wollenberg et al., 2018b). Indeed, current UK guidance advises that the use of topical antibiotics in children with atopic eczema should be reserved for cases of clinical infection in localised areas, and used for no longer than 2 weeks (NICE, 2007). These recommendations appear to be formed on the basis of a dearth of evidence, in the form of good quality randomised controlled trials, to support the clinical benefit of the use of topical antibiotics on balance with the associated risk of an increase in drug resistance. Thus, to fully evaluate the role of the Aron regimen in the treatment of severe cases of infected (or uninfected) atopic eczema, controlled investigations into the clinical outcomes (change in the eczema area and severity index, for example) following treatment with combined TCS and antibiotic therapies are required. Furthermore, the long term implications and risks associated with wide spread, frequent applications of a potent TCS to the skin, albeit heavily diluted, need to be fully evaluated.

5.4 Conclusion

The findings of the work reported in this Chapter established that the chemical stability of betamethasone dipropionate and fusidic acid the Aron mix formulation displayed a significant change in drug content over two months, though the pH of the formulation remained unchanged. This is an important consideration when determining the expiry date for the extemporaneously prepared formulation and it may be more beneficial for pharmacists to supply smaller quantities of the product more frequently, though this will be associated with a greater cost burden for the patient. Considerable variability in batch-to-batch drug content was observed, raising quality concerns over the compounding the formulations in a community setting by trained healthcare professionals, or indeed by patients and carers themselves, as sometimes recommended by Dr Aron.

On evaluation of the *in vitro* drug transport experiments with varying proportions of Diprosone cream and Diprobase cream, a proportional relationship was observed between the degree of dilution of Diprosone cream in Aron mix 1-4 and the decrease in total betamethasone dipropionate transport (Q_{26}) and flux across silicone membrane. Formulation investigations indicated that the drug was present in Diprosone cream at subsaturated concentrations. As Diprobase cream is listed as a compatible base in which to dilute Diprosone cream, the margin of difference observed was thought to be attributable to the formulation effects of Fucidin cream where excipients such as glycerol or Polysorbate-60 (Tween 60) or indeed fusidic acid may alter the solubility of betamethasone dipropionate in the Aron mix, thus decrease drug thermodynamic activity and flux to unpredictable extents. Comparatively, dilution of Fucidin cream in complex bases dissimilar to the product (varying ratios of Diprobase cream and Diprosone cream) reduced fusidic acid flux and Q_{26} to a lesser extent than the degree of dilution. It was postulated that this was because of (i) the high degree of fusidic acid saturation in the initial product, (ii) the introduction of excipients with solubilising or antisolvent effects into the topical antibiotic formulation and (iii) the potential effect of the binary drug mixture where the presence of, and ratio with, betamethasone dipropionate in the formulation impacted on fusidic acid solubility in the mixed formulations, altering the solubility of fusidic acid in Aron mix 1 and 5-8 to differing extents. Future work to ascertain the drug solubilities in the mixed formulation are required to investigate this theory.

Following the single application of Aron mix 9 to *ex vivo* human skin, total betamethasone dipropionate delivery to the skin was significantly reduced in a manner proportional to the degree of dilution of Diprosone cream in Aron mix 9. The permeation profile for fusidic acid in Fucidin cream was indicative of a high degree of drug saturation in the formulation, with an extended duration of steady state flux compared to Aron mix 9. Overall, total fusidic acid delivery to the skin from Aron mix 9 decreased to a lesser extent than the degree of Fucidin cream dilution in the product. This trend was consistent with the findings of the *in vitro* drug transport experiments and suggests that whilst Diprosone cream was a suitable diluent for Diprosone cream and proportionally decreased thermodynamic activity of the drug in the formulation, the same cannot be expected for Fucidin cream. Instead total fusidic acid delivery to the skin was potentially influenced by a decrease in thermodynamic activity of potential penetration enhancers such as glycerol in Fucidin cream compounded with the formulation effects postulated following the *in vitro* drug transport study.

The findings of the work reported in this Chapter described trends in the complex mix of TCS, topical antibiotic and emollient that were consistent with the findings of the work presented in Chapter 2 and Chapter 3. Dilution of a saturated formulation (Elocon cream or Fucidin cream) or subsaturated formulation (Dermovate cream or Diprosone cream) with emollient products dissimilar to the base resulted in an altered drug delivery profile, compared to the application of the product alone, as a result of a multitude of complex formulation effects.

Whilst it was established that heavily diluting the TCS product delivered significantly less drug to the skin following a single application of the Aron mix, further studies on the potency, efficacy and long term implications associated with wide spread, frequent application of a heavily diluted potent TCS should be conducted. Additionally, dilution of the topical antibiotic appeared to deliver effective drug concentrations (above the MIC) to the epidermal and dermal target sites following a single application of Aron mix 9, however these findings will need to be confirmed *in vivo* with a full appreciation for the prevalence of fusidic acid resistant *S. aureus* in the community alongside the stability of the product over the recommended period of use.

Chapter Six:

General discussion

The overarching aim of this thesis was to develop a body of evidence to address the prioritised treatment uncertainty ‘what is the best and safest way of using topical corticosteroids for eczema with respect to alternating with other topical treatments?’ Emollients are the cornerstone of maintenance therapy for atopic eczema and it is recommended they are applied liberally and frequently to manage dry skin conditions; TCSs are routinely prescribed with emollients for inflammatory control and it is recommended they are applied sparingly to the affected area once to twice daily (Eichenfield et al., 2014; National Institute for Health and Care Excellence, 2018b; Wollenberg et al., 2018a). In cases of infected eczema, patients may also be prescribed a topical antibiotic for short term application to the affected areas only. Whilst the manufacturers’ recommendation for the safe and effective use of TCSs and topical antibiotics is made with a good understanding of the clinical efficacy of the formulation, they are not, however, made with consideration to the application with other topical products.

Despite the widespread prescribing of TCSs and emollients, there exists a remarkable lack of consensus between healthcare bodies on the optimum application protocol for the products, with recommendations made on the basis of clinical ‘expert’ opinion rather than evidence-based findings (Voegeli, 2017). For example, current recommendations differ on the time interval between product applications, with suggestions ranging from ‘as soon as absorbed’ (Penzer, 2012) to 60 minutes (Flohr & Williams, 2004). Further disparity is evident when considering the order in which products should be applied, with propositions that the order of application is in fact unimportant (Moncrieff et al., 2013), patient preference should determine which product is applied first (NICE, 2007), TCSs should be applied before the emollient (National Eczema Association, 2019) or that the formulation of the emollient should be considered (cream based emollients 15 minutes before, but ointment based emollients 15 minutes after, the TCS; Ring et al. (2012)). Additionally, relatively recent international guidance on the treatment of atopic dermatitis fails to address the issue (Eichenfield et al., 2014; Wollenberg et al., 2018a). Whilst it is recommended that topical corticosteroid therapy should continue alongside topical antibiotic treatment (National Institute for Health and Care Excellence, 2018b), to date there have been no reported studies evaluating the impact of one formulation on the performance of the other, or indeed whether the physicochemical properties of one drug may be altered in the presence of a second drug.

The division of opinion, or lack of, highlights the scope for improvement of clinical recommendations and patient education, but realisation of this goal first requires a clear understanding of the formulation effects on drug delivery to the skin when different application protocols and product combinations are employed.

When TCSs, topical antibiotics and emollients are mixed either *in situ* (on the skin surface) or extemporaneously, the degree of drug saturation and drug thermodynamic activity in the mixed formulations may change relative to the TCS or topical antibiotic alone. Simultaneous to this, it is feasible that mixing of the products may also reduce the thermodynamic activity of penetration enhancing excipients formulated in the TCS or topical antibiotic formulations. In a further complex scenario, should emollient formulations contain excipients with potential penetration enhancing effects, such as isopropyl myristate and urea present in Hydromol Intensive cream, introducing these excipients into the TCS or topical antibiotic formulation may enhance drug delivery to the skin, relative to the marketed products alone. Ultimately, a combination of these effects will collectively govern the extent to which drug delivery to the skin may be altered for a particular product combination. A greater understanding of the complex interplay that exists between TCSs, topical antibiotics and emollients will therefore support in the development of clinical guidance addressing the uncertainties amongst healthcare professionals and patients about the safest way in which topical products can be used together to ensure clinical efficacy, adherence to treatment and patient safety (Batchelor et al., 2013).

Considering the wide range of TCS, topical antibiotic and emollient combinations available and the variety of approaches currently recommended for application of these products, ascertaining the impact on drug delivery to the skin exclusively through clinical efficacy studies is unfeasible and offers a limited mechanistic understanding of formulation effects on TCS and topical antibiotic delivery to the skin. The *ex vivo* drug permeation model has demonstrated a good correlation with clinical bioequivalence data for topical products (Franz et al., 2009); thus this model was adopted for the experimental work presented in this thesis. A two component TCS and emollient system was first employed to gain an understanding of the fundamental formulation effects that may occur when two topical products were applied to the skin at similar times. Thus, the work presented in Chapter 2,

Chapter 3 and Chapter 4 evaluated the impact of employing currently recommended clinical application protocols for TCSs and emollients on the *ex vivo* percutaneous absorption and skin retention of TCSs. Four clinical application protocols were selected to reflect the range of current clinical guidance: the application of the TCS before an emollient (with a five minute or thirty minute interval) or after an emollient (with a five minute or thirty minute interval). Two commonly used TCS formulations were investigated to elucidate trends, if any, in TCS and emollient combinations: Elocon cream (0.1 % w/w mometasone furoate; potent UK classification) and Dermovate cream (0.05 % w/w clobetasol propionate; very potent UK classification). The rationale for selecting (very) potent TCS formulations associated with inherently smaller safety margins was to ascertain whether particular emollients could significantly increase or decrease drug absorption to the skin, thus further increase the risk of side effects or reduce TCS performance, respectively. To discern emollient effects on drug delivery to the skin, a range of frequently prescribed emollient formulations were selected to reflect products containing excipients that may potentially act as penetration enhancers, relatively simple emollient formulations and those offering a range of occlusiveness.

Evaluation of the *ex vivo* drug permeation and skin retention data revealed that applying an emollient at similar times to Elocon cream or Dermovate cream altered total drug delivery to the skin to varying extents, compared to the application of the TCSs alone. The extent to which total drug delivery increased or decreased, compared to the application of the TCSs alone, was partially governed by (i) formulation changes *in situ* and (ii) the application protocol employed.

It is noteworthy that different emollients were selected for investigation with Elocon cream (Diprobace cream, Diprobace ointment and Hydromol Intensive cream) and Dermovate cream (Diprobace ointment, Hydromol Intensive cream and Doublebase gel), based on the findings of *in vitro* drug transport studies (presented in Chapter 2 and Chapter 3). However, direct comparison of the emollients common to both TCSs (Diprobace ointment and Hydromol Intensive cream), revealed no commonality in the emollient effect on TCS drug delivery to the skin, rather more intricate formulation specific effects were occurring. This was best exemplified by the behaviour of Elocon cream in the presence of Hydromol Intensive cream where total mometasone furoate delivery to the skin invariably increased irrespective

of the application protocol employed, when compared to Elocon cream alone ($p < 0.05$; Chapter 4). Comparatively, the same emollient applied with Dermovate cream resulted in an invariable decrease in total clobetasol propionate delivery to the skin, compared to the TCS alone ($p < 0.05$; Chapter 3). Isolation of the formulation design of the TCSs alone strongly suggested that (i) mometasone furoate was saturated or nearly saturated in Elocon cream, thus at maximum thermodynamic activity in the TCS formulation and (ii) clobetasol propionate was present at a sub-saturated concentration in the Dermovate cream thus at sub maximum thermodynamic activity. Thus, *in situ* mixing of the respective TCSs with Hydromol Intensive cream may have altered the degree of drug saturation in the formulations to differing extents, governed by the drug solubilities in the new mixed systems. It was then important to consider the role of emollient excipients with penetration enhancing capabilities such as urea in Hydromol Intensive cream. To isolate the changes potentially occurring in the mixed layer, a premixed TCS and emollient model (1:1) was employed. A significant increase in pseudo steady state drug flux (J_{ss}) from the premixed Elocon cream and Hydromol Intensive cream system was attributed to a significant increase in the apparent partition co-efficient (Kh) and an unaltered apparent diffusion co-efficient (D/h^2) of mometasone furoate, when compared to Elocon cream alone. It was postulated that the introduction of urea to the TCS formulation (10 % w/w urea in Hydromol Intensive cream) was partially responsible for the observed effect and in agreement with this theory, a similar increase in total drug delivery to the skin was evident following the application of a urea spiked Elocon cream formulation to skin (5 % w/w urea to model the concentration in 1:1 premixed formulation). These findings confirmed the theory that penetration enhancing capabilities of emollient excipients can be realised following rapid *in situ* formulation changes. Comparatively, a premixed system of Dermovate cream and Hydromol Intensive cream resulted in a significant decrease in J_{ss} attributable to a decrease in apparent Kh of clobetasol propionate. Mixing of the sub saturated Dermovate cream formulation with Hydromol Intensive cream is likely to have reduced the driving force of the drug and thermodynamic activity of propylene glycol in the TCS formulation to the extent that the penetration enhancing capability of urea was not realised. This further demonstrate the variety of complex formulation factors that warrant consideration when selecting TCS and emollient combinations as a treatment package.

Within emollient groups, a general trend emerged (with the exception of Hydromol Intensive cream) where the application of Elocon cream or Dermovate cream five minutes after an emollient resulted in significant decreases in total drug delivery to the skin, J_{ss} , and apparent Kh , when compared to the TCSs alone. As with Hydromol Intensive cream, it was thought that dilution of the TCSs with emollient excipients dissimilar to the TCS base increased or decreased the drug solubilities in the mixed formulations to differing extents. In practicality, determining the solubilities of the drugs in the mixed marketed formulations was challenging without knowledge of the exact composition of the TCS and emollient formulations. However, it was postulated that the application of the TCSs after an emollient resulted in *in situ* formation of a mixed TCS and emollient layer which potentially reduced drug delivery to the skin through several mechanisms. Firstly, incorporating emollient excipients such as castor oil and isopropyl myristate with relatively high solubilising capabilities for mometasone furoate and clobetasol propionate may have reduced the degree of drug saturation in the mixed formulations, thus J_{ss} , to varying extents when compared to the TCSs alone. Secondly, dilution of the TCSs with an emollient was also likely to reduce the thermodynamic activity of hexylene glycol in Elocon cream or propylene glycol in Dermovate cream, two TCS excipients which may act to favour the partitioning of the drugs out of the vehicle (Barry, 1987; Mollgaard & Hoelgaard, 1983). Finally, a possible counter effect was inclusion of emollient excipients with poor drug solubilising capacity, such as liquid paraffin or water, which may have acted in an anti-solvent capacity and increased thermodynamic activity of the drugs in the mixed formulations. Whilst the final mechanism may transiently enhance drug delivery to the skin, in thermodynamically unstable systems drug crystallisation may ensue over time accompanied by a loss in drug thermodynamic activity in the formulation. Interestingly, a significant decrease in apparent Kh of mometasone furoate was attributed to rapid drug crystallisation when Diprobace cream was applied with Elocon cream, potentially induced by introduction of a large proportion of water from Diprobace cream to the saturated TCS formulation. The relative contribution of each mechanism described was likely to have governed the extent to which apparent Kh of mometasone furoate or clobetasol propionate was reduced, highlighting the complexity of predicting emollient formulation effects on TCS delivery to the skin in a clinical setting.

In addition to the multitude of formulation effects observed thus far, resulting in a reduction in the apparent Kh of either drug when Dermovate cream or Elocon cream were applied after an emollient, an intriguing general trend of significant reductions in apparent D/h^2 of clobetasol propionate was observed only in cases where Dermovate cream was applied after an emollient, compared to Dermovate cream alone or the respective TCS and emollient premixed systems. It was postulated that this effect was attributable to the presence of a thin unmixed residual layer of the emollient formulation on the surface of the skin creating an additional barrier to drug permeation. In this scenario, drug partitioning and diffusion into the stratum corneum was likely to be further reduced compared to the application of Dermovate cream alone. Further studies to evaluate the relative contribution of the residual emollient layer to a decrease in clobetasol propionate D/h^2 are required to fully characterise the role of the emollient 'barrier' and explain why this effect was absent when Elocon cream was applied after an emollient.

When a TCS was applied five minutes before an emollient, no discernible trend in total drug delivery to the skin was observed across emollient groups, with total drug delivery generally increasing (Elocon cream before Diprobase cream or Hydromol Intensive cream), decreasing (Dermovate cream before any investigated emollient) or unchanged (Elocon cream before Diprobase ointment), compared to the TCSs alone. In part, the application of the TCSs before an emollient is likely to have resulted in *in situ* formation of a mixed layer and similar mechanistic changes in the behaviour of the drugs as observed for the reverse order of application. Despite the variability in emollient effects on drug delivery to the skin, a general trend of increased apparent D/h^2 of either drug was observed across most emollient groups when the TCSs were applied five minutes before the emollient. It was postulated that below the newly formed TCS and emollient mixed layer remained a thin unmixed TCS layer, from which the drugs permeated under occlusive conditions created by the layer above. In further support of this theory, an increase in apparent D/h^2 of either drug from premixed formulations, thus in the absence of a residual TCS layer on the skin surface, was largely absent.

Regardless of the exact mechanism through which drug delivery to the skin was altered when either TCS was applied five minutes before or after an emollient, increasing the time interval to thirty minutes

was not sufficient to mitigate emollient effects on drug delivery to the skin, compared to the applications of the TCSs alone. These findings contradict current clinical recommendations that an emollient should be applied 15 – 30 minutes before the application of a TCS (National Institute for Health and Care Excellence, 2018a; Primary Care Dermatology Society, 2019) or the opinion that that the application of emollients and TCSs should be separated by thirty minutes, with the order of product application being unimportant (Moncrieff et al., 2013). Further *in vivo* studies should be conducted to confirm whether the application of multiple products to the skin at similar times will impact on the clinical efficacy of the TCS and this evidence base will be beneficial to inform future clinical recommendations.

To then investigate whether the trends observed with TCS and emollient formulations were applicable to further complex mixtures of topical products, a topical antibiotic was introduced to the TCS and emollient system. The Aron regimen was identified as a model therapy for investigation which employs a three-component system of a topical corticosteroid and a topical antibiotic heavily diluted with an emollient to produce one tailored, extemporaneously compounded, formulation (the Aron mix). Unlike Elocon cream or Dermovate cream, the Aron mix is not subject to the regulatory safeguards of licensed medicinal products thus no drug stability investigations, *in vitro* formulation performance testing or *in vivo* clinical efficacy data are currently available. Dr Aron's rationale behind heavily diluting the TCS and topical antibiotic is to allow uninterrupted therapy (and more frequent applications) to prevent the risk of 'steroid rebound' or recolonisation of the skin by *S. aureus*. It is, however, clear from the work reported in earlier Chapters that diluting a TCS with an emollient does not always proportionally decrease TCS delivery to the skin, especially when the diluent is dissimilar to the TCS formulation base. Thus, the body of work reported in Chapter 5 presented an opportunity to confirm whether the altered critical quality attributes observed with mixed TCSs and emollients formulations were also evident in a dual drug complex mixture (Diprosone cream, Fucidin cream and Diprobase cream).

Following a finite application of the Aron mix to *ex vivo* human skin, total betamethasone dipropionate delivery to the skin was significantly reduced (6 fold) in a manner proportional to the degree of dilution of Diprosone cream in the formulation (6.6 fold), compared to the application of Diprosone cream alone. As Diprobase cream is the base vehicle for Diprosone cream, this observation was explained by a

decrease in betamethasone dipropionate thermodynamic activity proportional to the degree of Diprosone cream dilution in the Aron mix. The slight difference between the degree of dilution and reduction in total drug delivery was attributed to the presence of the excipients, and potentially the active pharmaceutical ingredient, in Fucidin cream creating a more complex base for dilution of the TCS. Comparatively, total fusidic acid delivery to the skin from the Aron mix decreased to a lesser extent (5.4 fold) than the degree of Fucidin cream dilution in the product (13 fold), compared to Fucidin cream alone. Investigations to elucidate the formulation design of Fucidin cream indicated that fusidic acid drug particles were present in the formulation, strongly suggesting the drug was at maximum thermodynamic activity in the topical product, similar to the formulation design of Elocon cream. On introduction of an emollient product (Diprobase cream) and TCS product (Diprosone cream) to Fucidin cream, formulation changes similar to those reported with Elocon cream mixed with Diprobase cream or Diprobase ointment are likely to have occurred. Thus, total fusidic acid delivery to the skin was potentially governed by the relative changes in the degree of fusidic acid saturation in the mixed formulation and the thermodynamic activity of potential penetration enhancers such as glycerol in Fucidin cream.

Overall, the work presented in this thesis delivers a body of evidence previously unreported to suggest that applying multiple topical products to the skin can induce complex formulation changes *in situ* when products are mixed on the skin surface, or indeed in extemporaneously prepared premixed systems such as the Aron mix, resulting in an altered performance of the medicinal products. Stability investigations did not support the typically recommended shelf life for the extemporaneously prepared Aron mix (two weeks to one month) with a significant decrease in betamethasone dipropionate and fusidic acid content evident after only 7 days at the recommended storage temperature (< 95 % of initial drug contents; 25 °C). Applying topical products as per the clinical guidance has the potential to significantly alter the expected drug delivery to the skin and presumably clinically efficacy of the TCSs and topical antibiotic. Considering the multitude of complex formulation effects that may occur with different product combinations, it is not possible to predict the exact formulation effects on TCS or topical antibiotic delivery to the skin for a wide range of products. For TCSs and emollients in particular, allowing up to

thirty minutes between product applications in an attempt to allow the products to be ‘fully absorbed’, as recommended, is not sufficient to mitigate emollient effects on TCS drug delivery to the skin. Indeed, it appears that greater time intervals between product applications, or prioritised application of a single product to the affected site may be necessary. In further disagreement with clinical guidance that the application of a TCS to well moisturised, thus hydrated, skin can increase the delivery of a TCS, the work presented in this thesis found that applying an emollient before a TCS can in fact significantly reduce TCS delivery to the skin.

To build on the presented evidence base, further *in vitro* and *in vivo* investigations are recommended. For dual drug products such as the Aron mix an evaluation of the impact, if any, of binary drug mixtures on altered drug thermodynamic activities and release characteristics from Aron mix when the products are mixed at various ratios coupled to *ex vivo* skin permeation studies will enable further elucidation of the mechanistic effects occurring in these complex mixtures. The presented evidence base may be expanded, by use of the *in vitro* and *ex vivo* models employed in this thesis, to include topical calcineurin inhibitors (tacrolimus and pimecrolimus), immunomodulating agents which are prescribed for the treatment of moderate to severe atopic eczema alongside emollient therapy. To fully appreciate the clinical impact of the changes observed, future *in vivo* studies evaluating the effect of frequent applications of a heavily diluted potent TCS on skin thinning and systemic side effects are required, alongside investigations on the antimicrobial activity of fusidic acid in the Aron mix and frequency of fusidic acid resistance in the patient population. It is also recommended that *in vivo* healthy volunteer studies are conducted to establish whether biophysical measurements (skin blanching as a measure of TCS bioavailability, for example) are equally impacted by the clinical application protocols employed and product combinations. Additionally, this will enable an assessment of the power of the currently employed *ex vivo* model to predict the extent of change in *in vivo* physiological effects, supporting the more practical *ex vivo* screening of a wide range of TCS and emollient formulations. Furthermore, the formulation specific trends observed may be exploited to develop dual products (individual TCS and emollient formulations) with knowledge of their performance when combined and recommendations to be applied together, as a novel approach to tailor the potency of TCS treatment in a clinical setting.

Chapter 6

Finally, the findings of this thesis pose a vital question of whether data pertaining to the altered bioavailability of a topical medicinal product, in the presence of emollients or other topical products, should be provided to regulatory bodies on submission of the product for approval. Collectively, these outcomes will fill an area of unmet clinical need and serve to answer the valid concerns posed by healthcare professionals, patients and carers who are regularly involved in the treatment of inflammatory skin conditions.

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