Investigations of the Bioavailability/Bioequivalence of Topical Corticosteroid Formulations containing Clobetasol Propionate using the Human Skin Blanching Assay, Tape Stripping and Microdialysis

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This thesis is dedicated to the following:

To Professor Isadore Kanfer and Mrs. Josephine Kanfer.

In loving memory to my late grandmother,Tong Lai Kuen, and grandfather, Leung Yung.

To the Au family.

ABSTRACT

Currently, clinical trials in patients are required by most regulatory authorities for the assessment of bioequivalence of topical products where the drug is not intended for systemic absorption. Hence there is a dire need for suitable methods for the assessment of bioavailability and bioequivalence of such products since clinical safety and efficacy studies are expensive, time-consuming and require very large numbers of patients. Except for topical corticosteroid products where the human skin blanching assay/vasoconstrictor assay has been approved by the US FDA for bioequivalence assessment of those products, no other method has been "officially" approved for use in those investigations. However, a few alternative methods such as tape stripping and microdialysis have been pursued and considered to have the potential for use in bioequivalence/bioavailability studies.

The human skin blanching assay was used to assess the bioequivalence of commercially available topical products containing 0.05% clobetasol propionate. Both visual and chromameter data were obtained and a commercially available topical corticosteroid product, Dermovate® cream was used as both the "Test" and the "Reference" product. The results indicated that both visual and chromametric assessments were comparable to each other and that either could be used for the assessment of the bioequivalence of topical products containing clobetasol propionate. The screening procedure was optimized to identify potential "detectors" for inclusion in the bioequivalence studies. This resulted in fewer subjects being required in a bioequivalence pivotal study, still having the necessary power to confirm bioequivalence using the human skin blanching assay.

Another objective of this research was to re-visit tape stripping and other possible alternative methods such as dermal microdialysis and to optimize these procedures for bioequivalence assessment of topical formulations where the drug is not intended for systemic absorption.

In the past few decades, tape stripping has been used to investigate bioavailability/bioequivalence of various topical formulations. This technique involves the removal of the *stratum corneum* to assess drug penetration through the skin. A draft FDA guidance for tape stripping was initially published but was subsequently withdrawn due to high variability and poor reproducibility. This research project used an optimized tape

stripping procedure to determine bioavailability and establish bioequivalence between three commercially available formulations containing 0.05 % m/m clobetasol propionate. Furthermore, tape stripping was validated by undertaking a study to assess the bioequivalence of a 0.05% topical cream formulation (Dermovate® cream) using the same cream as both the "Test" and "Reference" product, in which bioequivalence was confirmed. The findings highlight the potential of tape stripping as an alternative method for the assessment of bioequivalence of clobetasol propionate formulations and may possibly be extended for use in other topical products.

Microdialysis is another useful technique that can assess the penetration of topically applied substances which diffuses through the *stratum corneum* and into the dermis. Microdialysis has previously been successfully used for *in vivo* bioavailability and bioequivalence assessments of topical formulations. However, the drugs which were under investigation were all hydrophilic in nature. A major problem with the use of microdialysis for the assessment of lipophilic substances is the binding/adherence of the substance to the membrane and other components of the microdialysis system. As a result, this necessitates the development of a microdialysis system which can be used to assess lipophilic drugs. Intralipid® 20% was investigated and successfully utilized as a perfusate to recover a lipophilic topical corticosteroid, clobetasol propionate, in microdialysis studies. Hence, the bioavailability of clobetasol propionate from an extemporaneous preparation was determined in healthy human volunteers using microdialysis. These findings indicate that *in vivo* microdialysis can be used to assess lipophilic drug penetration through the skin.

A novel approach to investigate drug release from topical formulations containing 0.05% clobetasol propionate using *in vitro* microdialysis was also undertaken. The *in vitro* findings were found to be in agreement with the results obtained using tape stripping to assess bioequivalence of the same commercially available products, namely Dermovate® cream, Dovate® Cream and Dermovate® ointment. These results indicate the potential to correlate *in vitro* with *in vivo* data for bioequivalence assessment of such topical dosage forms.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AUC	area under the curve
AUCcorr	area under the curve (corrected tape stripping data)
AUCuncorr	area under the curve (uncorrected tape stripping data)
AUEC	area under the effect curve
BA	bioavailability
BE	bioequivalence
CI	confidence interval
CP	clobetasol propionate
CV	coefficient of variation
DOCA	desoxycorticosterone acetate
FDA	Food and Drug Administration
HPLC	high performance liquid chromatography
HSBA	human skin blanching assay
IL	Intralipid®
IVIVC	*in-vitro-in-vivo* correlation
LOD	limit of detection
LOQ	limit of quantitation
MD	microdialysis
RIA	radioimmunoassay
RP	reversed phase
RR	relative recovery
RSD	relative standard deviation
SC	*stratum corneum*
SD	standard deviation
SEM	standard error mean
SPE	solid phase extraction
TEWL	transepidermal water loss
TPN	total parenteral nutrition
TS	tape stripping
UV	ultraviolet
VCA	vasoconstrictor assay

1. CHAPTER 1

1.1. TOPICAL CORTICOSTEROIDS

Soon after the introduction of hydrocortisone, the first effective topical corticosteroid for the treatment of inflammatory dermatoses, a range of corticosteroid derivatives have been developed and marketed for the treatment of inflammatory skin conditions. The large varieties of topical corticosteroids have an extensive range of potencies, concentrations and vehicles (see Table 1.1). The choice of topical corticosteroid therapy depends on the nature, severity and location of the dermatological condition to be treated [1,2].

Whereas the use of topical hydrocortisone and its acetate ester derivative was not associated with significant side effects, the introduction of other and more potent topical corticosteroids was accompanied by more serious side effects. Thus, researchers have attempted to create topical corticosteroid derivatives which still retain the same efficacy but with a significant reduction and even elimination of some side effects [3].

Since the site of action is considered to be in the dermis, topical corticosteroids are only minimally absorbed following application onto normal, healthy skin. However, the penetration of topical corticosteroids may be altered when applied under occlusion using a plastic covering or when applied to broken skin where the dermis may be breached, resulting in enhanced penetration [2]. In such situations, considerable absorption may occur resulting in suppression of endocrinal function and associated undesirable systemic effects [2,4]. Various other factors that affect the absorption of topically applied corticosteroids include application to different anatomical skin sites, age, skin disease, skin hydration and drug concentration [5,6].

Over the years, the development and utilization of topical corticosteroids have encouraged researchers to study the uses and side effects associated with a specific chemical structure and/or ester derivative in order to determine its potency and biopharmaceutical properties. These studies have been performed by applying various techniques and assessment methods which involve skin and/or blood assays.

1.1.1. Classification of the clinical potency of topical corticosteroid preparations

Since there are many topical corticosteroids available on the market, a classification system has been developed to differentiate between the different corticosteroid derivatives according to their anti-inflammatory activities. This system of classifying topical corticosteroid molecules is recommended mainly for clinicians to provide information on the relative anti-inflammatory potency of the various topical products. This system has the advantage of indicating not only the relative potency of a specific proprietary preparation but also has the ability to classify both local and systemic side effects of corticosteroids assuming the potency and side effects run parallel to each other. The human skin blanching assay (HSBA) also known as the vasoconstriction assay (VCA) and clinical efficacy trials have been used as standard methods for evaluating the relative anti-inflammatory potency of various topical corticosteroid proprietary preparations. The HSBA assesses the degree of vasoconstriction following application of the topical corticosteroid which affects the cutaneous capillaries in healthy human volunteers where normal human skin is used

In northern Europe a four-category system is used but may not be sufficient to indicate significant biological differences amongst the various corticosteroid preparations available. This system regards class I as mildly potent and class IV, very highly potent [7]. In the U.S., a six-category system (Stoughton's system) was set up utilizing the HSBA as well as clinical efficacy trial data where the topical corticosteroids were used in patients suffering with skin diseases. In this system, the most potent was designated class I and the least potent was class VI [8]. Whenever a new topical corticosteroid is developed and does not fit into any of the divisions, a new roman numeral class is added which will shift all the previously designated preparations by one numeral.

In some instances, the bioavailability of a particular corticosteroid may be enhanced by formulation which can result in a change in potency of that particular corticosteroid. For example, the introduction of an optimized ointment of betamethasone dipropionate which significantly increased the bioavailability of the corticosteroid to the skin compared to the previously available formulation, a new class had to be created with this formulation being promoted to a class I classification. Instead of having six classes, there are now seven classes [9,10]. The current classification system is shown below (Table 1.1)

Table 1.1. Potency ranking of some topical corticosteroid preparations and their strengths utilizing Stoughton's system [10]

Classification and Substance	Formulation Type(s)	Strength (%)
Class I (superpotent)		
Clobetasol propionate	Ointment, cream	0.05%
Betamethasone dipropionate	Ointment, cream	0.05%
Class II (potent)		
Mometasone furoate	Ointment, cream	0.1%
Fluocinonide	Ointment, cream, gel	0.05%
Class III (potent)		
Fluticasone propionate	Ointment	0.005%
Flucortolone	Cream	0.25%
Class IV (midstrength)		
Hydrocortisone valerate	Ointment	0.2%
Desoximetasone	Cream, gel	0.05%
Class V (midstrength)		
Betamethasone dipropionate	Lotion	0.05%
Fluocinolone acetonide	Cream, oil	0.025%,0.01%
Class VI (mild)		
Betamethasone valerate	Lotion	0.05%
Alclometasone dipropionate	Ointment, cream	0.05%
Class VII (least potent)		
Dexamethasone	Cream	0.1%
Hydrocortisone	All types	0.5%, 1%, 2.5%

1.1.2. Clobetasol 17-propionate

In this thesis, the focus is on clobetasol 17-propionate (CP) which falls under the category of high-potency corticosteroids i.e. Class I (US classification system). CP has been used since 1973 for the short-term topical treatment of inflammatory and pruritic manifestations of moderate-to-severe corticosteroid-responsive dermatoses [11]. It is also indicated for plaque and scalp psoriasis (moderate to severe) [12].

CP was the first potent topically active corticosteroid investigated at an optimal concentration of 0.05% in a cream and ointment formulation [13].

The treatment of more severe skin disorders using CP with or without the inclusion of other drug substances were compared in several clinical studies. To-date, CP has demonstrated excellent recovery, rapid relief and reduced relapses of different skin conditions and symptoms [3,14,15]. It was also proven to be the first topical corticosteroid that demonstrated satisfactory results in the treatment for psoriasis [13,15].

Although CP is a commonly used potent corticosteroid which has been on the market since the early '70s, not much research has been emphasized on its properties and this dearth of information promoted a need to further investigate this particular molecule. It was therefore chosen as the topical corticosteroid drug candidate for this research in order to gain more insight into this drug's transdermal behaviour in the skin and its bioavailability.

1.1.2.1. Physicochemical properties

1.1.2.1.1. Chemical structure

CP (CAS No. 25122-46-7) [16] is a 21-chloro-9α-fluoro-11β-hydroxy-16β-methyl-3,20-dioxopregna-1,4-dien-17α-yl propionate, and it is a synthetic corticosteroid that has a molecular weight of 467.0 g/mol with the empirical formula, $C_{25}H_{32}ClFO_5$ [4,17].

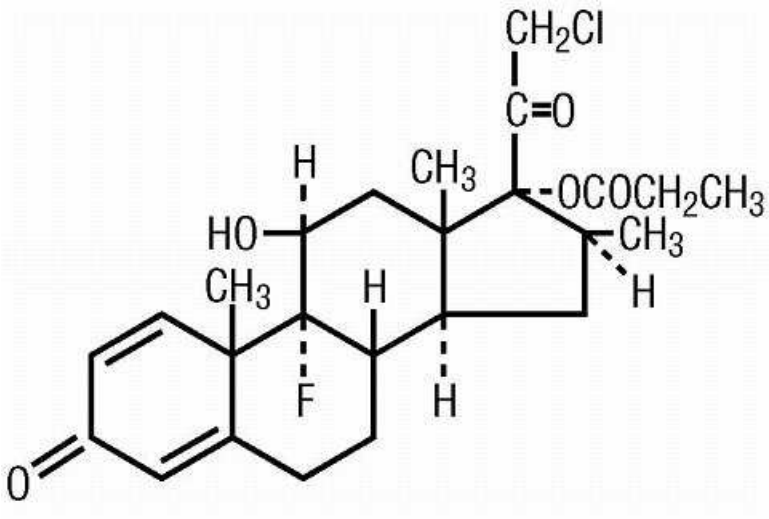

Figure 1.1 Chemical structure of clobetasol 17-propionate

CP is a white or almost white, crystalline powder [4].

1.1.2.1.2. Structure-activity relationships

The skin possesses a variety of enzymes that facilitate a range of metabolic activities [18-23]. Identifying the different enzymes and their activities facilitates the design and development of a drug with required optimal activities. The level of activity of a synthetic topical corticosteroid depends on three factors:

1. Better penetration through the skin.
2. Slower degradation by enzymes.
3. Greater affinity to the receptors in the cells.

This can be optimized by adding, removing or altering the functional groups on the steroid nucleus [24]. This indicates that the relationship between the drug's structural and functional activity is important.

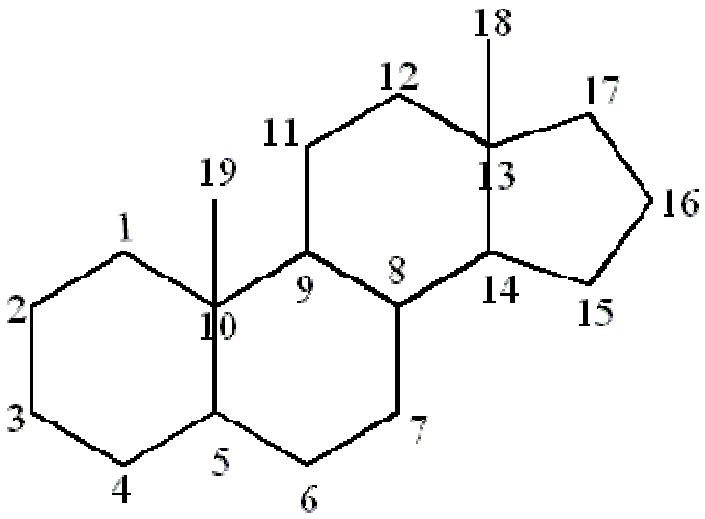

Figure 1.2. Basic steroid structure

The topical corticosteroids are based on the typical steroid nucleus structure (Figure 1.2) that comprises of 17 carbon atoms arranged into 4 rings. There are three 6 membered rings and one 5 membered ring fused to form the basic nucleus. Structural features considered desirable for topical activity are the double bonds at C1 and C4, a C3 keto group, β-methyl groups at C18 and C19, and a C20 keto group [25].

Referring to the structure of CP (Figure 1.1), the introduction of a double bond at the C1-C2 position led to an increase in glucocorticosteroid and anti-inflammatory activity and a decrease in mineralocorticoid properties [6,24,26,27]. Fluorination on carbon 9-α produces the largest increase in glucocorticoid activity, but also enhances the unwanted mineralocorticoid effects [24,26]. The hydroxyl group at carbon 11 is required for inflammatory activity [6,27] and allows the drug to be topically active [24]. Esterification of the hydroxyl group on carbon 17 with lipophilic halogenated forms on the different sites of the molecule enhances skin penetration and effectiveness thus increasing potency, slows down degradation by biological enzymes and increases the affinity of the drug to the receptors in the cells [28,29]. Enhanced penetration is obtained by optimising lipophilicity based on alteration of the free-OH groups [6,24,27,28]. Typical derivatives include the acetates, propionates, butyrates, valerates, pivalates and acetonides. The addition of these esters do not alter the intrinsic activity of the active substance, however, they increase the lipophilicity of the molecule which enhances the compound penetration through the skin by providing a more favourable partition coefficient and becoming more effective [6,25,30,31]. Esterification of the CP molecule at carbon 17 with a propionate group resulted in enhanced potency. The chlorination of the 21-hydroxyl group also resulted in an increase in lipophilicity and enhanced activity [26,32].

As stated above, the slowing down of drug degradation also plays a role in altering the level of activity of a corticosteroid. The degradation of a corticosteroid molecule involves all or some of the following chemical reactions; oxidation of the 11-hydroxyl group, reduction of the C4-C5 double bond, reduction of the keto-oxygen C20, hydroxylation at C6 and degradation of the carbon side chain at C17 [33]. In order to slow down the degradation process in the body, it was found that the introduction of 16α- or β-methyl groups will protect the corticosteroid against metabolizing enzymes [24,26]. From further research [24,29], the C17 esters were found to be more resistant to degradation by cutaneous esterases than the C21 esters. Being more resistant to degradation, the chance of reservoir formation on the skin is higher which will allow the drug to act for a longer period of time thus increasing the potency.

1.1.2.1.3. Solubility

CP is relatively insoluble in water (3.86 mg/L at 25 °C) [16]. It is slightly soluble in benzene and diethyl ether, soluble in chloroform and methanol, freely soluble in acetone and in dichloromethane, and sparingly soluble in ethanol (96 %).

1.1.2.1.4. Melting point

It has a melting point of ~196 °C.

1.1.2.1.5. Optical activity

The specific optical rotation in a 1% w/v solution in 1,4-dioxan, calculated with reference to the dried substance, is between +96° to +104° [4].

1.1.2.1.6. Partition coefficient (Log P) and dissociation constant (pKa)

The partition coefficient, Log P (octanol-water) value is 3.5. Since CP is a neutral compound it does not ionize and thus does not have a dissociation constant (pKa) [16].

1.1.2.2. Pharmacology

1.1.2.2.1. Pharmacokinetics

Pharmacokinetics explains the time course of drug concentration in the body. The

recognition of clearance and volume of distribution has provided an important link to the physiological determinants of drug disposition. Mathematical models of absorption, distribution, metabolism and elimination have been extensively applied, and their predictions agree well with actual observations. Pharmacokinetics is used to describe the process controlling drug concentration at any time after one or more doses [34].

Pharmacokinetics after topical application of drugs differs from pharmacokinetics after all other routes of administration in that the site of action (the skin) is accessible to immediate drug analysis. The development of new topical glucocorticoids has two aims: 1) to produce preparations which assure sufficiently high concentrations at the site of action in the skin, and 2) to keep systemic corticosteroid load as low as possible to reduce the risk of systemic effects [35].

The pharmacokinetic properties of topical corticosteroids still remain poorly understood. In general, corticosteroids which enter the systemic circulation are metabolized in the liver and in other tissues, and are excreted in the urine. The synthetic corticosteroids have lower protein-binding affinity which in turn causes slower metabolism and this may explain their increased potency [27]. Most topical corticosteroids and their derivatives can be absorbed through the skin and penetrate into dermal blood vessels. Some of the molecules are metabolized in the skin sites and excreted as occurs when they are systemically administered.

It has been established that the rate of penetration through the *stratum corneum* (SC) is the rate-limiting step in the percutaneous absorption of the topical corticosteroids. According to Bronaugh *et al* [36], some compounds are metabolized extensively during skin absorption whereas for many, metabolism may be small or undetectable. Cutaneous metabolism occurs at the epidermis where certain similar enzymes as those found in the liver (including a cytochrome P450 system) may be involved and induced. Topical corticosteroids may undergo hydrolysis sulphate conjugation within the epidermis [6].

It has been found that betamethasone-17- and -21- valerates undergoes hydrolysis by esterases found in the skin. It is shown that the 17-ester topical corticosteroids are resistant to both liver and hepatic esterases whilst the 21-esters are rapidly hydrolyzed to the free steroid

alcohol. Hence, this resistance of enzymatic hydrolysis of the 17-ester, as in the case with clobetasol 17-propionate, leads to a more pronounced reservoir effect in the skin [29].

1.1.2.2.2. Mode of action

Inflammation is a dominant feature of many skin diseases, including dermatitis, psoriasis and eczema [1,2,4]. The inflammation process involves the recruitment and activation of inflammatory cells and changes in structural cells. This process is characterized by an increased expression of cytokines, chemokines, growth factors, enzymes, receptors and adhesion molecules [37]. Hence, topical corticosteroid use is based on their primary therapeutic effect i.e. anti-inflammatory activity.

The mode of action of corticosteroids is still not clear, however there are postulations on their anti-inflammatory activities. One hypothesis that has been widely accepted proposed that the inflammatory process results from the release of hydrolytic enzymes of lysosomes and that the glucocorticoids stabilize lysosomal membranes, preventing the rupture of the organelles [38,39]. Greaves *et al* [40] reported that prostaglandins were recovered from several types of inflammatory reactions and are capable of mediating most of the components of inflammation. Hence, the possibility arises that corticosteroids may owe their anti-inflammatory activity in part to the inhibition of the prostaglandin biosynthesis process. It has been suggested by Lewis *et al* [41] that corticosteroids inhibit the release of prostaglandin by preventing the transport of prostaglandin from inside the fat cell to the extracellular space where it would normally act on blood vessels. It has also been postulated [42,43] that corticosteroids block arachidonic acid release from phospholipids by enhancing the production of lipocortin, this in turn inhibits the activity of phospholipase A_2. The activity of topical corticosteroids is thought to be due to the binding of the drug to steroid receptors located in the cytoplasm or on the outside of the nuclear membrane to form complexes that enter the cell nucleus. These complexes then bind to DNA and modify mRNA transcription and the subsequent synthesis of proteins, this effect is ultimately responsible for the anti-inflammatory activity of the corticosteroid [6,44,45]. As mentioned earlier, clobetasol 17-propionate is a highly potent synthetic corticosteroid, and it has been found that high-potency corticosteroids appear to bind more effectively to the receptors [11].

It has also been reported that corticosteroids inhibit DNA synthesis in many types of cells and therefore assist in the treatment of proliferative inflammations such as keloids [24]. The anti-inflammatory effects of the corticosteroids may, in part, be explained by the above postulations since the arachidonic acid cascade generates several inflammatory mediators.

1.1.2.3. General side effects

There are several safety concerns with the use of topical corticosteroids especially that of Class I. Often the misuse and abuse of a topical corticosteroid leads to numerous local and systemic side effects. Factors that contribute to the occurrence of these side effects include the amount applied, percentage of body surface covered, frequency of application, application to thin skin or naturally occluded areas, age of patient, skin quality (i.e. diseased), and steroid potency [46]. The majority of the side effects are reversible. Appropriate management will be either stopping the use of a specific topical corticosteroid or switching to an alternative corticosteroid with lower potency [3]. According to reports in the published literature [14,47], CP demonstrated greater occurrences of side effects than other topical corticosteroids and is therefore recommended for short term therapy only.

1.1.2.3.1. Local side effects

The onset of local side effects from topical corticosteroids is due to application on the same skin site over a long period of time at relatively high concentrations [37]. The more common local side effects consist of atrophy, *striae*, *purpura*, acne, and telangiectases. The less commonly occurring side effects are local hypertrichosis, hypopigmentation, and allergic contact dermatitis [3,37,46].

Dermal atrophy in both the epidermis and the dermis was found to be the most common side effect of topical corticosteroid therapy [10,37,48]. This results in increased transparency, shininess of the skin and *striae* [10]. Skin atrophy is mainly caused by suppressive effects on cutaneous cell proliferation and protein synthesis by the corticosteroid [48]. Corticosteroids also stimulate human dermal microvascular endothelial cells, leading to the occurrence of telangiectasia which is characterized by an abnormal dilatation of capillary vessels and arterioles [10,48]. *Striae* are visible linear scars that develop with an initial

inflammation and oedema of dermis, followed by the deposition of dermal collagen along the lines of mechanical stress. Development of *striae* generally accompanies the occurrence of atrophy [49,50]. Topical steroids can induce an acneform eruption which is due to the degradation of the follicular epithelium, resulting in extrusion of the follicular content [51,52].

It has been reported that steroids can induce hypertrichosis (growth of vellus hair) by means of an unknown mechanism [3,10]. Acne and hirsutism have been reported to be pharmacological effects of glucocorticoids. However, these effects are caused by metabolic degradation products resulting in the loss of the side chain at C17 forming 17-ketosteroid compounds which tend to be androgenic. A positive use of a potent corticosteroid is the reduction of the dosage administered thereby reducing the quantity of 17-ketosteroids being formed [53].

1.1.2.3.2. Systemic side effects

The potential systemic adverse effects upon topical administration include hypothalamic-pituitary-adrenal (HPA) axis suppression, retarded growth in children, and hyperglycemia [10,46,47]. Many studies have shown that high-potency topical agents can suppress the HPA axis. The application of as little as 2g a day of CP 0.05% cream can cause a decrease in morning cortisol levels after only a few days. In some cases, the suppression of the HPA axis brings about the elevation of endogenous cortisol levels which manifests physical features to that of Cushing's syndrome. Clinical features of Cushing's syndrome include round face, hypertension, depression, central obesity and thin extremities to name a few [10,46,47].

These systemic side effects usually occur as a result of an increase in steroid penetration and absorption through the skin when corticosteroids are applied to large surface areas, under occlusion using higher concentrations or more potent derivatives, especially in children [54-56]. Although patients find CP preparations to be more effective in treating stubborn skin diseases, it is recommended that patients should be warned by their physicians to avoid using more than 50g a week [47].

1.1.2.4. Precautions

There is always a risk of systemic absorption following the application of topical corticosteroids. Potent topical corticosteroids should not be used with any occlusive dressing nor should they be applied to large areas of the body. Long-term topical treatment should best be avoided, especially in children. Topical corticosteroids should also not be used for the treatment of ulcerative conditions, rosacea and for pruritis. When used in combination therapy with an antimicrobial substance, hypersensitivity reactions can occur [27]. Failure to heal may be evidence of allergy rather than exacerbation of the condition. Use of topical corticosteroids in uncontrolled skin infections should be avoided [12].

1.1.2.5. Contraindications

Patients who present with rosacea, acne vulgaris, peri-oral dermatitis, peri-anal and genital prurities should avoid using formulations containing CP. The same applies to patients with skin lesions caused by infection with viruses, fungi or bacteria; dermatoses in children under one year of age, including dermatitis and napkin eruptions. Use of CP in pregnant women and in patients with hypersensitivity towards corticosteroids should also be avoided. These contraindications were mentioned in the patient information leaflet of Dermovate® cream (published: June 1981).

1.1.2.6. Dosage and directions for use

As indicated in the patient information leaflet of Dermovate® cream, the formulation must be applied sparingly to the affected area once or twice daily until improvement occurs. As with other highly potent topical steroid preparations, therapy should be discontinued when control is achieved. If a longer course is needed, it is recommended that treatment should not be continued for more than four weeks without the patient's condition being reviewed. Repeated short courses using CP formulations may be used to control exacerbations.

1.1.2.7. Commercially available formulations

Topical formulations containing CP on the South African market include creams, ointments and scalp lotion (see Table 1.2 below). All these topical preparations contain a concentration of 0.05 % m/m CP [57].

Table 1.2. Table of commercially available CP topical formulations in South Africa.

Product name	**Manufacturer**	**Concentration**
Dermovate® cream	Glaxo Wellcome, South Africa	0.05 % m/m
Dermovate® scalp application	GlaxoSmithKline, Bryanston, South Africa	0.05 % m/m
Dermovate® ointment	Sekpharma, South Africa	0.05 % m/m
Dovate® cream	Aspen Pharmacare, South Africa	0.05 % m/m
Dovate® ointment	Aspen Pharmacare, South Africa	0.05 % m/m
Xenovate® cream	Aspen Pharmacare, South Africa	0.05 % m/m
Xenovate® ointment	Aspen Pharmacare, South Africa	0.05 % m/m

2. CHAPTER 2

2.1. PERCUTANEOUS ABSORPTION

The skin, the largest organ of the body, is a possible route for the absorption of chemical agents, although this is usually not intended. Before any topically applied agent can act either locally or systemically, it must penetrate the "barrier layer" of the skin known as the SC. Penetration of this layer not only produces therapeutic action of applied drugs, but also possible local or systemic toxicity and injuries by chemical agents or antigenic substances [58,59]. The skin's impermeability depends upon its integrity, thus damaged or diseased skin is often very permeable. In view of its thickness and chemical structure, it is also a site where numerous drugs can accumulate and are only very slowly removed by absorption. This occurrence is known as the "reservoir effect" where it permits the development of prolonged effects after a single topical application.

The skin is capable of metabolizing various chemical compounds. In order to study the percutaneous absorption of a compound, knowledge of the metabolic capability of the skin is important. It allows the investigator to understand the transformation of the parent compound into its metabolite form and this facilitates the determination of bioavailability and the associated permeation properties of the topically applied compound. However, percutaneous absorption is a complex process where it is more than just diffusion through the SC. It involves sequential steps of several transport processes. First, the molecules must be adsorbed onto the SC, diffuse through it, move into the viable epidermis, diffuse through the epidermis and dermis until they reach the capillary bed, and finally be transferred to the circulating blood. The rate-limiting step in percutaneous absorption is the diffusion through the SC [58].

A great deal of experiments have been performed to explain skin structure, physiology, barrier properties, and the mechanisms by which substances enter and cross the skin. In the previous 20 to 30 years, the scientific study of percutaneous absorption has moved from emphasizing descriptive detail to correlating physicochemical factors to try and understand the types of molecules that may penetrate the protective barrier especially through the influence of vehicles [59].

2.1.1. The structure and function of human skin

The skin is a vital organ and its most important function is to act as a barrier preventing the entry of exogenous substances and retain essential endogenous substances in the human body. The skin is not just the largest organ in the body, but possibly the most complex owing to the variety of cell types contributing to its structure. The function of the skin is primarily to provide protection from physical and chemical damage, UV radiation, and free radical attack. Its other functions include thermoregulation and also acting as a sensory (allow the sensation of feeling) and endocrine organ (for example, the synthesis of vitamin D).

The skin is made up of two distinct layers: an outer, unvascularized epithelial layer (epidermis); and an inner vascularized layer (dermis). The epidermis is further divided into several layers which is characterized by the various stages of differentiation of cells [60]. The most superficial layer, the SC of the epidermis, is recognized as the major barrier. Although much thicker than the epidermis, the dermis offers little resistance to drugs and water [61].

Since the term “epidermis” usually includes the SC together with the various other layers described above, the term “viable epidermis” is used to exclude reference to the SC layer.
The thickness of the outermost layer is ~10μm, whereas the other layers are ~100μm and ~100-200μm, respectively [62].

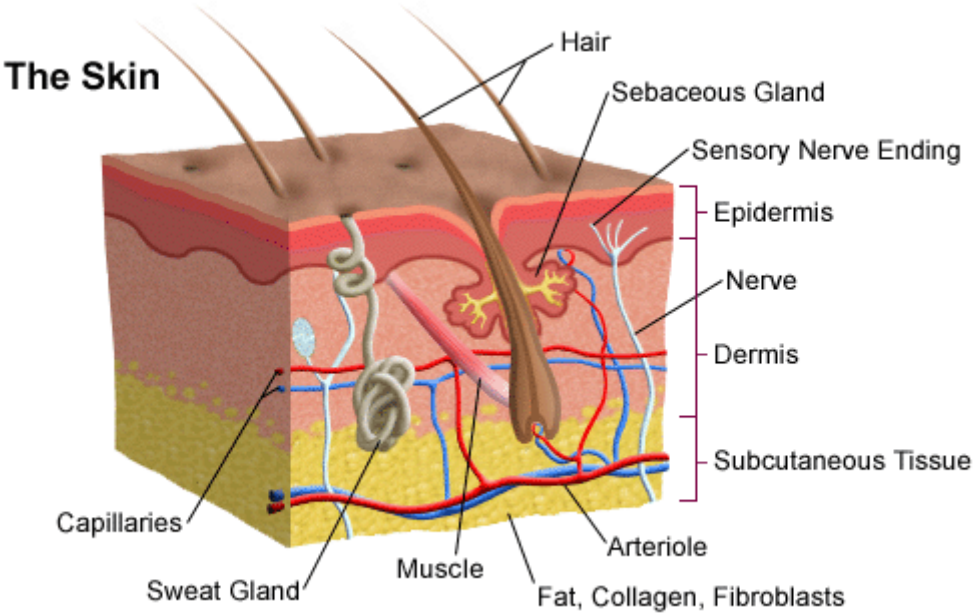

Figure 2.1 Structure of the skin (http://www.methodisthealth.com/tmhs/basic.do?channelId=-1073830178&contentId=1073790565&contentType=HEALTHTOPIC_CONTENT_TYPE, accessed on 04-10-2009)

2.1.1.1. Epidermis

The epidermis is chiefly made up of approximately 95% of keratinocytes and the rest are melanocytes, Langerhans cells and Merkel cells. The avascular, stratified epidermis is approximately 100 – 150 µm thick and is divided into four layers. This multi-lamellar structure represents the different stages of cell differentiation which are the *stratum basale*, *stratum spinosum*, *stratum granulosum* and the SC [63,64].

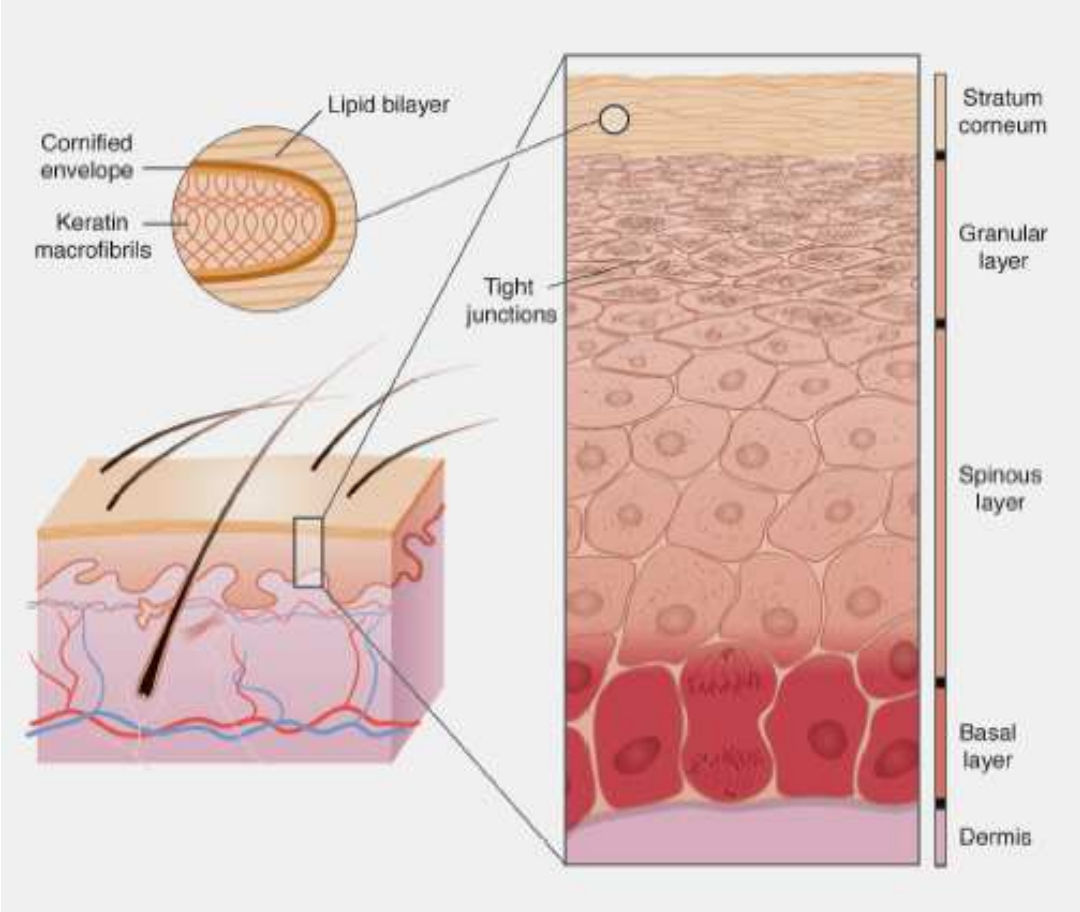

Figure 2.2. A representation of epidermal cell differentiation [65]

2.1.1.1.1. *Stratum basale* (basal layer)

The *stratum basale* is a monolayer of cubic basal cells which are attached to the basement membrane via hemidesmosomes. This layer is made up of epidermal stem cells and transiently amplifying cells derived from them. These cells have a high nucleo-cytoplasmic ratio and contain cell organelles such as keratin filaments (tonofilaments), and are involved in hemidesmosome formation which links neighbouring and overlying cells. Two types of keratins (K14 and K15) are expressed in the basal cells [64].

2.1.1.1.2. *Stratum spinosum* (spinous layer)

The *stratum spinosum* cells have a spiny appearance in histological sections due to the large quantity of desmosomes. In addition to the typical cell organelles found in the basal

layer, the *stratum spinosum* also contains lipid-enriched lamellar bodies called Odland bodies, keratinosomes and membrane-coating granules. The lamellar bodies are approximately 0.2 – 0.5 µm in diameter with parallel stacks of lipid-enriched disks enclosed by a tri-laminar membrane. In cross sections, each lamellar displays a major electron dense band divided centrally by a minor electron dense band. Their appearance marks the dual aspects of epidermal differentiation, namely protein and lipid synthesis. There is a noticeable increase in cellular keratin filaments in this layer compared to the basal cells. Keratins 1 and 10 are the biochemical markers for this layer. In the upper layers of the *stratum spinosum*, the cells begin to flatten and elongate before migrating to the *stratum granulosum* [64].

2.1.1.1.3. *Stratum granulosum* (granular layer)

Keratohyalin granules characterize this layer and these granules are composed of profilaggarin, loricin, and a cysteine-rich protein as well as keratins 1 and 10. They become progressively larger in the upper granulocytes, indicating a quantitative increase in keratin synthesis. The filaggrin subunits of profilaggrin play the role of aggregating and aligning the keratin filaments. Keratin filaments in upper granular layers have extensive disulfide bonds and are highly phosphorylated compared to cell layers below. The progressive cell differentiation is accompanied by increased protein synthesis and lipogenesis, evident by the presence of large numbers of lamellar bodies which reach their highest density in the uppermost granulocytes (about 20% of the cell cytosol). The uppermost cells in the *stratum granulosum* terminally differentiate into corneocytes, during which the lamellar bodies are secreted to the extracellular domains [64].

2.1.1.1.4. *Stratum corneum*

The SC is composed of corneocytes (terminally differentiated keratinocytes) and secreted contents of lamellar bodies which are generated in the basal layer of the epidermis. As cells move towards the surface they lose their nuclei and develop keratohyalin to form an inactive layer of dead, flattened, interlocked cells. During this migration, the lipid content also undergoes modification [66]. Lipid is contained within intracellular lamellar bodies

inside the *stratum granulosum.* As the cells move towards the SC, this lipid is extruded out of the lamellar bodies and into the intercellular spaces to form lipid bilayers around and between the corneocytes [63,67-69]. The lipid composition in the SC is a mixture of cholesterol, ceramides, cholesteryl esters, a small fraction of cholesterol sulfate and free fatty acids [70]. The arrangements of intercellular lipids and the degree of cohesion between cells seem to be vital for the maintenance of an effective epidermal barrier. As the cells migrate towards the skin surface, the intercellular lipids become fragmented, the cohesive forces lessen and desquamation occurs [61].

This layer consists of eight to sixteen layers of flattened, stratified and fully keratinized dead cells. Each cell is approximately 34 – 44 µm long, 25 – 36 µm wide and 0.15 – 0.20 µm thick. The life span of a cell on the surface of the skin is two to three weeks [71]. The barrier function of the SC has a water permeability of 1000 times less than the other membranes [72].

2.1.1.2. Dermis

The dermis has two distinct layers i.e. the papillary layer (which is adjacent to the epidermis) and the reticular layer (which provides structural support) [71]. The dermis is made up of collagen, elastin and glycosaminoglycans as well as fibroblasts that extend the extracellular matrix [64]. This layer also consists of a blood supply network which is made up of a deep plexus of arteries and veins. A second network of capillaries is located on the sub-papillary region of the dermis where small branches of capillaries are sent towards the surface layers of the skin. The capillaries only reach up to 150 – 200 µm before the surface and do not enter the epidermis [71].

2.1.1.3. Skin appendages

Hair and glands are known to be distributed unevenly in the skin on the human body except on glabrous skin. Glabrous skin is hairless (and without hair follicles) and is found on fingers, palmar surfaces of hands, soles of feet, lips, labia minora and the penis. Hair follicles are found at different densities on the body. A majority of the hair follicles in man

are found on the face (600-800cm^{-2}) whereas hair is also found over the rest of the body (60-80cm^{-2}) as well. The amount of appendages on the total skin surface has been estimated to be up to 0.1% [73]. A recent study had shown that the highest follicular density and orifices are on the forehead whereas the least was found on the forearm. However, the amount of skin appendages contradicts the former hypothesis of the skin appendages measuring not more than 0.1% of the total skin surface area [74].

Sebaceous glands that are found in the upper third of the hair follicle secrete sebum into the hair follicle which eventually ends on the surface of the skin. Eccrine sweat glands possesses a coiled section in the lower dermis, and make up 1/10 000 of the total skin surface area [71].

2.1.2. Routes of penetration

Percutaneous delivery of a drug can occur via several possible routes (Figure 2.3). One of the ways is via the transepidermal route which involves diffusion through the SC and the viable cells of the epidermis, and finally through the upper layers of the dermis into the microcirculation [72]. The degree of diffusion is usually limited by the intact SC. However, the rate determining step for very lipophilic drugs lies in the essential aqueous nature of the viable epidermis [75,76]. Other percutaneous routes include via the skin appendages i.e. through sebaceous glands and eccrine glands.

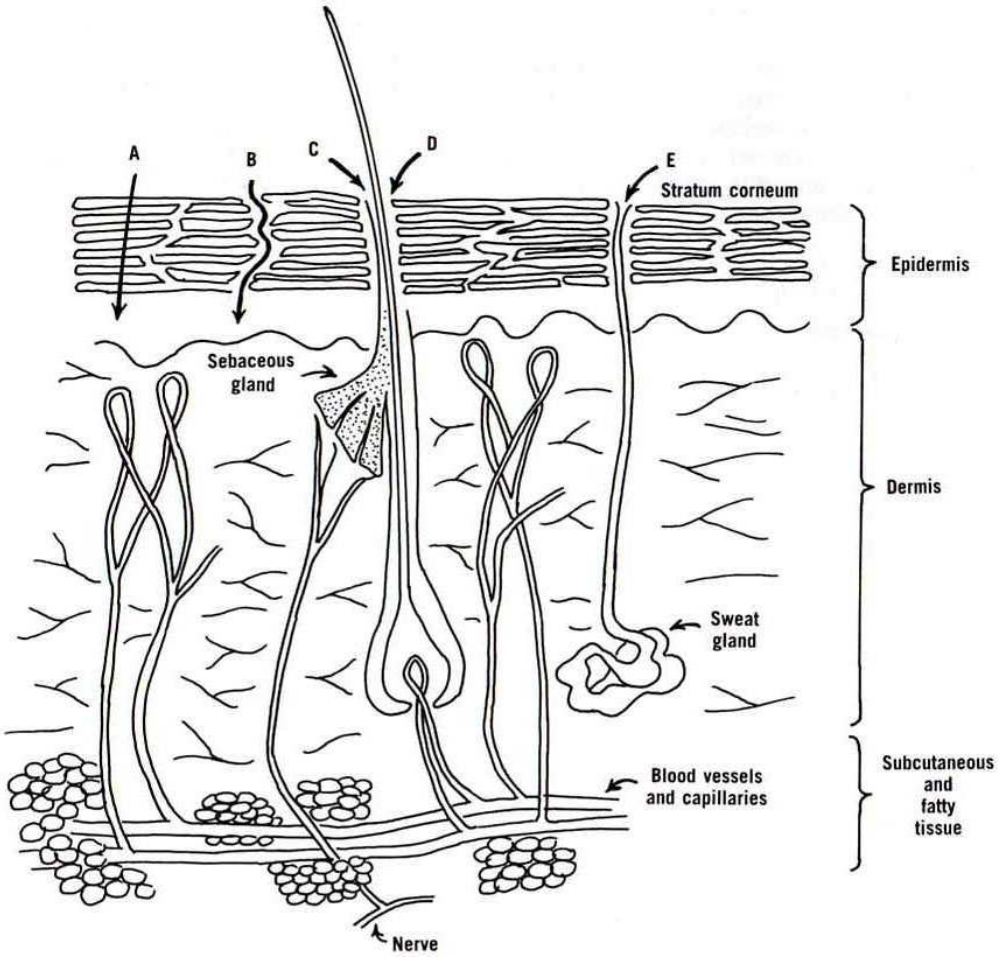

Figure 2.3. Possible routes of percutaneous delivery of drugs A) Transcellular, B) Intercellular, C) through sebaceous ducts, D) transfollicular, and E) through sweat ducts [77]

2.1.2.1. Intercellular route

This route involves the passage of drugs through the SC between the lipid channels between the cells. The lipoidal nature of the lipid channels favours the passage of lipophilic molecules. Since a majority of topical drugs on the market are hydrophobic, this becomes the main entry into the skin [71].

2.1.2.2. Transcellular route

The permeation of drug substances occurs through the hydrophilic keratinized cells. It has been shown that hydration of the skin increases the penetration of polar molecules more

than non-polar ones [78].

2.1.2.3. Skin appendages

The permeation of drugs across the skin includes diffusion through the epidermis and skin appendages such as hair follicles and eccrine glands which form shunt pathways. These skin appendages occupy only 0.1-0.5% of the total skin surface and thus the contribution of this pathway is generally not considered to be significant except in a few cases [58,79].

Previous studies have found increased penetration rates of topical substances in skin areas with high follicular density [80,81]. Otberg *et al* [74] have demonstrated that each anatomical site has its own arrangement of hair follicle characteristics, which suggests that the variations and distributions of skin appendages in the different body sites are also vital for the evaluation and quantification of the penetration process for the different topical drugs and their vehicles/formulations.

2.1.3. Enhancement of percutaneous absorption

Over the years there has been a general perception that the bioavailability of topically administered drugs is very low. In order to improve topical bioavailability, it is necessary to employ enhancement strategies. The ideal characteristics of a dermal permeation enhancer would be that it should be pharmacologically inert, non-toxic, immediate in action, reversible in action, chemically and physically compatible and cosmetically appealing. Owing to all these characteristics, it is doubtful that any enhancer will have all of these properties and compromises are generally made with benefit to risk calculations [82].

To understand the percutaneous absorption of a specific drug, one must know the type of formulation and content of certain excipients in the formulation. The formulation affects the way in which the drug penetrates through the skin. It is important to formulate an active ingredient in a vehicle so as to allow contact and then penetration into healthy or diseased skin for the desired result. The ability of a drug in a topical formulation to penetrate the skin and exert its effect is dependent on two physical events. Firstly the drug must be able to diffuse out of the vehicle onto the skin surface and then it must penetrate this natural barrier

en route to the site of action. Depending on either process, it could affect the overall effectiveness of the topical dosage form. These two processes are related and are thus dependent on the physical properties of the drug, vehicle and the skin barrier.

The drug-vehicle factors that need to be considered when formulating a topical preparation have been suggested by Poulsen [83]:

I. Effects of vehicles on the integrity of the skin barrier or permeability.
II. Particle size of poorly soluble suspended drugs.
III. Chemical nature of the drug.
IV. Partition coefficient of the drug between the skin and vehicle.
V. Drug concentration in the vehicle.

In many studies [84-86], it has been found that vehicles do affect the therapeutic potency of a topical corticosteroid. It is important that an appropriate base must be used in order to obtain maximal clinical response. Vehicles may provide beneficial effects on diseased skin such as emolliency, occlusion or astringent properties. However, the combination of the vehicle and the drug must be compatible with each other to exert a non-deleterious effect.

Topical corticosteroids are available in a variety of vehicles. Depending on the type of vehicle used in the particular formulation, each steroid will respond differently in its ability to penetrate and be biologically active [11,87]. A variety of vehicles are available for topical formulations such as ointments, creams, lotions, gels, liquids and sprays.

2.1.3.1. Effect of concentration of the active ingredient

It is known that the higher the concentration of a single topical corticosteroid component, the more potent it would become since a larger amount will likely penetrate into the skin. However, the result is not always linear with corticosteroids and may result in wastage i.e. a 10-fold increase in concentration may not necessarily yield a 10-fold increase in absorption [6,88].

2.1.3.2. Hydration of the epidermis

Emollients are often found in topical corticosteroid formulations which includes propylene glycol, oils of vegetable and animal origin, and fluid silicone [11].

The rationale for including an emollient is to re-hydrate and soften the SC which creates a higher likelihood for the penetration of both hydrophilic and hydrophobic substances [58]. The emollient assists in restoring intercellular lipids and improving water retention by forming a barrier over the skin which results in an occluding effect [59,89]. Baker [84] has demonstrated that various pharmaceutical vehicles may influence the rate of penetration of therapeutic substances through the skin via their occlusive potential, i.e. their ability to suppress transepidermal water loss. This occlusion re-hydrates the SC and enhances the penetration of therapeutic substances [90]. In fact, occlusion following application of a topical corticosteroid formulation using an occlusive dressing is a commonly used practice to improve drug penetration through the SC.

2.1.3.3. Increasing the solubility of the active ingredient

A way in which excipients can modify skin permeability is to shift the solubility parameter of the skin in the direction of that of the diffusing drug. An increase in the solubility of the drug in the outer layers of the skin will also lead to an increase in the rate at which the drug diffuses through the skin, i.e. the flux [91]. Simple solvent types such as propylene glycol and ethanol are known to improve the solubility of many relatively insoluble drug compounds.

The inclusion of propylene glycol into a formulation generally enhances the penetration of topical corticosteroids due to increased solubility of the active substance [3,92]. Studies have shown that dissolved drug particles penetrate faster than that of undissolved crystal or microcrystal forms [5,59]. On the basis of the results obtained from studies performed by Ostrenga *et al* [92,93] the following were suggested:

I. The concentration of the diffusible drug in the vehicle should be optimized by ensuring all the drug is in solution.

II. Minimum amount of solvent should be used to dissolve the drug completely and yet yield a favourable partition coefficient with the skin.
III. The excipients should affect the permeability of the SC in a positive manner.

Dempski *et al* [94] showed findings in their study that corresponded well with some of the results of Ostrenga *et al* [93].

Only a fraction of a corticosteroid has been shown to penetrate into the SC when dissolved in ethanolic solution following the spreading of the solution over the skin surface. The ethanol keeps the corticosteroid in solution in its dissolved form and prevents interaction occurring between the corticosteroid and the protein-lipid-water matrix of the SC. However, after some time, when most of the ethanol has evaporated and/or diffused into the skin, a layer of corticosteroid (in its undissolved form) is left behind in the SC [5,90]. Consequently, when including ethanol in a vehicle, the initial enhancement of penetration of the corticosteroid is off-set after the disappearance of the ethanol. It is also interesting to note that finer drug particles or particles in solution penetrate the skin more effectively than drug particles in the form of large or insoluble crystals [95].

2.1.3.4. Penetration enhancement using chemicals

Penetration enhancers are chemical compounds which should be pharmacologically inactive but can partition into and interact with the SC constituents. It can be used alone as a pre-formulation application or incorporated into the formulation to reduce the resistance of the skin to drug diffusion [96-98]. A penetration enhancer may also alter the thermodynamic activity of the SC resulting in enhanced drug flux [91,99].

Baker [84] showed that solvents which have been proposed as pharmaceutical vehicles can alter the integrity of the SC due to modification or dehydration, allowing therapeutic substances to move easily across the skin surface in any direction.

Surfactants are frequently included in topical preparations and have demonstrated their capability of enhancing the penetration of topically administered compounds by altering the

skin's barrier function [100]. Normally, anionic surfactants tend to be more effective than cationic surfactants, and non-ionic surfactants are less effective than the previous two [59]. Among the anionic surfactants, the laurate ion has been reported to have the best penetration activity and penetration effects on other solutes [101,102]. The anionic surfactants promote absorption possibly by a denaturing action on epidermal proteins [103,104]. This denaturing effect is caused by anionic surfactants interacting with the SC causing an increase in the local water concentration with consequent swelling and expansion of the tissue and possibly reducing the cohesion between the lipid bilayers [103,105]. The main mechanism by which certain cationic surfactants lead to enhanced skin permeability is to act on the keratin fibrils of cornified cells resulting in disruption of the cell/lipid matrix. Non-ionic surfactants cause fluidization of lipid components of the SC enhancing absorption via penetration through the cholesterol monolayers [100].

The largest class of penetration enhancers seems to act by fluidizing the lipid channels. These include dimethyl sulphoxide (DMSO) at high concentrations, decylmethyl sulphoxide, ethanol and azone. The most commonly used penetration enhancer is ethanol due to its recurrent use in cosmetics and pharmaceutical formulations [106]. These excipients are known to influence the SC at the molecular level, and several modes of action can be observed. Depending on the structure of the penetration enhancer, it may cause a decrease in the intermolecular interaction between the polar head groups or a laxity of the carbon chains found in the lipid bilayers of the SC. The disorder induced by these perturbations permits an increase in the percutaneous penetration of active substances [106].

2.1.3.5. Physical penetration enhancement

Penetration enhancement of all drug molecules may not be easily achieved with chemicals alone. Specific physical penetration techniques may be a better alternative for improving permeation. Possible alternative approaches to augment the flux of a substance through the skin include the use of electrical techniques such as iontophoresis [107] and electroporation [108], or the application of ultrasound [109].

Iontophoresis is a technique which requires passing a small electrical current across the skin (electro-repulsion and electro-osmotic flow) which promotes drug penetration into the skin [110]. This assists the delivery of ionized drug molecules and peptides at a faster rate [107,111,112]. Fundamentally, the charged molecule is forced into the SC as it is repelled from the electrode of similar polarity. The advantage of iontophoretic delivery is that the flux of the diffusing drug molecule can be controlled by altering the applied current, thus tailoring therapy for specific conditions. After the termination of electrical current application, the alteration of skin permeability is maintained for a brief period and the normal barrier function is generally subsequently restored. However, it has been shown that iontophoretic permeation enhancement is more suitable for ionized polar compounds [113,114]

The application of ultrasound can alternatively be used to enhance permeability of permeants through the skin. The mechanism through which ultrasound is thought to act may be by increasing the fluidity of the barrier domains and kinetic energy of the permeant molecules with subsequent heat generation within the SC. However, the use of this technique destroys skin structures if the frequency and intensity of application are extensive [109,115]. This technique can also be harmful when used improperly. As a result, the proper frequency, power level, and duration should be extensively studied before it can be considered as a safe practice. Ultrasound transdermal enhancement may be a solution to one of the major problems facing the field of transdermal drug delivery i.e. lag time and low drug penetration. In addition to its enhancement capabilities, it may control transdermal penetration rate and function as a responsive delivery system [116].

3. CHAPTER 3

3.1. BIOAVAILABILITY AND BIOEQUIVALENCE

Bioavailability (BA) for systemically delivered oral products can be defined as the measure of both the rate and total amount (extent) of drug reaching the general circulation from an administered dosage form [117]. This assesses the process by which the drug is released from the dosage form and moves to the site of action and therefore, the drug's absorption, distribution, metabolism and the elimination processes can be determined [118].

BA studies are conducted to determine a systemic exposure profile by measuring the concentration of drug and/or metabolite in the systemic circulation over time. Often, the concentration of the active moiety or moieties is determined by collecting and analyzing samples of biological fluids such as plasma and urine. The major parameters which represent the rate and extent of drug absorption are [119]:

1. C_{max} – The peak plasma drug concentration is used to measure the rate of drug BA.
2. AUC – the area under the plasma level-time curve is a measure of the extent of drug BA
3. T_{max} – the time of peak plasma concentration, corresponds to the time required to reach maximum drug concentration after drug administration.

As a result, therapeutic dosage regimens can be established which is vital for the development of new drugs. The systemic exposure profile of drug formulations can then serve as a benchmark for subsequent bioequivalence (BE) studies [118].

BE implies that the drug is absorbed from a test dosage form at a comparable rate and extent to the innovator product. Demonstrating BE amongst drug products often necessitates the measurement of drug and/or metabolite levels either in the blood or urine over a fixed period of time following drug administration. Comparison of therapeutic performances in two formulations containing the same active substance is a critical means of assessing the possibility that a similar medicinal product can be used as an alternative to the innovator product. Assuming that in the same subject a similar plasma concentration time course will

result in similar concentrations at the site of action and thus a similar effect. Pharmacokinetic data can be used instead of therapeutic results to establish equivalence between products i.e. BE [118,120].

In order to show BE, it can be measured in at least three ways: chemically, biologically, or therapeutically [121]. However, for topically applied products, standard BA studies used to establish BE of orally administered drugs are often difficult to carry out because blood levels are too low and/or they may provide an inappropriate measure of BE. Topical doses tend to be so small that serum and/or urine concentrations are often undetectable unless a suitable highly-sensitive analytical assay can be developed. Furthermore, systemic availability may not properly reflect cutaneous BA for medications intended to treat local skin disorders.

Assessment of BE of topical formulations may be established through a well-controlled clinical trial or through the measurement of a pharmacological or pharmacodynamic end point e.g. the human skin blanching assay (HSBA) (as described in chapter 4). Other techniques used to assess BA/BE include punch biopsies, tape stripping, dermal microdialysis, *in vitro* dissolution studies and animal studies. However, much work needs to be done in these particular areas before such approaches can find general acceptance.

The advantage for patients using generic substitution of certain medications is that a generic product is identical to the innovator product in terms of BA, safety and efficacy but is more affordable. A generic product may be used as a substitute for a prescribed drug if it complies with the regulatory requirements and law in a particular country and meets certain criteria, i.e. the generic product must show that it is BE before being placed on the market. Hence, there is a need for appropriate BE testing methods for topical formulations.

Factors found to influence the assessment of the BA and BE of topical products include biological factors in the case of BA testing and formulation factors in the case of BE testing. Formulation factors refer to the effect that different excipients and manufacturing methods have on the diffusion of the active drug to the site of action. Biological factors include sex [122], age [123,124], anatomical sites [81,125,126], race [127,128] and diseases [129,130].

3.1.1. BE assessment methods for topical products

BE assessment of topical products has been studied using several different techniques. However, the only internationally acceptable technique other than the use of clinical trials conducted in patients is the human skin blanching assay (HSBA) for topical corticosteroid products. A major problem in the study of dermal BE has been the interpretation of results from various investigators. It is very difficult to draw valid conclusions and comparisons due to the diverse test systems exploited *in vitro* and *in vivo*, anatomical locations, various times and modes of application, and the experimentation on the different types of animal species [59,131].

The development of *in vitro* systems for the estimation of the percutaneous delivery of drugs across the skin has proven to be useful when used accurately and properly [132]. In such systems, it becomes plausible to conduct research on toxic compounds without involving the ethical issues associated with the use of human volunteers. However, a problem arises when attempting to correlate data between animals and humans [133,134]. It is difficult to know how closely dermal penetration in animals mimics penetration through human skin. Thus in percutaneous absorption studies, the exposure/risk assessment scenarios do not take into account the variation in the permeability of animal vs. human skin, and the metabolic/pharmacokinetic/dose response relationships between animals and humans. Predictive models based on animal data are nevertheless a necessity for estimating tissue dose and target tissue dose response in humans [135].

Numerous methods have been attempted to assess BE of topical products by using *in vitro* and *in vivo* study test systems.

3.1.1.1. *In vivo* methods

3.1.1.1.1. Biological/pharmacological response

The HSBA (*vide infra* chapter 4) is an extremely sensitive physiological marker for the presence of minute amounts of corticosteroids in the skin. This assay is based on the ability of topically applied corticosteroids to cause a blanching (pallor) effect on healthy human skin

at the site of application.

3.1.1.1.2. Tape stripping

A dermatopharmacokinetic approach, *vide infra* chapter 6, has been proposed for the BE testing of topical drug products by comparing drug content kinetics in the human SC. This kinetic approach is based on drug delivery into the rate-limiting barrier of the skin i.e. SC. It uses the SC as the sampling matrix in combination with a validated bioanalytical assay for quantifying the analyte in the stripped skin sample. This method has proven to be an effective and promising tool but has currently not been recommended by regulatory agencies for use to assess BE in spite of published data that indicate its potential application. The major advantages of this technique are the use of non-radiolabeled substances and hence a less sensitive analytical method can be used due to the adequate concentration found in stripped skin samples.

3.1.1.1.3. Dermal microdialysis

Dermal microdialysis (MD) (*vide infra* chapter 7) is a technique that measures topically applied substances in the extracellular space beneath the exposed skin site. It is a slightly more invasive technique than TS, which necessitates the superficial insertion of a MD fibre under the skin via the use of a cannula. The drug diffuses from the surrounding tissue across a semi-permeable membrane by passive diffusion into the MD fibre which acts as the receptor medium (perfusate). The perfusate is then collected at various time intervals for analysis. The advantage of using dermal MD is the suitability of the technique to measure the analyte before it enters the systemic circulation or acts locally in the epidermis layer. The major drawback is the need of a highly sensitive analytical method to quantitatively determine the analyte.

3.1.1.1.4. Other *in vivo* assessment techniques

With the exception of topical corticosteroids, the only other means of demonstrating BE of

topical dermatological product to an innovator's product is through the use of comparative clinical trials with a BE endpoint. However, these clinical efficacy trials are time consuming and costly. Additionally, to gain adequate statistical power to show BE, it is necessary to use a large number of patients [132]. Often, hundreds of patients are required in such studies due to the fact that in many clinical endpoint studies, the topical products assessed do not have a specific dosage i.e. the amount of the active ingredient applied on a day-to-day basis and between patients can be quite different. Additionally, while the patient is outside of the investigators direct control, the evaluation of clinical response in this type of study may last for weeks [136].

A pharmacokinetic endpoint is normally preferable to a pharmacodynamic or clinical endpoint for better accuracy, sensitivity, ease of study conduct, cost and timing. A decision tree (Figure 3.1) may be useful in determining the type of study required for any particular product [136].

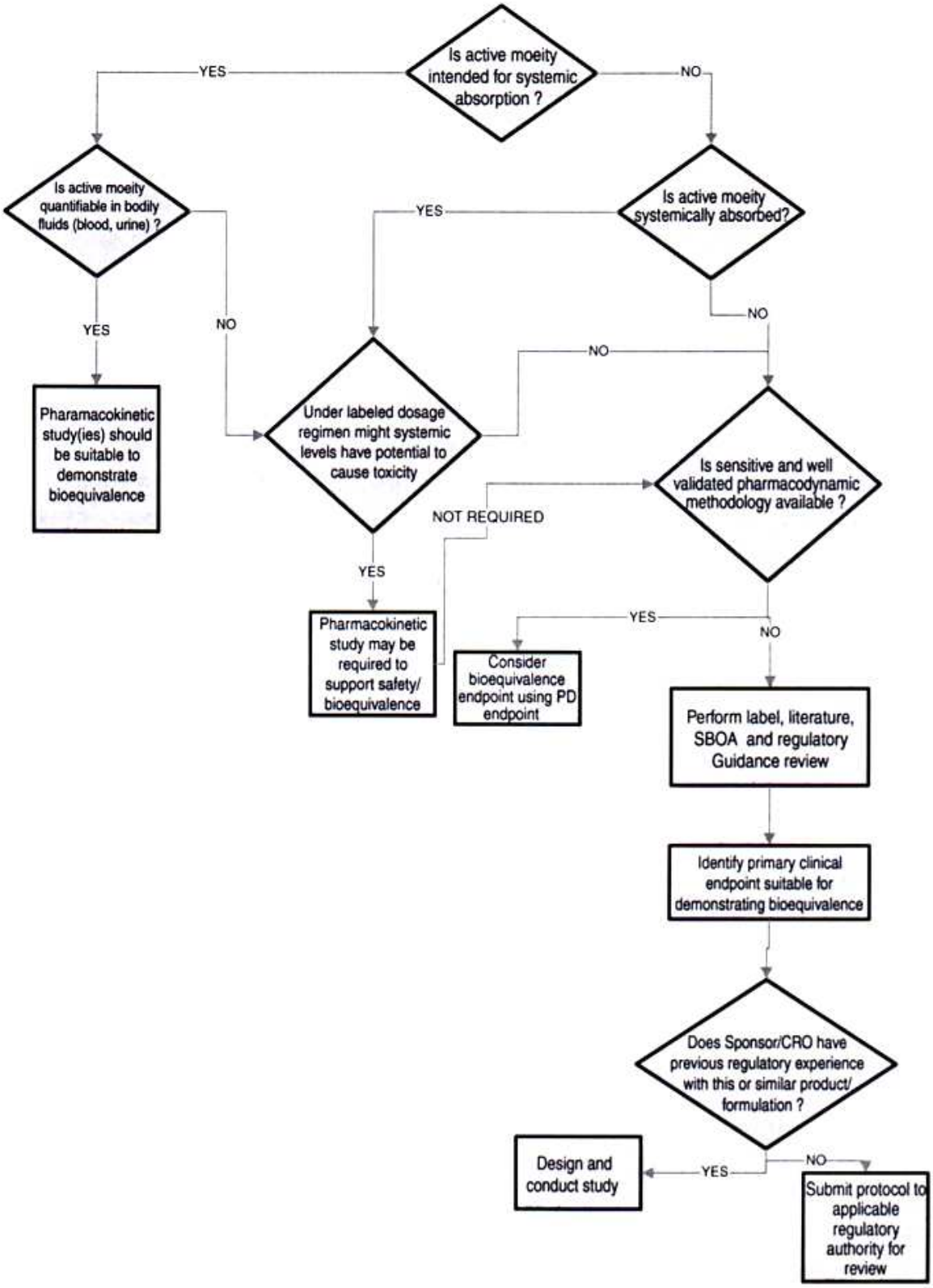

Figure 3.1. A decision tree to determine whether a clinical endpoint BE study is required [119]

Other possible methods for assessing the concentration of the analyte in the skin following topical application include surface biopsy, surface scraping, sebum collection, fluid collection from suction blisters, shave biopsy, and punch biopsy [132].

Although these above listed methods have proven to be useful, the major drawback is the invasiveness of the technique. These procedures were not only found to be painful by the subjects but also did not result in compelling data to validate their use for the assessment of BE.

It is thus clear, that whereas well-established methods for the determination of BE of extravascularly administered drugs exist, there is a dearth of methods for use with topical dosage forms not intended to be absorbed into the systemic circulation.

3.1.1.2. *In vitro* methods

The initial development of dermatological and transdermal drugs requires knowledge about the percutaneous permeation and absorption (systemic) of the drug. The ultimate testing of a new drug or formulation is *in vivo* human testing. However, a compromise on safety, relevance and cost needs to be made, thus alternative test procedures are desirable. *In vitro* experimental techniques have progressed over the years and are yielding valuable data on routes of absorption, barriers to absorption, degree of systemic absorption after topical application, and the effects of various physical and chemical parameters.

The standard method for measuring drug permeation across the skin is the use of a diffusion cell system (Figure 3.2). The classic diffusion cell utilizes a skin section which acts as the membrane that divides the donor and receptor compartments. The rate of drug penetration from the donor chamber to the receptor chamber is determined by quantifying the amount of drug permeated over time with the use of an analytical method e.g. high-performance liquid chromatography. The source of skin [137-139], skin layers used [140,141] and the properties of the receptor solution [142] can have a significant effect on the permeation. The shortcoming of this type of methodology is thought to be that it does not truly reflect the role of the skin *in vivo* e.g. sink conditions are absent because there is no blood supply system

and there is also a lack of biological enzymes. Attempts have been made to develop vehicles acting as "sinks" or receptor skin phases and the results have established fair correlations [143-146]. In this type of study, it is wise to do a material balance study i.e. analyse the drug contents in the skin layer and the formulation to obtain data on drug partitioning from the donor chamber to the skin layer and from the skin layer into the receptor phase. This will determine a rough estimate of the partitioning effect through the skin *in vivo* [147].

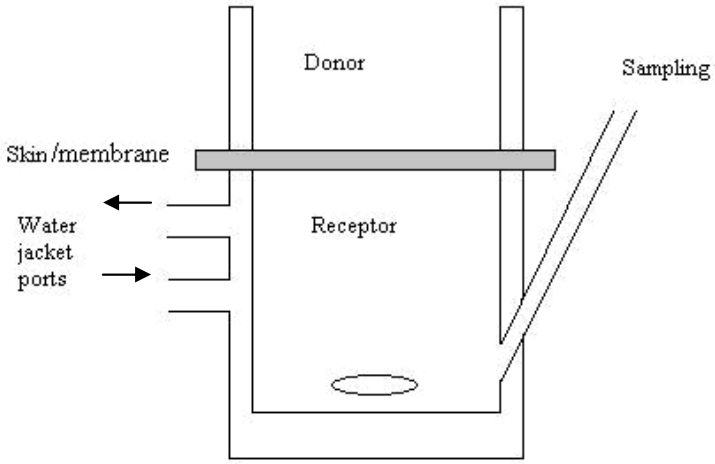

Figure 3.2. A schematic representation of a diffusion cell [91]

An alternative method to determine drug permeation through the SC is to use ATR-FTIR spectroscopy. In this system, the SC is sandwiched between a reflectance and a drug donor solution, the diffusion of the substance into the deeper layers of the SC is monitored by the appearance and increase of a drug-specific infra-red absorbance as a function of time. However, this technique is limited to measuring IR-active substances only [148,149].

In vitro penetration studies can imitate transdermal drug absorption only if the penetration process is rate limiting *in vivo* as well as *in vitro*. Not only the permeation of substances through the SC, but also the microcirculation of the skin may be rate-limiting for certain substances [146]. Several researchers [150-152] have proven that data obtained from such *in*

vitro systems are comparable with those *in vivo.* However, there are only a few experiments where the data correlated well. The advantage of an *in vitro* system is the total control of environment which permits the demonstration of the significance of individual factors in determining percutaneous absorption of a particular substance. Another advantage is applicability of the *in vitro* system which allows the screening of new products and hazardous chemicals [59].

4. CHAPTER 4

4.1. THE HUMAN SKIN BLANCHING ASSAY (HSBA) also known as the VASOCONSTRICTOR ASSAY (VCA)

The determination of the BA/BE of oral dosage forms involves the comparison of drug concentrations found in biological fluids (blood plasma/serum/urine) following administration of the dosage form. However, this approach cannot be used for medicinal products not intended for absorption into the systemic circulation, such as topical dosage forms used for local action.

Topical corticosteroid products have been extremely effective for the treatment of various skin disorders such as eczema, psoriasis and keloids, amongst others [153-157]. The advent of generic topical corticosteroid products necessitated the development of a suitable technique for the assessment of BE of such products which resulted in the publication of a Guidance by the USA Food and Drugs Administration (FDA) in 1995 [158].

Various techniques have been studied to assess the effectiveness of topical corticosteroids. These include the vasoconstrictor assay [132,159-161], ultraviolet [162-164] and croton oil inflammation suppression studies [165-167], psoriasis assay [168,169] and adhesive TS methods [5,14,170-175].

The HSBA is a reliable and convenient assay for the comparison of the BA and BE of topical corticosteroids products [176] where a great advantage is that it can be preformed on healthy human subjects. This assay, initially introduced by McKenzie and Stoughton in 1962 [159], relies on the unique ability of topical corticosteroids to produce a blanching response (a skin whitening effect) on the skin following application and is illustrated in Figure 4.1. This blanching response relates to the amount of corticosteroid which has penetrated into the skin [158].

This method was initially utilized for the potency ranking of corticosteroids. However, it was also found that the method has limitations and does not necessarily rank the corticosteroids in the same order when compared to other assessment methods [177]. In practice, however,

topical corticosteroids are usually applied to diseased skin thus penetration of the drug will differ due to less resistance by the SC barrier. As a result, information on topical BA [177] obtained from studies on healthy skin may not reflect the penetration of the drug into diseased skin. Notwithstanding, the HSBA has been found to be very effective for the determination of the effect of formulation on the activity and efficacy of topical corticosteroid products and to examine the comparative bioavailabilities of such topical preparations [95] as indicators of the efficacy of those products, i.e. for BE assessment.

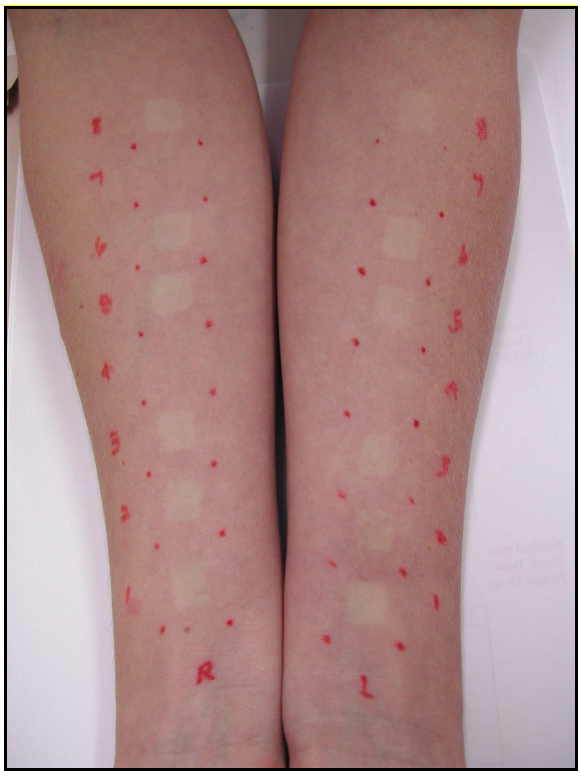

Figure 4.1. A typical blanching response after application of Dermovate® cream (0.05% CP) [178]

Initially skin blanching was investigated using a simple "Yes/No" assessment to establish whether a blanching response was present or absent after application [179-181]. However, this type of assessment does not provide useful information on the degree of BA of the product and it is thus not suitable for the assessment of BE by comparing BA between different topical corticosteroids and/or their formulations.

The HSBA was subsequently optimized to facilitate comparisons by evaluating time-response profiles, potencies and formulations of topical corticosteroids. The optimizations

included the application of appropriate statistics, establishment of requisite duration of application of the drug or drug product (dose duration) and observation intervals of time following application at which the response could be assessed and also a scoring system to facilitate visual assessment [158,161,182-184]

4.1.1. Mechanism of skin blanching

The exact mechanism of blanching produced by topical corticosteroids remains unresolved. Several investigators have suggested that a relationship exists between noradrenalin and corticosteroids [185-187]. However, some investigators have found that although noradrenalin may be involved, there are also other existing factors that may account for the blanching response. Solomon *et al* [187] postulated that there are several explanations for the cause of skin blanching, as follows:

1) corticosteroids, similar to sympathomimetic agents, directly affect sodium transfer systems across the smooth muscle cellular membranes causing contractions of the smooth muscles of vessels [188]

2) locally bound stores of norepinephrine are released by corticosteroids thereby causing smooth muscle contraction

3) various enzyme systems including those involved in the release of bradykinin, norepinephrine and serotonin are affected by corticosteroids which upsets normal vascular tone. It has thus been suggested that steroids mediate the release of norepinephrine in normotensive subjects only [187]. It has also been proposed that glucocorticoids may act by opposing various natural vasodilators such as histamine, bradykinin and prostaglandins [189,190]. Haynes [53] stated that the slow development of vasoconstriction provides sufficient time for major alterations to occur in the tissue under the influence of glucocorticoids and suggested that since they act to regulate synthesis of proteins, the altered vascular tone may be the result of a basic change in the proteins in the blood vessels or some components of the tissue that affect the blood vessels.

Another mechanism which could cause a skin blanching response may be the effect of melanocytes. Edwards *et al* [191] studied the effect of corticosteroids on melanosome aggregation in frog skin. They found that cortisol reversed skin darkening by isoproterenol,

caffeine as well as melanocyte-stimulating hormone (MSH). The results suggested that cortisol binds specifically to a novel type of receptor that has not yet been characterized on the melanophore and which may influence certain steps between the adenylate cyclase and melanosome movement thereby causing the skin whitening effect. It has been postulated that the cause of hypopigmentation may be due to interference by steroids with the synthesis of melanin by smaller melanocytes, leading to patchy areas of decreased pigmentation [3,10]

4.1.2. Methods for the evaluation of skin blanching

4.1.2.1. Visual assessment

The initial methodology for the assessment of topical corticosteroids formulations involved the visual assessment of the degree of skin blanching following application to the skin. Visual assessment remained for some period of time as the most commonly used tool when applying the HSBA to compare skin blanching activities between different topical corticosteroids and also formulations [161,192-194] as well as for the assessment of BA/BE. However, a Guidance [158] document was issued by the US FDA in 1995 wherein an instrumental method using a chromameter was recommended although the visual assessment method was also retained.

Colour is a matter of perception of electromagnetic radiations in the wavelength range of 400 to 700 nm by the eye, followed by subjective interpretation by the brain. Colour vision is trichromatic combining the blue, red and green registrations. Although the human eye is sensitive enough for the discrimination of small colour changes in skin blanching, different people usually draw upon different references and may express the exact same colour in different words. As a consequence, this is perceived as an apparent weakness when using the human eye to evaluate skin whitening owing to the observer's subjectivity. A further criticism on visual assessment relates to the inability to validate the eye as one is able to do when using an instrument [195-197]. However, if observers undergo sufficient training and gain experience in visual evaluation of skin blanching, reproducibility and reliability of visual assessment can be established [198-200].

Visual assessment of skin blanching involves the use of a scoring system to measure skin

blanching intensity. As mentioned previously, the initial scoring system only used a "Yes/No" assessment, which simply indicated whether or not a skin blanching response occurred following application of the topical corticosteroid, i.e. using a score of 1 or 0, respectively. The scoring system subsequently evolved to a graded response based upon the following criteria; absent, faint, faint – moderate, moderate – strong and strong – intense blanching using the scores of 0, 1, 2, 3 and 4, respectively. This remains the commonly used visual assessment scoring system based on the 0 – 4 scale introduced by Barry and Woodford [201,202]. The data are reported in terms of the percentage of the total possible score calculated as follows [161];

The maximum score per site	= 4
The number of independent observers	= n
The number of sites per preparation per arm	= S
The number of subjects	= V
Total possible score (TPS)	= 4 x n x S x V

Percent total possible score (%TPS) = (Actual score / TPS) x 100 (equation 4.1)

4.1.2.2. Chromameter assessment

An instrumental method involving a tristimulus colorimeter was subsequently introduced as an objective and thus "preferred" method. The Minolta® chromameter, which is a portable instrument that uses tristimulus colorimetery involving reflectance spectroscopy was adapted for use to measure skin blanching. This approach had subsequently been used for the objective measurement of skin color [203,204]. The chromameter functions by emitting a white light (using a pulsed xenon arc lamp) onto the chosen area of assessment and measuring the intensity of reflected light through three particular wavelength filters (analyzed at wavelengths of 450, 560 and 600 nm) or using a photodiode array in more recent instruments. The detected signal is converted into three coordinates: L* (luminosity), a* (the amount of green or red), and b* (the amount of yellow or blue). These three coordinates record color in a three-dimensional color system recommended by CIE (Commission International de l'Eclairage) [196,197,205]

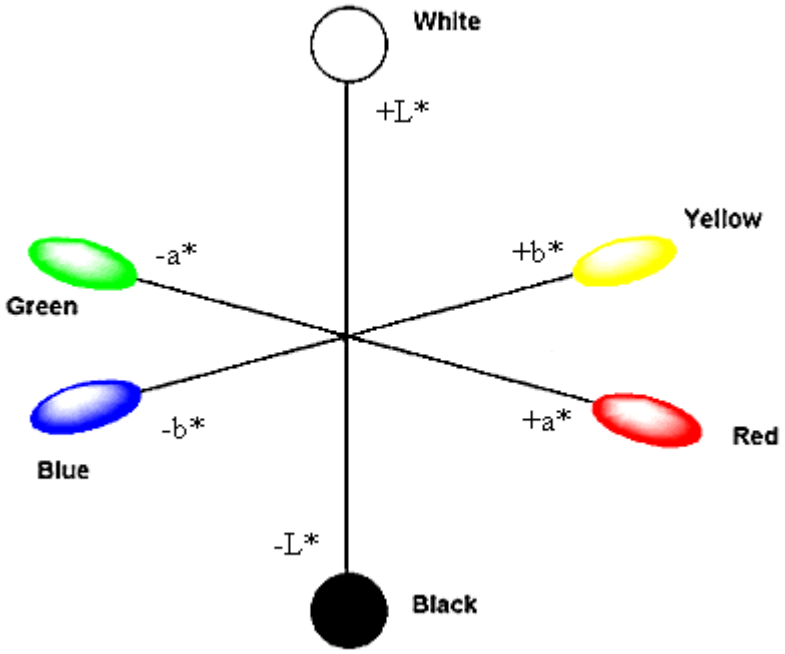

Figure 4.2. The L*a*b* color space (adapted from Waring [206])

The skin blanching response is measured relative to the color change in the skin. As the skin blanching response develops, the skin becomes lighter and its redness fades. As the skin becomes more pale the L* scale increases, a* scale decreases and b* scale increases very slightly. It [207] has been shown that the L* and a* coordinates are more discriminative than the b* coordinate in determining skin blanching responses, thus the latter coordinate is omitted from data analysis. However, following release of the FDA guidance, only the a-scale data has been recommended for use in the statistical analysis [158]. This is possibly due to better correlation with visual skin blanching data found by Pershing *et al* [160].

The chromameter can offer reliable and repeatable results provided that certain drawbacks are avoided such as manipulation of the measuring head of the instrument which can affect the quality of the data produced. Skin compression by the measuring head and the angle alignment of the chromameter play a role in obtaining repeatable data [198,205,206]. To obtain optimal results, each subject's assessment site as well as ambient temperature should ideally be controlled. It is also important for the operator to hold the chromameter head in such a way that variation in pressure is avoided [198]. The presence of hair and variations in skin glossiness related to the amount of water and lipid on the skin surface, scarring, uneven skin tone, etc. can influence the data obtained [208]. As a result, it is important to avoid these

areas of the skin to achieve reliable and reproducible data.

4.1.3. Study Designs

4.1.3.1. Types of studies (pilot and pivotal)

The FDA Guidance recommends that two *in vivo* studies, i.e. a pilot and pivotal study, be conducted in order to determine BE between topical corticosteroid products. A pilot study provides information on an appropriate dose duration required for the subsequent BE testing in a pivotal study. The pilot study utilizes a dose duration-response approach which controls the dose of topical corticosteroid being delivered by comparing different times of exposure of the product on the skin (dose duration is the period of time that the formulation/product is left in contact with the skin). This study is usually conducted only using the reference product.

Dose durations required for the pivotal study as recommended by the Topical Corticosteroid FDA guidance [158] are ED_{50}, D_1 (half of ED_{50}) and D_2 (two times ED_{50}), where ED_{50} is the dose duration at which 50% of the maximum blanching response is achieved. The ED_{50} is chosen since it represents the portion of a dose-response relationship plot where the optimum discrimination of relevant differences can be detected. Using longer dose durations may dampen the assessment of relatively small but significant differences in blanching between a test and a reference product. Furthermore, using shorter dose durations will influence the reliability and repeatability of the assessments.

The development and validation of a dose-response curve is therefore essential to determine ED_{50}, D_1 and D_2. These values are determined from an E_{max} model, in accordance to the relevant FDA guidance [158], shown as follows:

$$E = E_0 + \frac{E_{max} \times D}{ED_{50} + D} \qquad \text{(equation 4.2)}$$

where E = effect elicited

E_0 = baseline effect in the absence of ligand

E_{max} = maximum effect elicited

ED_{50} = dose duration (D) at which effect is half maximal

The pivotal study is then conducted where a comparison between the responses of a test and reference product is investigated for BE using the ED_{50}. Furthermore, the Guidance recommends that a subject must be a 'detector' in order for inclusion of their data for statistical analyses supporting *in vivo* BE assessment. Hence, subjects' responses are expected to meet the specified minimum D_2/D_1 ratio of AUEC greater or equal to 1.25 in the pivotal study, where AUEC is the area under the effect curve following the plotting of the particular response (visual response or chromameter response) versus time

4.2. PILOT STUDY

4.2.1. Experimental

4.2.1.1. Instrumentation

A Minolta Chromameter® (Model CR 400, Minolta, Osaka, Japan) that provides measurements based on three scales, the L-scale, a-scale and b-scale, was used. An Eppendorf pipette was used to dispense the accurate dose of topical formulation to each application site.

4.2.1.2. Formulations

Several Dermovate® creams, the currently marketed innovator product in South Africa, from the same batch, were purchased from a local pharmacy in Grahamstown. The preparations were kept away from direct sunlight and stored at room temperature controlled at 22 ± 1 °C. A new tube of corticosteroid cream was used for each study. The first gram of each tube of cream was discarded in case of any interaction between the closure of the tube and the corticosteroid formulation.

4.2.1.3. Subjects

Healthy male and female subjects in the age range of 18-50 years of age were screened for skin blanching response to the reference product, Dermovate® cream. The subjects were screened by applying ~11mg (SD ±0.17 mg – *vide infra* section 4.2.1.5*)* of the cream from a calibrated applicator at a demarcated site on the upper forearm for six hours unoccluded. The spot of cream was washed off after six hours and assessed for any skin blanching response. Those subjects who demonstrated a skin blanching response (referred to as a 'responder' in accordance with the FDA's guidance document [158]) to the reference product (Dermovate® cream) were included into the experimental study. According to the FDA Guidance, none of the subjects had received any corticosteroid treatment for the past 6 weeks, none had any skin disorders or allergy to the corticosteroid or the formulation, and none had skin defects that may interfere with evaluation of test sites [158]. Written informed consent was obtained from all the subjects before the study (*vide infra* appendix I).

From a number of subjects screened, 12 subjects were initially selected to participate in this pilot study. However, 2 of these responders dropped out due to alcohol consumption the night before and only one reserve subject out of three reserves were contactable to participate in the study. Hence, 11 responders in total were used for this pilot study.

4.2.1.4. Product application and removal

The flexor aspects of the two forearms were used for this experiment. Eight square application sites were demarcated with adhesive labels (Tower®, South Africa, Cape Town) avoiding areas with large blood vessels, scar or skin blemishes and approximately 4 cm away from the wrist and elbow regions on each forearm (as seen in Figure 4.3).

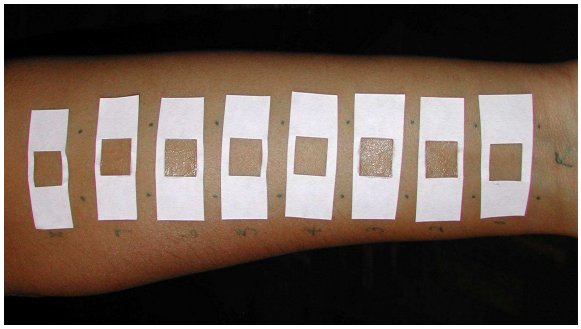

Figure 4.3. Demarcated forearm using adhesive labels

Adhesive labels were punched to produce a 1.1 x 1.1 cm square application site. Each application site was marked with two dots below each site with a template and an appropriate marker to allow correct placement of the chromameter during assessment. The chromameter head had also been marked with the corresponding marks to those on the sites. Approximately 12μL (equivalent to approximately 11mg) of the product was applied per site with a pre-loaded calibrated Eppendorf pipette. The 0.2ml Eppendorf pipette tips (Eppendorf, Eppendorf AG, Germany) were filled immediately prior to use to minimize any possible interaction between the corticosteroid and the plastic matrix of the tips. The tips were discarded after single use. The method of staggered application and synchronized removal was chosen. The application of the products on each demarcated site was done in a random manner so as to prevent bias during visual assessment. A glass rod was used to spread the product evenly over each site. The glass rod tip was cleaned with a paper towel to remove any remaining product before continuing onto the next application site. The application sites on the two forearms were protected with a non-occluding arm guard to prevent any smearing or removal of the creams during the entire cream application period (Figure 4.4). Before the removal of the product, the labels were first peeled off gently. The removal of the product was done by washing the forearm with mild soap and water and then blotted dry with a paper towel.

Figure 4.4. Protection of the application sites with a non-occluding armguard

4.2.1.5. Determination of the mass of product applied

Following the FDA Guidance [158], approximately 10 mg of product per 1 x 1 cm site is required. For an application site of 1.1 x 1.1 cm, it was calculated that about 12.1 mg of the product would be needed per site. The mass of preparation applied was determined by weighing the amount of the product being extruded from a 0.2 ml Eppendorf pipette tip at the different dial settings (Table 4.1). The different dial settings yielded the following volumes: 1 = 4µL, 2 = 8µL, 3 = 12µL, and 4 = 16µL with a 0.2ml tip. The different masses were weighed with a Mettler® Torledo weighing balance.

Table 4.1. The determination of an appropriate dial setting to yield the required mass of cream for application.

	Mass of cream weighed (mg)								
Volume (µL)	1	2	3	4	5	6	Average (mg)	SD (mg)	RSD (%)
4	3.6	3.8	3.8	4	3.7	3.7	3.76	0.13	3.46
8	7.7	7	7.2	7.7	7.7	7	7.38	0.35	4.74
12	11.1	11.2	10.8	11.2	11.3	11.2	11.13	0.17	1.53
16	14.8	15.8	15.6	13	14.6	14.7	14.75	0.99	6.71

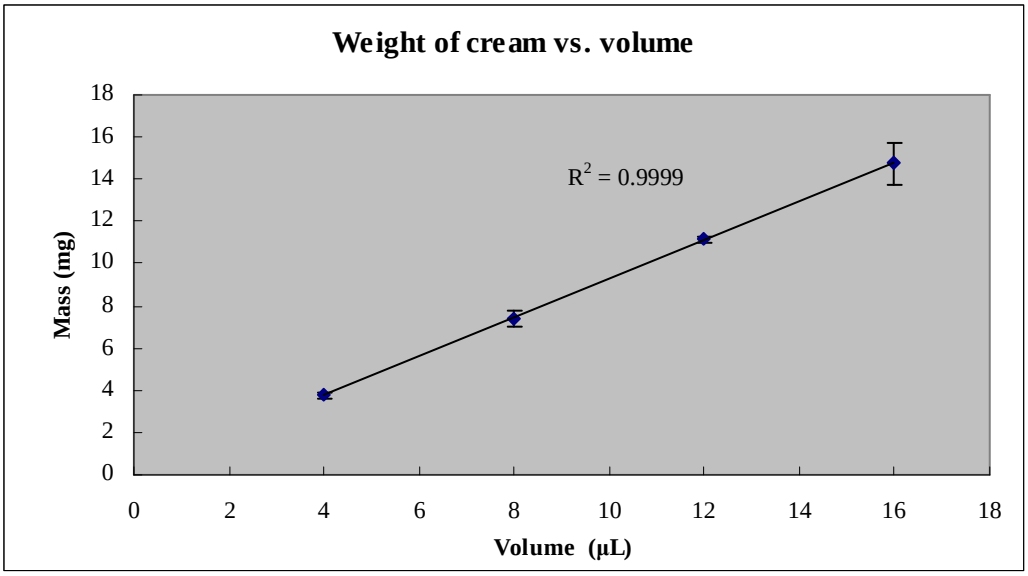

Figure 4.5. Mass of cream at the different volumes from the dial settings

As shown in Figure 4.5, the curve gave an R^2 value of 0.9999 indicating that the pipette releases a mass proportional to the dial setting/volume. The standard deviation was not large at the required amount i.e. the dial setting at 3 with a mass of 11 ± 0.17 mg, thus the results indicated that the Eppendorf pipette released a repeatable mass of the cream. This setup was considered suitable and was subsequently used for the application of the products in all further studies.

4.2.1.6. Assessment of the skin blanching response

The arms were evaluated visually by trained observers and with a mechanically-mounted chromameter (patent pending) at the various time intervals after the removal of the products to obtain a blanching profile. There were no oblique light sources. Standard lighting by overhead fluorescent lamps were used for all the studies. The arms were placed horizontally on a desk directly in front of each trained observer for the assessments. Visual scoring was done at a 0 – 4 scale where:

0 = no blanching, application site appeared identical to surrounding area of skin.

1 = faint blanching observed, but outline of application square was not seen.

2 = faint – moderate blanching, with one or two sides of the application square seen.

3 = moderate – strong blanching, the square outline of the application square is clearly

visible.

4 = strong – intense blanching, intense pallor observed over the entire square of application site.

Each site was be assigned a blanching score by comparing skin at the site of application to the adjacent skin unaffected by the product.

A mechanically mounted chromameter was used for the assessment of blanching over the whole application site. The chromameter was aligned into position directly over and covering the application site with the measuring head. The same orientation of the chromameter was maintained to get accurate readings for each assessment time for each site. Three consecutive readings were taken on each site without moving the chromameter. The three readings were averaged and the mean value reported. Prior to colour measurement, the instrument was calibrated with its own calibration tile.

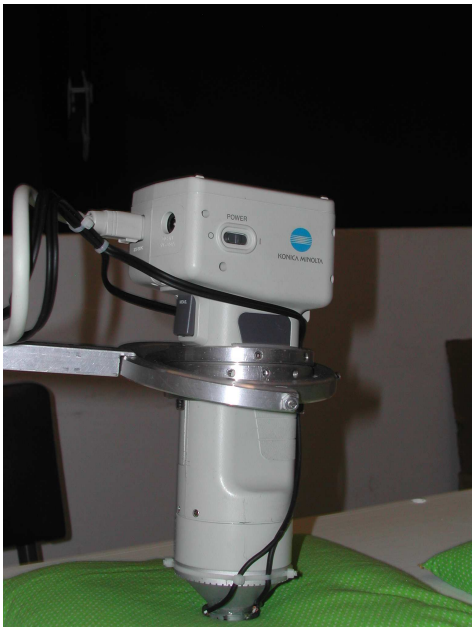

Figure 4.6. The Minolta® chromameter

All estimations of skin blanching responses using both visual and chromameter assessments were performed in a double-blind manner without reference to the application charts. Skin blanching responses were measured at 0, 2, 4, 6, 8, 10, 12, 14, 18, 22, and 26 hours post product removal.

4.2.1.7. Confinement of the subjects

Subjects that were accepted into the study were confined within the Biopharmaceutics Research Institute clinic for the initial part of the study i.e. the first day and night. On the final day of the study, the subjects were allowed to leave the clinic and return 20 minutes before their next scheduled assessment. This allows the subject's body to stabilise to the clinic's environment before their assessment. The clinic's environment was controlled to maintain the same temperature (± 22 °C), humidity, and lighting and environmental factors should therefore not have affected the blanching response of each subject. It also prevented the subjects from undertaking any restricted actions, and avoided any changes in ambient temperatures and atmospheric humidity.

4.2.1.8. Data and statistical analysis

Visual data were converted to %TPS as shown in section 4.1.2.1 above. The chromameter data only used the a-scale data as stipulated by the FDA guidance [158].

The Trapezoidal rule was used to calculate the AUEC values using visual and chromameter data vs. time in hours after the removal of the excess formulations. Statistical analyses were carried out using Graphpad® Prism 4 and Kinetica® software packages, both of which gave similar results. The t-test and goodness of fit test were performed, and the E_{max} model was established.

4.2.2. Results and discussion

Dose duration-response data of all 11 subjects (6 females and 5 males, ten of whom were Caucasians and one Asian) were obtained for Dermovate® cream. No adverse drug reactions or other clinical events were encountered during the conduct of this study. Only 11 subjects were used as previously explained.

After the different times of exposure (i.e. at 0.25, 0.5, 1, 2, 4 and 6 hours dose durations), the

blanching effect for Dermovate® cream peaked at 12 hours after product removal, decreasing thereafter. The visual mean score values and a-scale values were plotted against the time after product removal illustrating the pooled blanching response of the 11 subjects (Figures 4.7 and 4.8).

The graphs also show that as the dose durations increase, there is a corresponding increase in the skin blanching response. However, the skin blanching response becomes maximal at a certain point of time following application, thereafter any increase in dose duration will not result in any changes in the AUEC thereby losing discriminatory power to differentiate between products, if indeed differences do exist. Hence, the appropriate dose duration is extremely important for use in the pivotal study to determine BE between products.

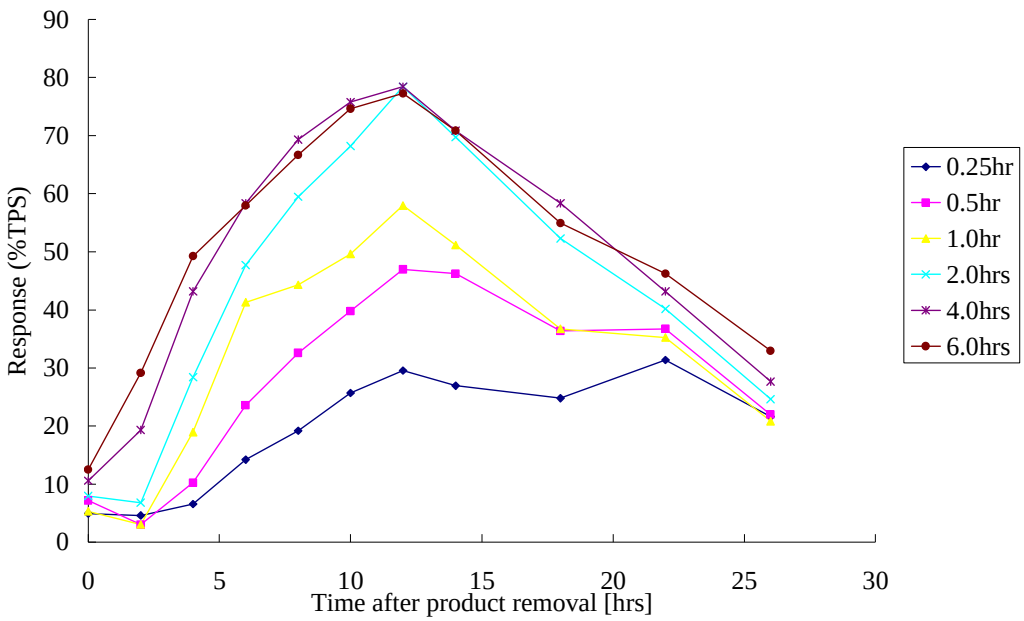

Figure 4.7 Blanching profile from visual assessment data

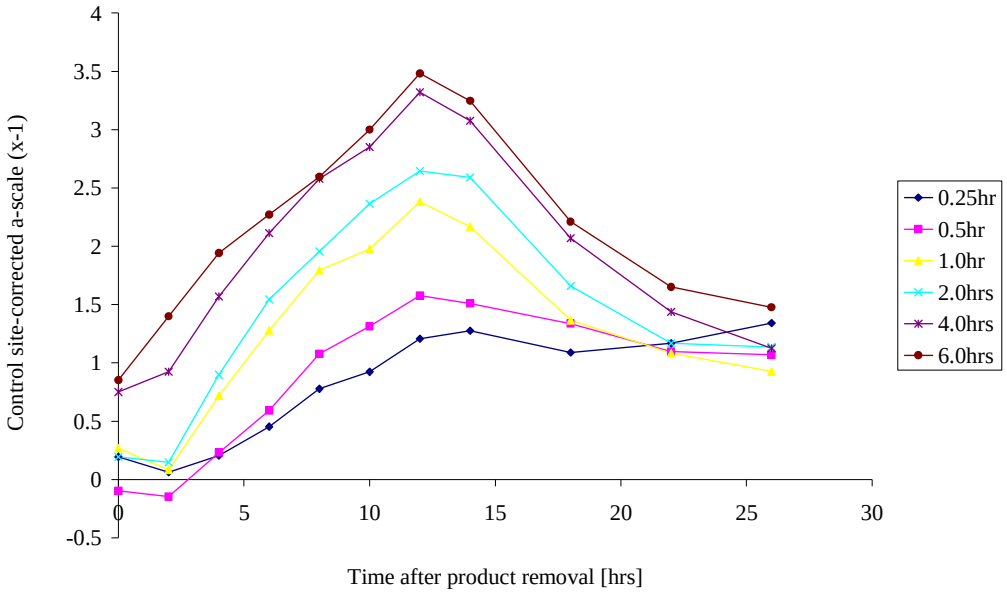

Figure 4.8. Blanching profile from chromameter assessment data

Pharmacokinetic models describe drug concentrations as a function of dose and time. This is the simplest model that describes drug effect over the whole range of concentrations based on the hyperbolic relationship found by the E_{max} model [34]. The hyperbolic E_{nax} model was used to determine the dose duration-response data as stated in the FDA guidance in this case [158]. This type of graph can predict 2 important properties, i.e. the maximum effect a drug can achieve and the absence of an effect when no drug is present. The product's data sets were analyzed with the naive pooled data method approach to fit the E_{max} model (see equation 4.2 above) [158].

The baseline effect was incorporated into the E_{max} model to reflect changes from baseline value depending on the nature of the effect and experimental design [34]. The AUEC values were calculated to yield a dose-duration versus AUEC profile of all the subjects to illustrate the response at the various different dose durations (Figures 4.9 and 4.10). Notice that both figures have different y-axes due to the different unit values obtained from the visual and the chromameter data.

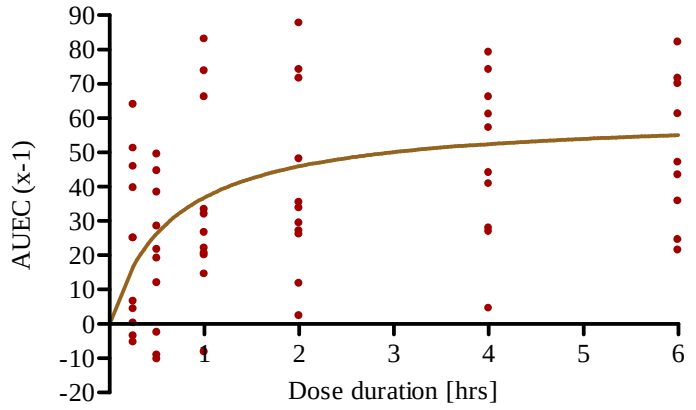

Figure 4.9. E_{max} model of the chromameter's a-scale data

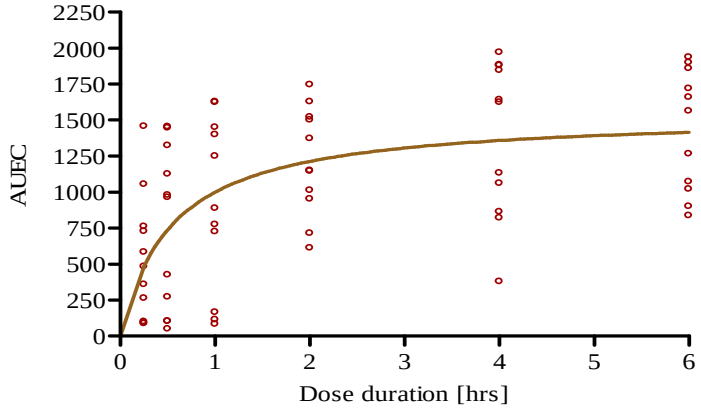

Figure 4.10. E_{max} model of the visual data

Table 4.2. E_{max} model parameters for both visual and chromameter data

Parameters	Visual	Chromameter
ED_{50} [hrs]	0.5457	0.6582
Standard Error	0.2101	0.3529
D_1 [hrs]	0.2729	0.3291
D_2 [hrs]	1.0914	1.3164
	Average of visual and chromameter data	
ED_{50} [hrs] (averaging visual and chromameter)	0.6025	
D_1 [hrs] (averaging visual and chromameter)	0.3012	
D_2 [hrs] (averaging visual and chromameter)	1.2049	
Goodness-of-fit (Spearman's correlation)	0.97	
p-value	$p < 0.05$	

The reason for choosing an ED_{50} value is that when testing is done at high dose durations, the change in response is minimal resulting in a loss of sensitivity and discriminatory power, as seen with the dose duration at 4 and 6 hours. When testing with lower dose durations, the reliability and repeatability, and even achieving a reasonable skin blanching response will become an issue. As a result, the dose duration where 50% of the response will be achieved will be at the most sensitive range and still yield reliable and reproducible data.

From the above Table 4.2, the dose durations (ED_{50}, D_1 and D_2 values) were utilized in the subsequent pivotal study. From visual inspection of the data, it can be seen that when comparing the graphs between the visual and chromameter data, the profiles are very similar to each other. The Spearman's correlation between the E_{max} models of both assessment techniques was determined and gave an r value of 0.97 indicating that these two graphs are statistically similar to each other. The p-value was calculated by the software and gave a value less that 0.05 showing that there is no significant difference between the two E_{max} models. From this, we can deduce that both evaluation techniques were comparable to each other and should be equally applicable for the establishment of the appropriate dose-response for subsequent use in the assessment of BE of CP creams.

4.3. PIVOTAL STUDY

4.3.1. Experimental

4.3.1.1. Instrumentation

Vide infra section 4.2.1.1

4.3.1.2. Formulations

The formulations used were the same as those mentioned in section 4.2.1.2 of the pilot study described previously. The same tube of cream was used as both the reference and test product in all the clinical trials. The test application was labeled as in-house batch no. WL2-01 and the reference application was labeled as in-house batch no. WL2-02.

4.3.1.3. Subjects

Subjects were screened as previously described in section 4.2.1.3 and also in appendix II. In addition, a slightly different screening procedure was used in this pivotal study. In pivotal studies, one requires a "qualified" subject to be a 'detector' as well as having been a 'responder'. In order to be a 'detector', the subject must be able to show skin blanching responses that are able to differentiate between the D_2 dose duration and D_1 dose duration. The equation used to evaluate the blanching responses is

$$\text{AUEC at } D_2 \,/\, \text{AUEC at } D_1 \geq 1.25 \qquad \text{(equation 4.3)}$$

The dose durations, D_2 and D_1, were obtained from the pilot study data, and were determined to be 72 minutes and 18 minutes, respectively. Three sites were demarcated on the left and right forearm. The reference product was applied onto two of the three sites on the volar aspect of each forearm using the D_1 and D_2 dose durations. The third site was reserved as a control site during the course of the screening test. A brief skin blanching profile was generated by plotting the a-scale chromameter data at 0, 6, 8, 12 and 24 hours post removal of the product.

A maximum of 12 subjects were used per clinical study due to time constraints on the use of the chromameter. The study was split into three separate days and a total of 34 subjects participated (12 on the first study, 11 on both the second and third studies). Subjects absent from the second and third trial were due to voluntary withdrawal from the study and alcoholic consumption, respectively.

4.3.1.4. Product application and removal

As mentioned above in section 4.2.1.4. in the experimental section of the pilot study.

4.3.1.5. Assessment of the skin blanching response

As mentioned above in section 4.2.1.6. The skin blanching responses were measured at 0, 2, 4, 6, 8, 10, 12, 15, 22, 25 and 30 hours after product removal.

4.3.1.6. Confinement of the subjects

Same as section 4.2.1.7 stated above.

4.3.1.7. Data and statistical analysis

The a-scale and %TPS data were determined as previously mentioned in section 4.2.1.8. Statistical analyses were carried out using Microsoft® Excel. The 90% confidence interval (CI) was calculated according to Locke's method [158,209].

4.3.2. Results and discussion [210]

No adverse reactions were observed during the conduct of this study. However, slight sensitivity towards the adhesive on the labels was seen. The adhesive caused slight redness and inflammation on the contact area for approximately one hour after removal of the label. This sensitivity reaction did not affect the visual assessment.

Out of the 34 subjects who participated in the pivotal study, 17 were Caucasian, 4 were Coloured, 3 were Asian, 5 were Indian and 5 were African. The study group included 22 females and 12 males in total. According to the Fitzpatrick skin type classification [211], the skin type ranged from type I to type V in the subjects. Subjects with skin type VI did not blanch or showed only very slight blanching and were therefore excluded from the study.

Figures 4.11 and 4.12 represent the mean visual blanching profiles for the CP creams obtained from the groups of 'detectors' and all the subjects, respectively. Figures 4.13 and

4.14 depict the mean chromameter blanching profiles for the CP creams obtained from the groups of 'detectors' and all the subjects, respectively. The four graphs showed very similar blanching profiles and all the profiles showed blanching that peaked at 15 hours after product removal. Comparison of the 'detectors' and of all the subjects revealed that the curves were quite similar to each other. This indicated that the exclusion of 'non-detectors' appears to have very little effect on the overall profiles.

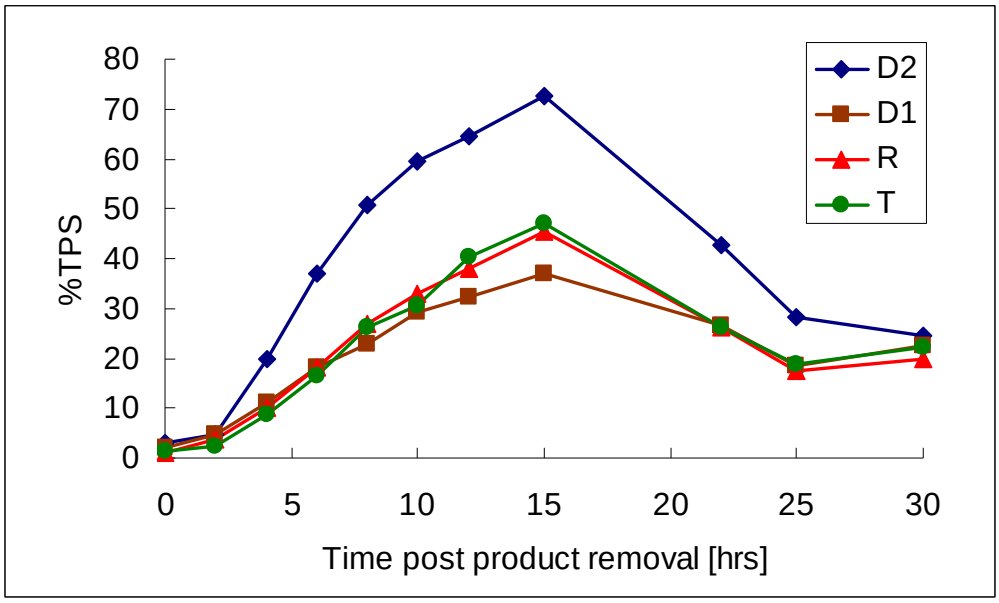

Figure 4.11. Visual data of 'detectors' (n=23)

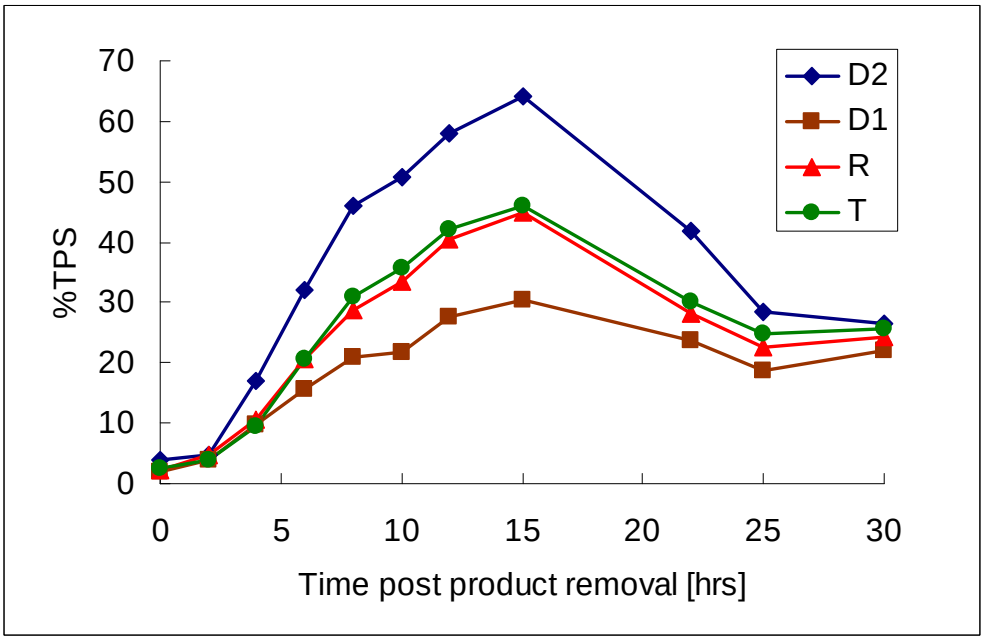

Figure 4.12. Visual data of all subjects (n=34)

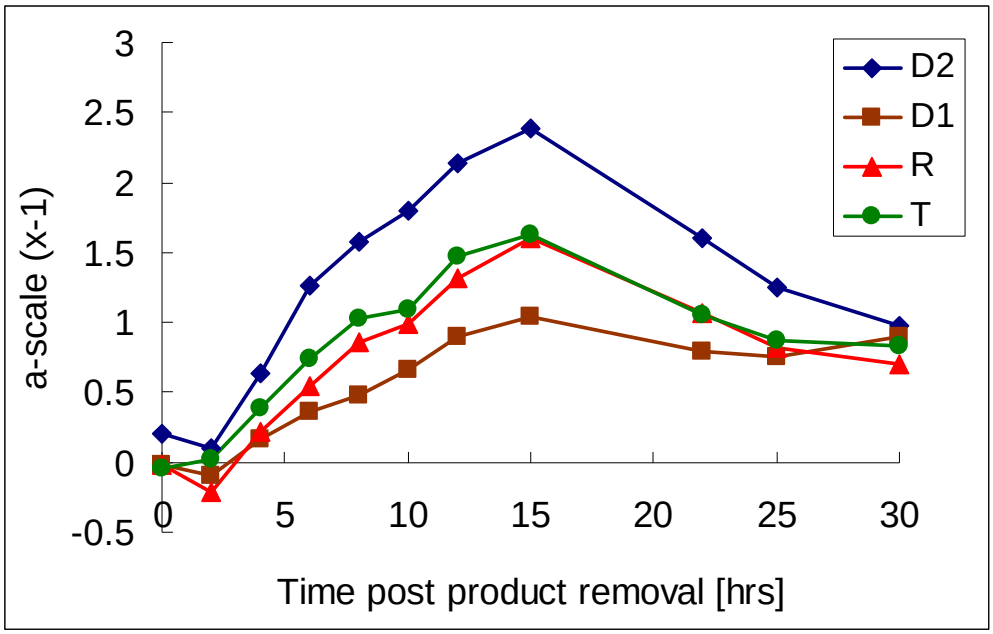

Figure 4.13. Chromameter a-scale data of 'detectors' (n =23)

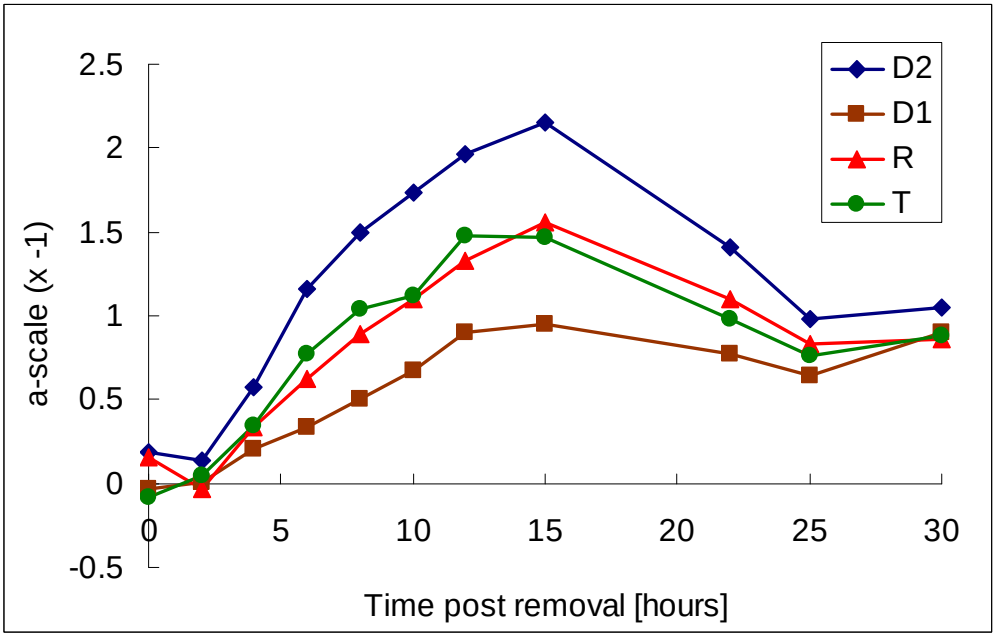

Figure 4.14. Chromameter a-scale data of all subjects (n=34)

As seen from Table 4.3 below, the visual data obtained from 'detectors' (n=23) fell within the BE range of 80 – 125% whereas the corresponding chromameter data slightly exceeded the upper limit of the range. However, when the data from all subjects (n=34) were used, both visual data and chromameter data were within the acceptance range. Overall, the visual data yielded a narrower interval compared to the chromameter data and using the data from

all subjects yielded a narrower range than using only 'detectors' data.

Table 4.3. 90% CI calculated using the Locke's method for visual and chromameter data

90% CI	Visual	Chromameter
Detectors:		
Group 1 (9 subjects)	94.2 – 125.9	67.7 – 156.5
Group 2 (7 subjects)	88.9 – 119.3	82.2 – 172.6
Group 3 (7 subjects)	99.0 – 138.6	76.2 – 141.5
Group 1 & 2 (16 subjects)	96.3 – 110.2	82.5 – 145.1
Group 1,2 & 3 (23 subjects)	99.3 – 111.6	86.5 – 129.3
All subjects:		
Group 1 (12 subjects)	97.0 – 118.8	75.8 – 141.3
Group 2 (11 subjects)	88.8 – 108.6	97.7 – 148.0
Group 3 (11 subjects)	96.5 – 121.7	71.0 – 114.4
Group 1&2 (23 subjects)	95.7 – 109.4	90.4 – 133.3
Group 1,2 & 3 (34 subjects)	97.9 – 109.2	90.2 – 120.7

Table 4.4. AUECs for visual and chromameter

	VISUAL		CHROMAMETER	
	Detectors (n=23)	All subjects (n=34)	Detectors (n=23)	All subjects (n=34)
TEST PRODUCT				
Mean AUEC	907.518	853.891	30.831	28.637
SD	453.674	431.579	16.138	16.715
CV%	50.0	50.5	52.3	58.4
REFERENCE PRODUCT				
Mean AUEC	891.803	829.534	28.302	27.460
SD	515.240	492.395	17.598	16.535
CV%	57.8	59.4	62.2	60.2

The acceptance criteria for BE of oral dosage forms must fall within the range of 80-125 % [118]. However, whether this acceptance range should be applied for the assessment of BE of topical products is moot.

In view of the relatively high variability [160,198,212] in percutaneous drug absorption amongst subjects and as reflected in Table 4.4 using visual or chromameter methods, consideration could be given to widening the acceptance range for the declaration of BE of topical formulations. Notwithstanding, blanching data from the 23 “detectors” in this study using the visual method and data from all the subjects using either visual or chromameter methods, resulted in the products falling within the BE acceptance interval. When, however, chromameter “detector” data were used, the products did not meet the criteria for the declaration of BE. It was, however, interesting to note that the chromameter data yielded wider BE intervals than the corresponding data obtained from the visual method. Furthermore, these results clearly indicate that the eye is a reliable evaluating tool for the assessment of skin blanching. In addition, whereas the FDA guidance recommended that 40 to 60 evaluable subjects are required for a typical HSBA, data obtained from this study indicate that this number may not be necessary. BE criteria were met with visual data using 23 “detectors” as well as data from all 34 subjects, where 90% CIs were similarly narrow for both sets. Furthermore, although the 90% CIs for chromameter data was wider for the 23 “detectors”, all subject data showed that 34 subjects were sufficient to demonstrate BE with a power of >95%. This further supports our earlier suggestion that less than 40 evaluable subjects can be adequate.

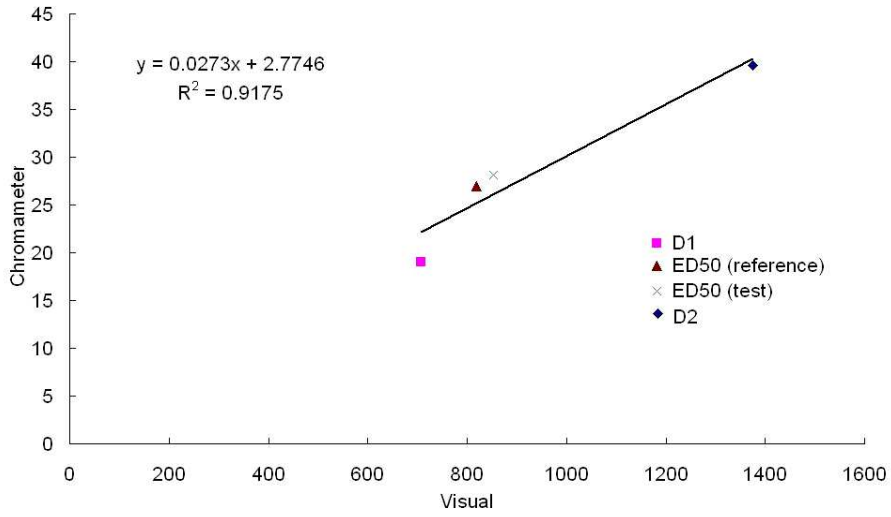

Figure 4.15. Correlation of chromameter and visual mean AUEC data of all subjects at dose durations of ED_{50} for test and reference products, and dose duration of D_1 and D_2 for the reference product

Several reports have been published on the use of these assessment techniques [195,207] and whilst the chromameter is currently perceived to be the method of choice [158], claims that the use of the visual assessment technique is more accurate have been reported [160,198,206,212]. The visual method involves subjective assessment of the intensity of blanching at the application site in comparison to surrounding untreated skin. It also requires considerable training and subsequent use of experienced observers and is difficult if not impossible to standardize and validate. An instrumental method largely overcomes the limitations of subjective assessment by providing objective measurements from a calibrated instrument, which can be validated by establishing the reproducibility of measurements. However, it should be noted that training and experience in the use of a chromameter is essential to obtain reproducible and reliable results [199,200]. Although the chromameter method seems to be a reasonable choice, the results from the present study show that visual and chromameter assessments are equally applicable for the BE assessment of topical corticosteroid products. Furthermore, the visual assessment data correlate well with chromameter results as demonstrated in Figure 4.15 with a R^2- value of 0.92.

4.4. CONCLUSIONS

In conclusion, the use of an optimized screening method (as explained in section 4.3.1.3) for

potential ‘detectors’ reduced the number of required subjects to establish BE between topical products containing a corticosteroid. Furthermore, the results have clearly shown reliability and appropriateness of using visual assessment. Hence, visual assessment is comparable to chromameter assessment for the evaluation of topical corticosteroid preparations in the human skin blanching assay.

These HSBA BE study data are used as the basis for comparing and correlating data from a BE study using TS.

5. CHAPTER 5

5.1. DEVELOPMENT AND VALIDATION OF A QUANTITATIVE HPLC METHOD FOR THE ANALYSIS OF CP

Various methods for the quantitative analysis of CP have been published in the literature, the most common method being HPLC coupled with UV detection although a few methods using HPLC together with mass spectrometry have also been reported [213-216]. Compendial methods for the analysis of CP also exist [4,17].

A radioimmunoassay (RIA) [217,218] has previously been used for the analysis of tritiated CP found in the plasma after topical application from an extemporaneous formulation containing the tagged molecule. However, this RIA method is currently not often used.

5.1.1. High-performance liquid chromatography (HPLC)

This technique utilizes a liquid mobile phase that is pumped under high pressure through a stainless steel column (stationary phase) for separating substances. The analyte is loaded on the head of the column via a loop valve and separation of a mixture occurs according to the relative lengths of time spent by its components in the column. All the components in a mixture spend a certain amount of time in the mobile phase on the column before exiting [219]. HPLC is usually coupled with a suitable detector for the quantitative analysis of substance(s).

A standard instrumental system for isocratic elution consists of (Figure 5.1):
1) a solvent reservoir,
2) a pump capable of pumping solvent up to a pressure of 4000 psi and flow rates up to 10ml/min (depending on the column type),
3) a loop injector fitted with a fixed volume loop between 1 and 200 µl (20µl is the standard)
4) a column, which is a stainless steel tube packed, usually, with a stationary phase, most commonly consisting of reverse-phase material such as octadecylsilane (ODS) coated silica gel with a particle diameter of 3, 5 or 10 µm.
5) a detector, which may be an ultraviolet detector, fluorescence detector or mass

spectrometer to name a few

6) A data capture system, which may be an integrator/chart recorder/PC with software for processing chromatographic data.

7) The column is connected to the injector and detector with tubing of relatively narrow internal diameter

8) The more advanced instruments have an automatic sample injection system and a column oven and are capable of mixing two or more solvents in varying proportions to produce a mobile phase gradient.

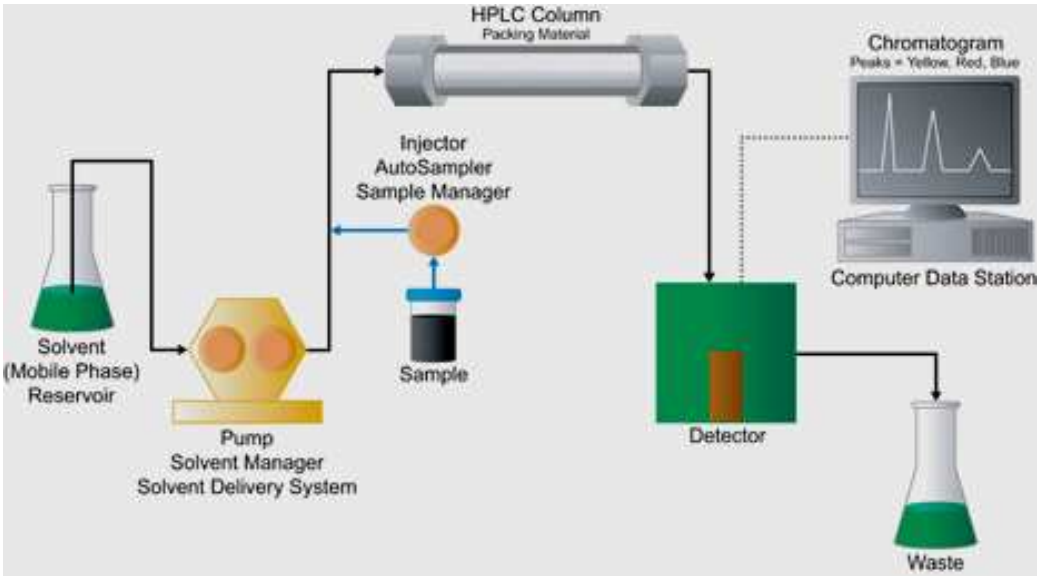

Figure 5.1. High-performance liquid chromatography system (http://www.waters.com/waters/nav.htm?locale=zh_TW&cid=10049055, accessed on 03-10-2009).

HPLC analysis can be readily controlled and ensures precise sample introduction for accurate quantitative measurement. It is also one of the most commonly used analytical techniques for drug analysis. A large variety of columns and equipment including pumps, detectors, sample injection systems and associated hardware are commercially available.

5.1.1.1. The column

The object of HPLC is to separate sample components within a reasonable time and the HPLC column is the component usually responsible for achieving this separation. HPLC columns containing ODS silica gel or related phases are used to obtain the required

selectivity for use in bioanalytical and formulation analysis. HPLC columns containing polymeric stationary phases are also available for certain applications. The polymeric phases are stable to extremes of pH and have the advantage of overcoming secondary interactions of analytes with uncapped silanol groups. However, these are expensive and have a tendency to swell when in contact with lipophilic mobile phases (can damage the column). These polymeric phases are best used with aqueous-based mobile phase [219].

A HPLC column is packed with a solid stationary phase with a liquid mobile phase flowing through it. If the compound does not partition substantially into the stationary phase, it will flow through the column at the same rate as the solvent. The length of time it takes a retarded substance to pass through the column depends on its capacity factor (K'). The capacity factor is a measure of the degree with which the substance partitions into the stationary phase from the mobile phase and can be derived by [219]:

$$K' = (t_r - t_0)/t_0 \qquad \text{(equation 5.1)}$$

where t_0 is the time taken for an unretained molecule to pass through the column, and t_r is the time taken for the compound to pass through the column [219].

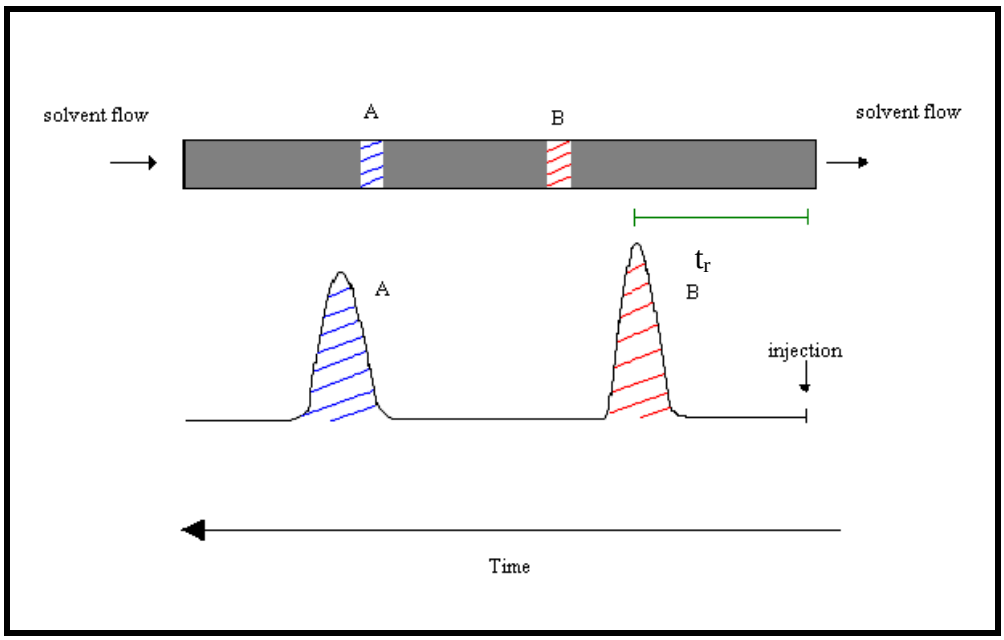

Figure 5.2. Schematic diagram of bands for two different compounds traveling through an HPLC column. The compound with the largest capacity factor emerges last.

The efficiency of a column, i.e. the effectiveness of the separation, can be assessed by the following methods:

The most common measure of efficiency of a chromatographic system is the plate number N. The efficiency of the column is assessed from the width of the peak at half its height and its retention time using equation 5.2:

$$N = 5.54 (t_r/W_{1/2}) \qquad \text{(equation 5.2)}$$

Column efficiency is expressed in theoretical plates per meter: N x 100/L

Hence, the broader the chromatographic peak is relative to its retention time the less efficient the column.

The greater the efficiency of a column, the better the degree of resolution produced between closely eluted peaks. The resolution between two peaks (R_s) is expressed as [219]:

$$R_s = 2 (t_{rB} - t_{rA})/(W_{bB} + W_{bA}) \qquad \text{(equation 5.3)}$$

Where t_{rB} and t_{rA} are the retention times of the different peaks

W_{bB} and W_{bA} are the widths of the different peaks at baseline

An R_s of 1.2 is an indication of complete separation between the different peaks. Any value less than 1.2 is indicative of unresolved peaks [219].

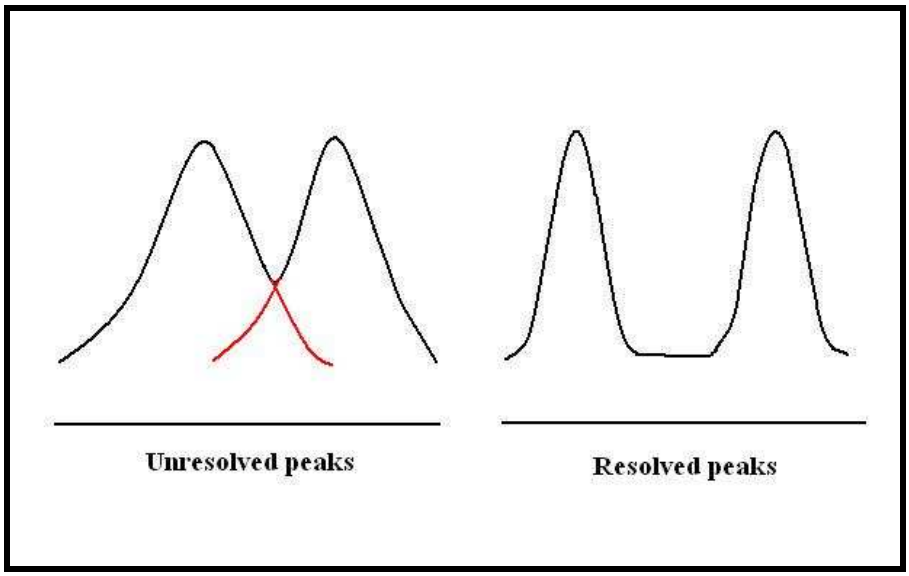

Figure 5.3. Illustration of unresolved and resolved peaks

Another factor which can result in poor efficiency of a column is where peaks are tailing thus having a high element of asymmetry. Equation 5.4 helps determine peak asymmetry [219].

$$\text{Asymmetry factor (AF)} = b/a \qquad \text{(equation 5.4)}$$

Where *a* is the leading half of the peak measured at 10% of the peak height, and *b* is the latter half of the peak measured at 10% of the peak height (Figure 5.4). The ideal value for symmetrical peak is in the range of 0.95 – 1.15 [219].

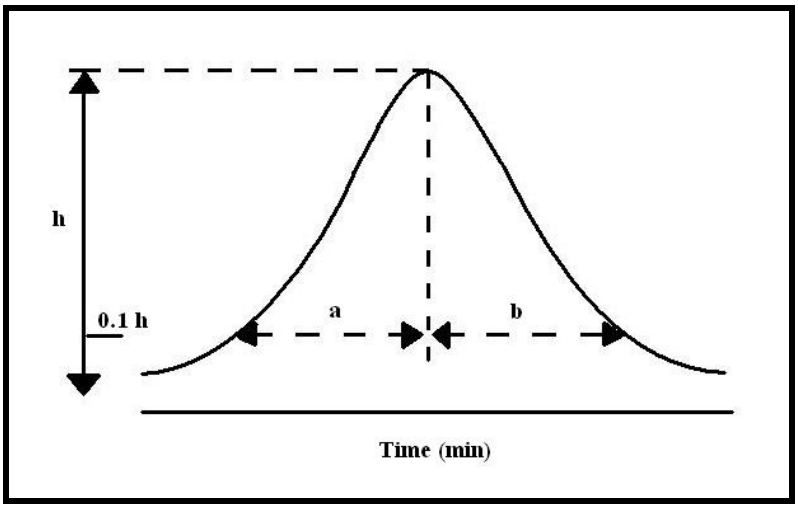

Figure 5.4. Determination of peak symmetry

5.1.1.2. Liquid chromatography modes

Normal phase chromatography is the oldest separation mode applied in column chromatography which is often governed by the interaction between adsorbent, solute and solvent molecules. This mode of chromatography employs polar stationary phases and non-polar mobile phases. All solids that are insoluble in common liquids can be used as the stationary phase provided they have high surface area. Normal phase chromatography is well suited for the separation of non-polar to medium polar substances. Silica and alumina are the adsorbents that are found used in HPLC [220].

Reversed phase (RP) chromatography utilizes non-polar stationary phases and a polar eluent which is the opposite of normal phase chromatography. Advantages of using RP chromatography includes compatibility with many biological samples, compatibility with electrochemical detectors, and the ability to control secondary chemical equilibria and gradient elution methods [220,221].

5.1.2. Method validation

Analytical methods used for the quantitative determination of drugs in samples play a significant role in evaluation and interpretation of BA, BE and pharmacokinetic data. It is necessary to utilize well-characterized and fully validated analytical methods to yield reliable results which can be satisfactorily interpreted. It is also important to highlight that each analytical technique has its own characteristics which will vary from drug to drug. Specific validation criteria are needed for methods intended for analysis of each analyte. Method validation includes all the procedures needed to demonstrate that a particular method for the quantitative measurement in a particular matrix is reliable. The parameters which are crucial to ensure the acceptability of the performance of an analytical method are stability of the drug in the matrix under study storage conditions, accuracy, precision, sensitivity, specificity, response function and repeatability [222-224]. Validation of the extraction method is also essential and best done in accordance with internationally acceptable guidelines [223].

5.1.2.1. Linearity

For quantitative purposes, the detector response should be linear for the amount of solute to be determined over a reasonable range of solute concentrations. Linearity should be evaluated by appropriate statistical methods e.g. regression line (using least squares method).

5.1.2.2. Range (standard curve)

The range is normally derived from linearity studies and depends on the application of the procedure. It is necessary to use a sufficient number of standards to adequately define the relationship between concentration and response, and must be demonstrated to be continuous and repeatable. The number of standards used will be a function of the dynamic range and nature of the concentration-response relationship. Concentrations of 5-8 standards (excluding blank standard) should define the standard curve. The standard curve should cover the entire range of expected concentrations [222].

5.1.2.3. Accuracy and precision

Within and between run accuracy and precision should be calculated using commonly accepted statistical procedures which can confirm the reliability of the analytical method. This can be accomplished by analysis of replicate sets of analyte samples of known concentrations from a particular matrix. Replicates of three concentrations representing the entire range of the calibration curve should be studied i.e. quality control (QC) samples. One near the lower limit of quantitation (LOQ), one near the centre, and one near the upper boundary of the standard curve. For a method to be considered valid, the mean value should be within ±5% of the actual value for analytes in a non-biological matrix or ±15 % in a biological matrix [222].

Inter-day precision should also be determined by analysing replicate sets of QC samples over a period of a few days. For the data to be valid, the above recommended acceptance criteria are used.

5.1.2.4. Limit of quantitation (LOQ) and limit of detection (LOD)

The LOQ is the lowest concentration on the standard curve which can be measured with acceptable accuracy and precision. The LOQ should be determined using at least 5 independent standard samples. The mean value should not deviate by more than ±10% in a non-biological matrix or ±20% in a biological matrix [222]. Typical signal-to-noise ratio is 10:1 [224].

The limit of detection is the amount of solute that can be distinguished with some level of certainty from the baseline noise. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit [224] .

5.1.2.5. Specificity

Specificity studies should be conducted to identify the impurities/degradants/matrix and the analyte(s) in the sample that is to be analysed. The analyte can be identified by comparing it to that of a reference standard.

At times, an extraction procedure has to be utilized. The principle of an extraction is to remove an analyte from a matrix which can interfere with the analysis. An extraction can be done by using a solvent in which the analyte is highly soluble but where the matrix interferants have limited solubility. Extraction methods are often applied to bioanalytical procedures and for concentrating trace amounts of analyte. If there is problem of interference from excipients following an extraction procedure, chromatography is often able to resolve the analyte from the interferants and permit quantitation.

There are two types of extraction methods that are most commonly used i.e. liquid or solid phase extraction. Solvent extraction methods provide simple and cost effective procedures for separating the analyte from excipients in matrices. However, for this to be possible, two immiscible solvents must be used thus reducing the choice of solvents. In a solid phase extraction (SPE), the analyte is retained on the solid phase extraction media and the interferants are eluted with a solvent with low eluting power. The analyte may finally be

washed out with a strong eluting solvent. SPE is often used in separating analytes from interferants when liquid-liquid extraction fails. However, an internal standard should always be used to compensate for any possible irreversible absorption of analyte onto the extraction media. Solid phase columns are , however, generally quite costly [225].

5.1.2.6. Recovery

The recovery, according to Braggio *et al* [226], is a "measure of the efficiency of the method in detecting all the analyte of interest present in the original sample", by comparing the amount found in a spiked sample (spiking occurs before extraction) with the extracted blank matrix sample and spiked thereafter. Recovery should be as high and as consistent as possible, especially within the concentration range.

The stability of the analyte during sample collection and handling, long-term and short term storage should be evaluated. This should be assessed in the matrix and container system used. Following the FDA guidelines for bioanalytical method validation [223], all stability experiments should use a set of samples prepared from a freshly made stock solution in the appropriate solvent and concentrations.

5.2. METHOD DEVELOPMENT AND VALIDATION

5.2.1. Experimental

RP-HPLC has often been used as the liquid chromatography mode for the analysis of CP. This system is coupled with a UV/visible spectrometer for detection. Table 5.1 is a list of HPLC conditions used to detect CP in the various *in vitro* and *in vivo* samples (extractions from plasma, formulations, solvents, etc.). The developments of suitable analytical methods to quantitate CP released from topical formulations have been based on some of the listed conditions (Table 5.1). However, the mobile phase compositions used by others may not give acceptable results if the stationary phase used is slightly different, thus optimization of analytical conditions is still required.

Table 5.1. Review of the analytical methods used for the determination of CP.

Column	**Mobile phase composition**	**Detection**	**Flow rate, injection vol., temp.**	**Reference**
4.6 mm x 15 cm column that contains packing L1	MeCN:NaH_2PO_4:MeOH (95:85:20) $NaHPO_4$ – 0.05M with pH=2.5	240nm	Flow rate: 1ml/min Inj vol: 10 µl Temp: ambient	[17]
4.6 mm x 15 cm, C18	MeOH:NaH_2PO_4:MeCN (10:42.5:47.5) $NaHPO_4$ – 0.065M with pH=5.5	240nm	Flow rate: 1ml/min Inj vol: 10 µl Temp: 30 °C	[4]
Nova-Pak® C18 column, 4 µm, 150 x 3.9 mm	MeCN:H_2O (1:1)	Scanned from 200 – 350nm, monitored at 240nm	Flow rate: 1 ml/min Injection vol: 100 µl Temp: ambient	[227]
Phenomenex® Luna C18, 5 µm, 150 x 4.6 mm	MeOH:KH_2PO_4: MeCN (10:40:50) KH_2PO_4 – 0.1M with pH=3	239nm	Flow rate: 1 ml/min Inject vol: 50 µl Temp: 20 ˚C	[215]
Purospher-Lichrocart®, 5 µm, 250 x 4.0 mm	Water: MeCN (40:60)	237nm	Flow rate: 1 ml/min Inject vol: 10 µl Temp: 25 °C	[228]
2 gradient system: Symmetry® C18, 3.5 µm, 75 x 4.6 mm Nova-Pak® C18, 4 µm, 150 x 3.9 mm	1.) MeCN: H_2O (18:82) 2.) MeOH: H_2O (38:88)	240nm	Flow rate: 1 ml/min Inject vol: 5 µl Temp: ambient	[229]

Hypersil® ODS, 5µm, 100 x 2.0 mm	MeCN: H_2O (55:45)	240nm	Flow rate: 1 ml/min Inj vol: 20 µl Temp: ambient	[172]
Phenomenex® Luna C8, 5 µm, 150 x 2.0 mm	MeCN: H_2O (46:54)	238nm	Flow rate: 0.5 ml/min Inj vol: 10 µl Temp: 22 °C	[230]
Nova-Pak® C18, 4 µm, 150 x 3.9 mm	MeCN: H_2O (50:50)	240nm	Flow rate: 1 ml/min Inj vol: 15 µl Temp: 22 °C	[231]

5.2.1.1. Instrumentation

The HPLC system used was equipped with a separation module (model 2690), a photodiode array (PDA) detector (model 2996), an online degasser, and an autosampler (Waters® Co., Milford, MA, USA).

Electronic pipettes (ePet, Biohit, Helsinki, Finland) and disposable tips were used for the preparation of standard solutions and quality control samples. A precision balance (model AG135, Mettler® Toledo, Zurich, Switzerland), ultrasonic bath (model 8845-30, Cole-Parmer Instruments, Chicago, USA), vortexer (Lab dancer vario, Ika®, Staufen, Germany), and a centrifuge (model 5414, Eppendorf Ag, Hamburg, Germany) were used for sample preparations.

In the solubility study of CP in the various pH solutions, the Junior Orbit Shaker (Lab-Line Instrument Inc., Illinois, USA) and a shaking waterbath maintained at 37 °C were utilized. Samples (~0.5 ml) were removed using 5 ml glass syringes and filtered through PVDF syringe filters (0.45 μm, Millipore Millex-HV, Millipore Co., Billerica, Massachusetts, USA) into amber HPLC vials (Waters® Co., Milford, Massachusetts, USA) Samples from the assay of topical formulations were also placed into amber HPLC vials and sealed with a cap before analysis.

5.2.1.2. Reagents, chemicals and materials

CP (98 %) and desoxycorticosterone acetate (DOCA) (98 %) were purchased from Sigma-Aldrich (Atlasville, South Africa). HPLC-grade acetonitrile (UV cutoff 200 nm) and methanol (UV cutoff 215 nm) were purchased from Romil Ltd. (Waterbeach, Cambridge, UK). HPLC-grade water was purified by reverse osmosis followed by filtration utilizing a Milli-Q® system (Millipore Co., Bedford, MA, USA). The water purification system comprised of a Milli-Q® Academic A10 with a Quantum™ EX Ultrapure Organex Cartridge equipped with Q-Gard® 1 Progard pre-treatment packs. All chemicals used were of analytical grade.

McIlvaine's buffer was prepared by dissolving 14.196 g disodium hydrogen phosphate (BDH Chemicals Ltd., Poole, England) in 1 L of Milli-Q® water to give a 0.01 M solution, and adjusted to the appropriate pHs using 0.05M citric acid solution (PAL Chemicals Ltd., Surrey, England). Citric acid solution was prepared by dissolving 10.52 g of $C_6H_8O_7$ in 1 L of Milli-Q® water. Saline was prepared by weighing 9 g of NaCl into 1 L of Milli-Q® water.

5.2.1.3. Column selection

A Luna C_8 minibore column with internal diameter of 2.00 mm and a length of 150 mm , containing 5 μm particles (Phenomenex, Macclesfield, Cheshire, England) was chosen for the analysis of CP and used together with a guard column (C_8, Phenomenex, Macclesfield, Cheshire, England).

5.2.1.4. Mobile phase selection

An important factor which influences the degree of retention of a particular compound, apart from the stationary phase, is the mobile phase. Using RP-HPLC, the more lipophilic a mobile phase the more quickly the compound will elute from the column. CP is a neutral compound and is non-ionisable [16], thus alteration of the pH of mobile phase was not necessary.

5.2.1.5. Internal standard selection

A number of corticosteroids were investigated for use as an internal standard. These were fluocinolone acetonide, hydrocortisone acetate, desoxycorticosterone acetate (DOCA), norethisterone acetate, ethinyl oestradiol, fluperolone acetate and mometasone furoate.

Utilizing the chromatographic conditions listed in Table 5.2, the most suitable internal standards found were DOCA and mometasone furoate. DOCA had a retention time of ~6 minutes and mometasone furoate ~10 minutes. The other internal standards either came too close to the solvent front or eluted at similar times to CP (not resolved).

5.2.1.6. UV Detection

The λ_{max} for CP and DOCA were determined from their UV spectra and are shown in Figure 5.5 and 5.6. CP and DOCA were both detectable at a wavelength of 238nm.

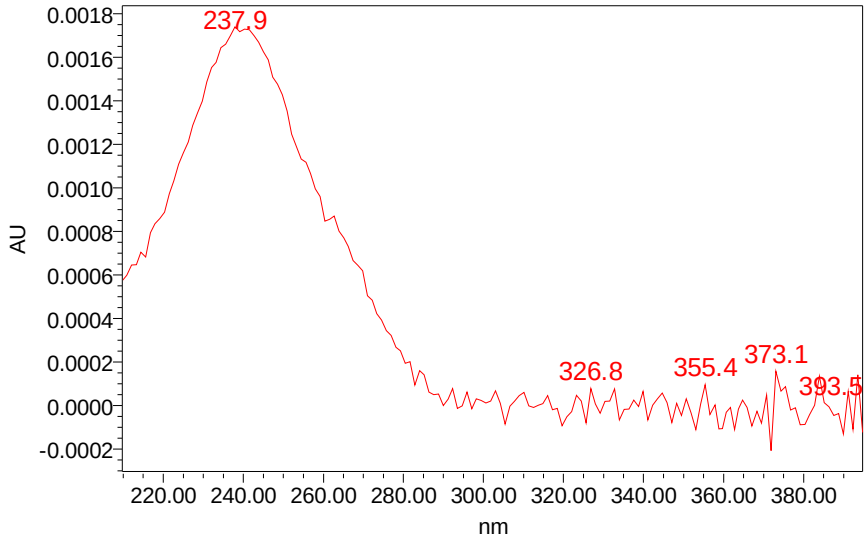

Figure 5.5. UV spectrum of CP

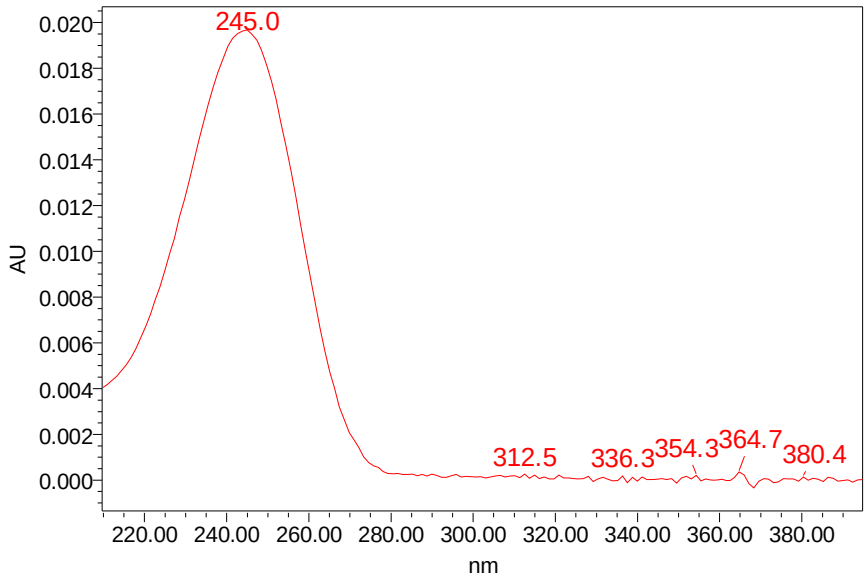

Figure 5.6. UV spectrum of DOCA

5.2.1.7. Preparation of mobile phase

The mobile phase was a mixture of acetonitrile and water (46:54 % v/v). Prior to use, the mobile phase was filtered with a 0.45 μm Durapore® (PDVF) filter (Millipore, Bedford, MA, USA) and degassed under vacuum with an Eyela Aspirator A-2S (Tokyo Rikakikai Co. Ltd., Tokyo, Japan)

5.2.1.8. Preparation of standard solutions

Stock solutions were prepared (100 μg/ml) by accurately weighing out approximately 10 mg of CP (reference standard) or DOCA (internal standard) on a precision balance (model AE163, Mettler Toledo, Zurich, Switzerland) and transferred to a 100 ml volumetric flask. The volumetric flask containing CP or DOCA was made up to volume with mobile phase. Standard solutions were prepared via serial dilutions of the CP stock solution (0.5, 1, 2.5, 5, 10, 25, 50, and 100 μg/ml) using mobile phase. The stock and standard solutions were sonicated in an ultrasonic bath and then shaken immediately after preparation. High, medium and low concentration quality control (QC) standards (75, 15 and 2 μg/ml) were also prepared in a similar fashion. A separate stock solution was used to prepare these samples. New stock and standard solutions were prepared daily, except for the precision study.

5.2.1.9. Chromatographic conditions

Table 5.2. HPLC-UV conditions

Column	Phenomenex® Luna C_8 (2) column, 150 x 2.1 mm, 5 μm
Mobile phase	Acetonitrile and water (46:54 v/v)
Flow rate	0.5 ml/min
Detection wavelength	PDA (but assessing samples at 238 nm)
Injection volume	10 μl
Retention time	~ 8 minutes
Column temperature	22 ± 0.5 °C

5.2.1.10. Method validation

5.2.1.10.1. Linearity

The linearity of the analytical method was evaluated over the concentration range of 0.5 – 100 µg/ml. The peak area ratios of CP/DOCA were plotted against CP concentrations.

5.2.1.10.2. Accuracy and precision

Intra- and inter-day accuracy and precision of the analytical method was assessed over 3 days using 3 QC standards. Accuracy was assessed by analyzing five replicate injections of each QC standard. The precision study assessed the same set of QC standards that were stored in the fridge (± 2-8 °C) over the 3 day period. .

5.2.1.10.3. Limits of quantification and detection

Standard stock solutions were diluted to obtain concentrations for the determination of the lower limit of quantification (LOQ) and limit of detection (LOD) based on a signal-to-noise (S/N) ratio of 10:1 and 3:1 respectively.

5.2.1.10.4. Stability

The stability of CP in solution (± 10 µg/ml; n = 3) was assessed over a period of 12 days stored under various conditions. CP solutions were made up in acetonitrile or in mobile phase (H_2O/ MeCN, 54/46).

5.2.1.11. CP solubility

The solubility of CP was investigated in McIlvaine's buffer and also in saline solution. The McIlvaine's buffer solutions were comprised of 0.05 M citric acid and 0.1 M disodium

hydrogen phosphate (pH of 2.5, 3, 4, 5, 6, 7 and 8). The samples were shaken at a speed of ± 100 rpm at 37 ± 1 °C in a water bath for 24 hours. This was repeated at room temperature (22 ± 1°C) using an Orbit Shaker.

Samples were prepared by accurately weighing out 10 mg of CP which was then transferred into a 20 ml test tube, 12 ml of the relevant solutions were then added. The test tube was then capped and placed directly onto the Orbit Shaker or into a test tube rack in the water bath. One millilitre of the sample was pipetted from the relevant test tube and placed into a glass syringe attached with a filter. The sample was then filtered directly into an amber HPLC vial and a 100 μL of methanol was included to avoid possible precipitation of CP. The final solution was then analysed for CP content. Samples were assessed over a period of 24 hours and collected at 2, 4, 6, 12, 18, and 24 hour intervals.

5.2.1.12. Assay of CP content and uniformity

The United States Pharmacopoeia [17] and British Pharmacopoeia [4] specifies that the CP content in a cream or ointment formulation should be within the range of 90-115% of the labeled CP amount. It is thus necessary to assay the CP content in each formulation prior to use in the studies to check the uniformity of the drug content in each dose. The assay was performed on three commercially available formulations, Dermovate® cream, Dovate® cream and Dermovate® ointment using 2g and 11mg quantities of each of the formulations. The larger mass was used to assay the products in accordance with the Pharmacopoeial monographs for CP whereas the lesser amount (11 mg) was used to determine the content of CP in each dose applied in the various studies. The formulations were dispensed into a volumetric flask or test tube using an Eppendorf pipette and weighed to obtain the mass of each sample. The assays were carried out as described in the British Pharmacopoeia [4]. The internal standard used was DOCA as opposed to beclomethasone dipropionate as per British Pharmacopoeia [4].

5.2.2. Results and discussion:

5.2.2.1. Method validation

5.2.2.1.1. Linearity

The CP standard solutions gave a linear response at the concentration range of 0.5 – 100 µg/ml with a correlation coefficient of 0.9998 (y = 0.3523x + 0.0708).

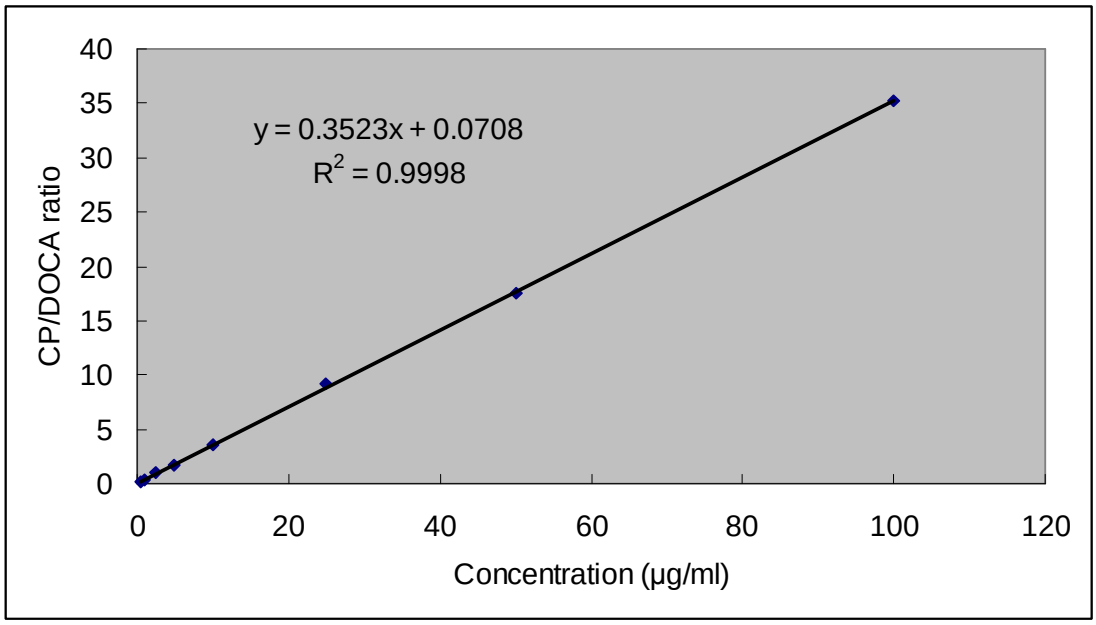

Figure 5.7. Calibration plot

5.2.2.1.2. Accuracy and precision

The accuracy of the analytical method for CP was found to be in the range of 94.8-105.8% with a relative standard deviation (RSD) of < 5% (as shown in Table 5.3). The results showed good intra- and inter-day precision as shown in Table 5.4.

Table 5.3. Intra- and inter-day accuracy

QC samples (n=5)	Day	Actual conc. (µg/ml)	Calculated conc. (µg/ml)	Accuracy	Inter-day RSD (%)
Low	1	2.12	2.25	106.13	1.85
	2	1.99	2.03	102.01	3.72
	3	2.03	2.09	102.96	1.93
Medium	1	15.90	15.07	94.78	0.18
	2	14.93	15.51	103.88	0.73
	3	15.23	15.64	102.69	1.73
High	1	74.2	72.43	97.61	2.54
	2	69.65	73.73	105.84	3.66
	3	71.05	72.30	101.75	4.05

Table 5.4. Intra- and inter-day precision

QC samples (n=5)	Day 1 Mean conc. ± SD (µg/ml) (RSD%)	Day 2 Mean conc. ± SD (µg/ml) (RSD%)	Day 3 Mean conc. ± SD (µg/ml) (RSD%)	Inter-day Mean conc. ± SD (µg/ml) (RSD%)
Low	2.19 ± 0.03 (1.28)	2.05 ± 0.10 (4.85)	2.06 ± 0.05 (2.38)	2.10 ± 0.08 (3.67)
Medium	15.08 ± 0.39 (2.60)	14.83 ± 0.30 (2.05)	15.40 ± 0.69 (4.49)	15.10 ± 0.29 (1.90)
High	72.64 ± 0.72 (1.00)	73.70 ± 1.08 (1.46)	73.43 ± 0.19 (0.26)	73.26 ± 0.55 (0.75)

5.2.2.1.3. Limits of quantification and detection

The limit of quantification (LOQ) for CP in mobile phase was found to be 0.5 µg/ml and the limit of detection (LOD) was found to be 0.25 µg/ml.

5.2.2.1.4. Stability

Figures 5.8 and 5.9 illustrate the degradation of CP over a period of 12 days under various storage conditions. It can be seen that CP is more stable in acetonitrile than in the mobile

phase which is a mixture of water and acetonitrile. This indicates that water enhances the degradation rate of CP compared to acetonitrile. Temperature does play a role in the slow degradation of CP, thus samples in the fridge were found to be more stable than those stored at room temperature.

CP samples in acetonitrile were sensitive to light.

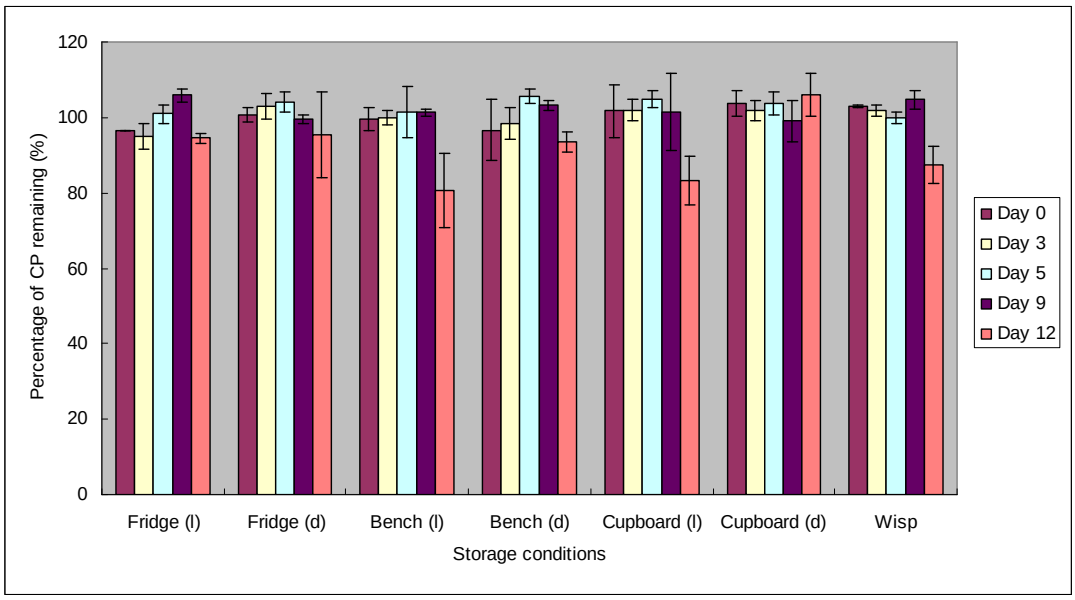

Figure 5.8. Stability of CP in acetonitrile under various storage conditions (l = light, d = dark)

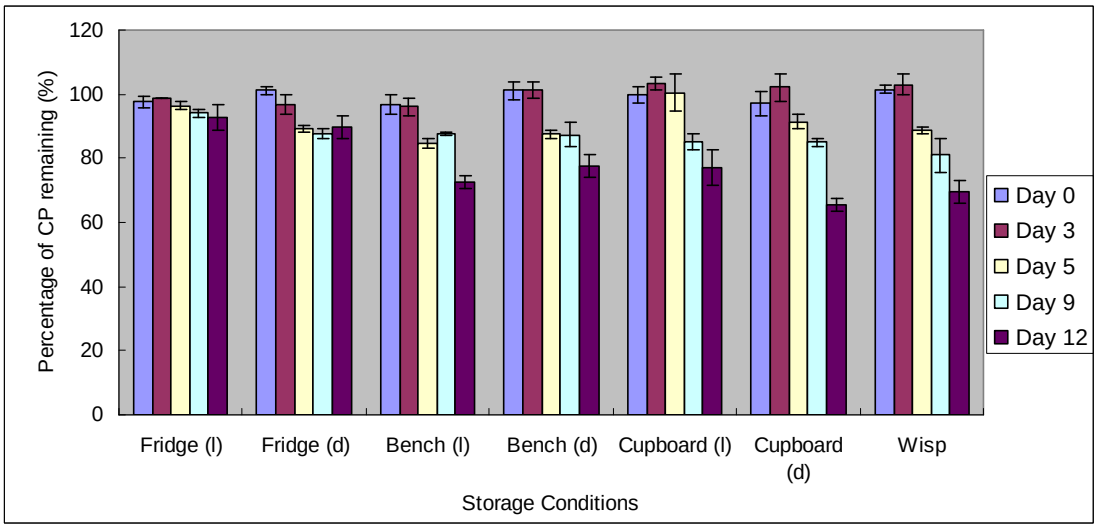

Figure 5.9. Stability of CP in mobile phase under various storage conditions (l = light, d = dark)

5.2.2.2. CP solubility

Figures 5.10 and 5.11 illustrate the solubility of CP in saline and in McIlvaine's buffer at the various pHs. CP has a higher solubility in saline solution (± 3.5 µg/ml) than in McIlvaine's buffer (highest is ± 2.6 µg/ml). Figures 5.10 indicates that CP is slightly more soluble in an acidic medium than in an alkaline medium, and that its solubility increases with an increase in temperature from 22 °C to 37 °C.

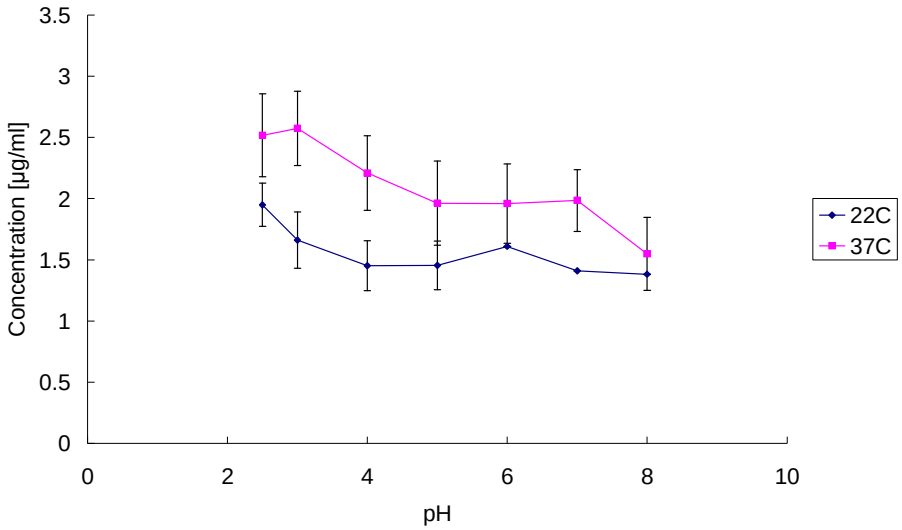

Figure 5.10. Solubility of CP in McIlvaine's buffer at the various pHs at 22 °C and 37°C °C

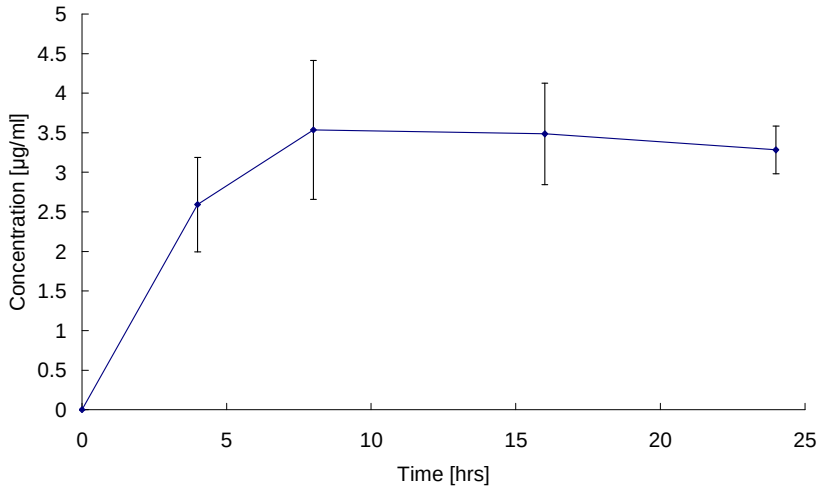

Figure 5.11. Solubility of CP in saline at 22 °C

5.2.2.3. Assay of CP content and uniformity in commercially available topical formulations.

The CP content of 2 grams of Dermovate® Cream, when expressed as a percentage of the theoretical amount of CP, was found to be 102.5 ± 0.7 % with a RSD of 0.7 % (n=3). However, when assaying at a smaller quantity equivalent to an application dose of 22 mg, the CP content was found to be 102.2 ± 41.2 % with a RSD of 40.25 % (n=3).

A 2 g quantity of Dovate® cream had a CP content of 92.5 ± 1.2 % with a RSD of 1.3 % (n=3). A 22mg quantity had a CP content of 102.58 ± 4.5 % with a RSD of 4.4% (n=3). Dovate® cream showed that the CP content was within the acceptable range as stated in the USP and the homogeneity of CP in this cream formulation showed uniform distribution.

The CP content found in the different masses of the ointment product, 2 g and 22 mg, were 103.8 ± 2.1 % and 121.8 ± 3.1 %, respectively. The RSD found within three extracted samples of each of the 2 g and the 22 mg masses were 2.0 % and 2.6 %, respectively. This indicates that CP was more uniformly distributed in the ointment formulation than the two cream formulations, Dermovate® and Dovate®. However, the 22 mg quantities of the ointment preparations had a CP content that was slightly higher than the limits specified by the USP and BP. The USP and BP allow for a CP content not less than 90.0% and not more than 115.0% of the labeled claim [4,17].

5.3. HPLC ANALYSIS OF TS SAMPLES

5.3.1. Experimental

5.3.1.1. Instrumentation

Eppendorf pipettes and disposable tips, a precision balance, ultrasonic bath, vortex, and a centrifuge were used for sample preparations. Extracted samples were also placed into 300 µl glass microinserts (Separations, Randburg, South Africa) where each was positioned in an amber HPLC vial and capped for analysis. The Waters Alliance® HPLC system was used to analyse tape strip samples.

5.3.1.2. Reagents, chemicals and materials

CP (a USP reference standard), HPLC-grade ethyl acetate (BDH Chemicals Ltd., Poole, England), acetonitrile, methanol, and water were utilized in these studies. The same conditions as mentioned in section 5.2.1.9 were used for the analysis of the samples and the injection volume was 20µL.

Scotch® Magic tape (no. 810, 24 mm x 50 m, 3M, Pymble, Australia) and Transpore™ dressing tape (no. 1527, 24 mm x 5 m, 3M, Isando, South Africa) were assessed.

5.3.1.3. Sample preparation

5.3.1.3.1. Extraction procedure

Each individual tape strip of approximately 2.2 x 2.2 cm dimensions was placed into a 1.5ml polypropylene centrifuge tube with the adhesive side of the tape facing towards the inside of the tube. Each tape was extracted with 500 µL of methanol and vortexed for 1 minute. The samples were centrifuged for 5 minutes at 12 000 rpm on an Eppendorf centrifuge (Model no. 5414, Eppendorf Ag, Hamburg, Germany). Approximately 100 µL of the sample was pipetted into a microinsert (Separation®, place of manufacturer, etc.) that was placed into an HPLC amber vial (Waters®, etc.) and the samples were analysed on an Alliance® HPLC system.

5.3.1.3.2. Preparation of standards

A stock solution of 1.25 mg/ml of CP was prepared by accurately weighing out ~25mg of CP into a volumetric flask and dissolving it in 20 ml of methanol. Calibration standard solutions of 0.01 - 1 mg/ml (0.01, 0.0125, 0.025, 0.0375, 0.05, 0.125, 0.25, 0.5 and 1 mg/ml) in methanol were prepared by serial dilution and made up to volume in 5ml volumetric flasks. Each tape strip was spiked with 10uL of a standard solution and left to air dry. The tape strips were then placed into a microcentrifuge tube and extracted according to the procedure

mentioned above. After extracting the spiked tape strips, the final concentration range was 0.2 - 20 µg/ml (0.2, 0.25, 0.5, 0.75, 1, 2.5, 5, 10 and 20 µg/ml).

A fresh, separate stock solution was used to prepare the quality control (QC) samples on a daily basis. Three QC standard solutions of 250, 75 and 30 µg/ml (high, medium and low concentrations) were extracted to give final concentrations of 5, 1.5 and 0.6 µg/ml.

5.3.1.3.3. Method validation

5.3.1.3.3.1. Linearity

Peak areas of CP were plotted against CP concentrations following the analysis of relevant samples (n=3). The data were evaluated using linear regression analysis over the concentration range of 0.2 – 20 µg/ml.

5.3.1.3.3.2. Accuracy and precision

Intra- and inter-day accuracy and precision of the extraction method was assessed over 3 days using the 3 QC standards. Accuracy was assessed by analyzing five separate tape strip extracts of each QC standard. Precision was assessed using the same QC standard tape strip extracts prepared on day 1 that had been stored in the fridge (± 2-8 °C) and evaluated over a period of three days. This also served as an indicator of stability.

5.3.1.3.3.3. Limits of quantification and detection

As shown in section 5.2.1.10.3, except extracted tape strip samples were assessed instead of CP solutions in this study.

5.3.1.3.3.4. Specificity

Extractions of blank tape strips and tape strips with adhered SC were assessed for specificity.

Both Scotch® and Transpore™ tapes were used for the TS and analysed for possible interferences.

5.3.1.3.3.5. Recovery

The extraction recovery of CP from the tape strips was assessed by analyzing spiked tape strips of high (~5 µg/ml), medium (~1.25 µg/ml) and low (~0.5 µg/ml) concentrations. The data from the extracted samples were compared with the standard samples in methanol without extraction.

5.3.1.3.3.6. Stability

The short-term stability of the extracted CP samples was assessed over a period of 24 hours under different temperature conditions. Samples were protected from light and were stored in the fridge or at room temperature. Extracted samples of high (40 µg/ml), medium (25 µg/ml) and low (5 µg/ml) concentrations were assessed.

5.3.2. Results and discussion:

5.3.2.1. Method validation

5.3.2.1.1. Linearity

The spiked tape strip extracts gave a linear response at the concentration range of 0.2 – 20 µg/ml with a correlation coefficient of 0.9994 (y =30921x – 7933.4).

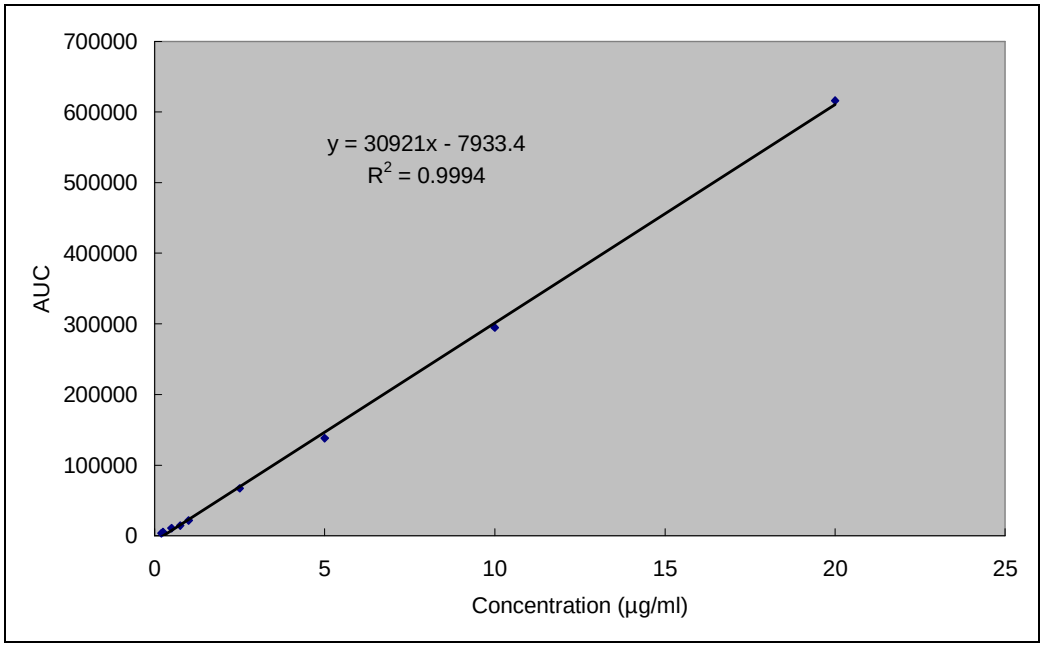

Figure 5.12. Calibration plot of extracted tape strip samples

5.3.2.1.2. Accuracy and precision

The accuracy of the method was found to be in the range of 99.9-105.4% with a RSD of <3% (as shown in Table 5.5). The results show good intra- and inter-day precision as shown in Table 5.6.

Table 5.5. Intra- and inter-day accuracy of TS samples

QC samples (n=5)	Day	Actual conc. (µg/ml)	Calculated conc. (µg/ml)	Accuracy	Inter-day RSD (%)
Low	1	0.64	0.67	104.69	0.86
	2	0.59	0.63	106.77	2.58
	3	0.57	0.60	105.26	1.62
Medium	1	1.59	1.60	100.63	2.07
	2	1.47	1.47	100.00	1.68
	3	1.44	1.49	103.47	2.24
High	1	5.03	5.00	99.40	1.85
	2	4.90	4.92	100.41	2.32
	3	4.80	5.06	105.42	1.09

Table 5.6. Intra- and inter-day precision of TS samples

QC samples (n=5)	Day 1 Mean conc. ± SD (µg/ml) (RSD%)	Day 2 Mean conc. ± SD (µg/ml) (RSD%)	Day 3 Mean conc. ± SD (µg/ml) (RSD%)	Inter-day Mean conc. ± SD (µg/ml) (RSD%)
Low	0.63 ± 0.02 (3.67)	0.60 ± 0.02 (3.04)	0.60 ± 0.01 (1.29)	0.61 ± 0.02 (2.58)
Medium	1.58 ± 0.04 (2.33)	1.48 ± 0.02 (1.47)	1.48 ± 0.03 (2.03)	1.51 ± 0.06 (3.97)
High	4.81 ± 0.10 (1.97)	4.66 ± 0.05 (1.00)	5.00 ± 0.10 (1.92)	4.82 ± 0.17 (3.48)

5.3.2.1.3. Limits of quantification and detection

The LOD and LOQ values were found to be 0.1 µg/ml and 0.2 µg/ml based upon signal to noise ratios of 3:1 and 10:1, respectively.

5.3.2.1.4. Specificity

Analysis of blank tape strips were required in order to determine whether interferences were present that could affect the analysis of CP. Interferences were found in the Transpore™ tape extract under UV detection as shown in Figure 5.13. Transpore™ tape showed impurity peaks over a run time of 22 minutes. Scotch® tape revealed fewer impurity peaks which eluted within 5 minutes as shown in Figure 5.14. The impurity peaks from Transpore™ tape interfered with the analysis of CP.

Methanol was used as the extraction solvent. Acetonitrile and ethyl acetate could not be used because the tape was simply liquefied when immersed into either of these solvents.

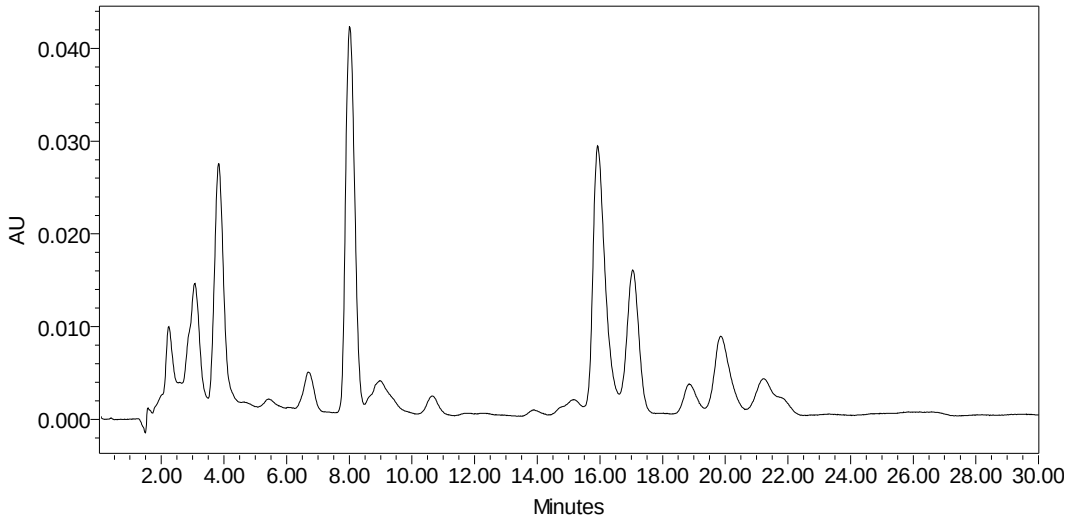

Figure 5.13. Extraction of blank Transpore™ tape strip

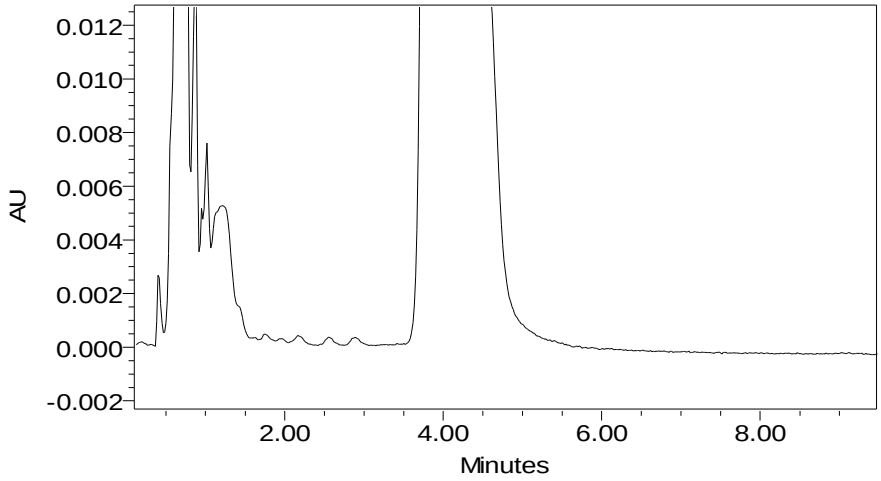

Figure 5.14. Extraction of a blank Scotch® tape strip

A variety of mobile phases and columns were tested to separate the CP peak from the interfering peaks when working with the Transpore™ tape. However, none of the alterations were able to resolve CP and thus Scotch® tape was chosen as the adhesive tape for the TS studies.

The tape strip sample which included the SC showed no interferences with the elution of CP as shown in Figure 5.15.

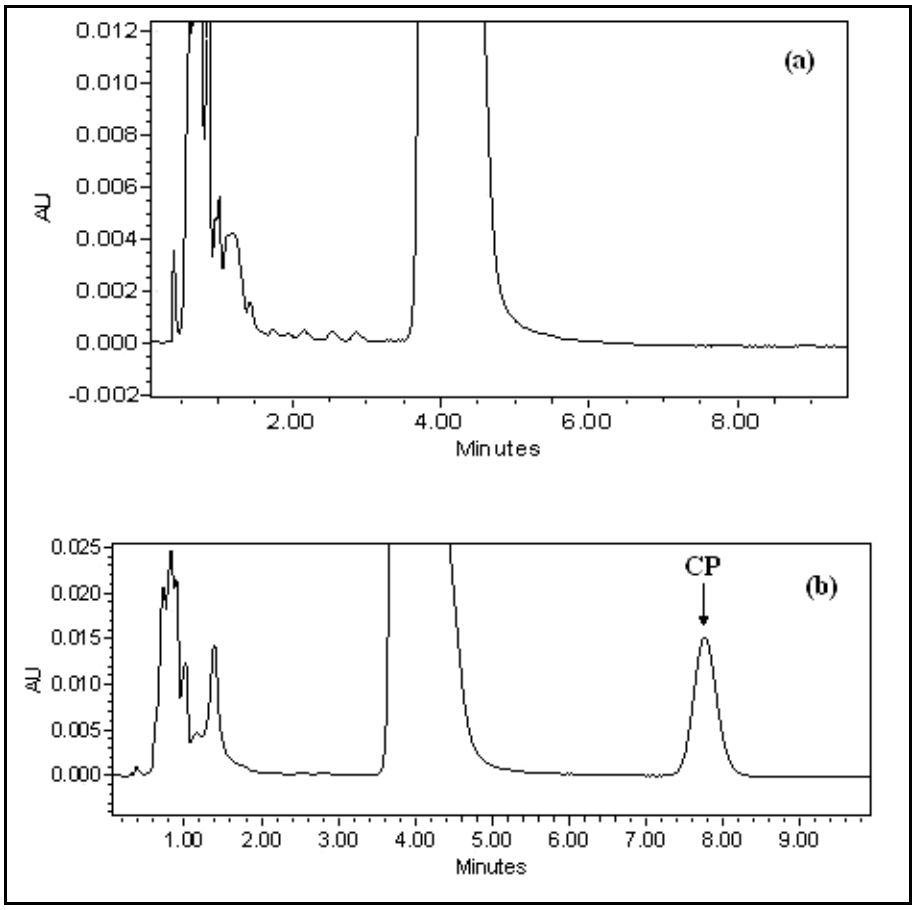

Figure 5.15. (a) Extraction of blank, stripped Scotch tape and (b) spiked, stripped Scotch® tape.

5.3.2.1.5. Recovery

The extraction recovery of CP from the tape strips was found to be in the range of 81.6 – 84.7%.

Table 5.7. Recovery of CP from tape strips

QC samples	Concentration of extracted sample (µg/ml)	Concentration of methanolic sample (µg/ml)	Recovery (%)
High	4.06	4.97	81.6
Medium	1.05	1.24	84.7
Low	0.34	0.41	82.9

5.3.2.1.6. Stability

The stability of CP in extracted tape strip samples were found to be stable for 24 hours regardless of whether or not they were stored in the fridge or at room temperature.

5.4. HPLC ANALYSIS OF MD SAMPLES

5.4.1. Experimental

5.4.1.1. Instrumentation

The instrumentation used was the same as that described in section 5.3.1.1

5.4.1.2. Reagents, chemicals and materials

The reagents and chemicals were the same as described in section 5.3.1.2. In addition, Intralipid® 20% (Fresenius-Kabi, Midrand, South Africa) and HPLC-grade hexane (Burdick & Jackson Laboratories Inc., Michigan, USA) were utilized in the MD studies. The same analytical method used to assay tape strips was used to assay MD samples.

5.4.1.3. Sample preparation

5.4.1.3.1. Extraction procedure

Thirty microlitres of CP in Intralipid® (IL) was placed in a 1.5ml polypropylene centrifuge tube and 60 µL of acetonitrile (containing 2 µg/ml DOCA) was added. The sample was vortexed and centrifuged for 20 minutes at 12 000 rpm on an eppendorf centrifuge. The supernatant was removed using a 100 µL pipette (pipetman, Gilson Inc., Middleton, USA) and placed into a 300 µL glass microinsert which was previously placed in an amber HPLC vial. Twenty microlitres of the sample was then injected onto the Luna C_8 column.

5.4.1.3.2. Preparation of standards

A 100 µg/ml stock solution was made by accurately weighing ~10 mg of CP or DOCA into 100 ml volumetric flasks.

The internal standard (DOCA) solution (2µg/ml) was made up to volume with acetonitrile. A serial dilution was made to obtain the relevant concentration which gave an AUC value corresponding to the AUC of CP approximately in the middle of the calibration concentration range.

The reference standard (CP) stock solution was first wetted with 0.5 ml methanol and then made up to weight with IL (density = 0.98 g/ml). The standard solutions were all prepared via serial dilution and were made up to a final mass of 5 g with IL.

All the solutions were sonicated or shaken immediately after preparation.

5.4.1.4. Method validation

5.4.1.4.1. Linearity

A calibration curve was constructed using the peak AUC ratios of CP/DOCA versus concentration over the concentration range of 0.5 – 100 µg/ml.

5.4.1.4.2. Accuracy and precision

See section 5.3.1.3.3.2 relating to TS.

5.4.1.4.3. Limits of quantification and detection

See section 5.3.1.3.3.3 relating to TS.

5.4.1.4.4. Specificity

Extractions of blank IL samples (unspiked with CP and DOCA), were assessed for specificity.

5.4.1.4.5. Recovery

The extraction recovery of CP from IL was assessed by comparing spiked IL samples with acetonitrile solution samples of the same concentrations. Three concentrations of high, medium and low were assessed.

5.4.1.4.6. Stability

See section 5.3.1.3.3.6 on the stability of TS samples. Since IL should not be stored in the fridge, Intralipid samples containing CP were stored at room temperature in the dark.

5.4.2. Results and discussion

Five different extraction solvents were assessed i.e. methanol, acetonitrile, hexane, and ethyl acetate, for the extraction of Intralipid samples. The results are shown in Table 5.8. Acetonitrile was found to be the most appropriate extraction solvent for IL using HPLC-UV.

Table 5.8. Liquid-liquid extraction of CP from IL using various solvents

Solvent	Miscible	Observations	Problems
Methanol	Yes	No separation: cloudy	Cannot introduce onto column
Acetonitrile	Yes	2 layers: have precipitate and oil phase at the bottom and clear supernatant	_
Hexane	No	2 layers: white, opaque layer at the bottom and clear supernatant	CP not found in hexane
Ethyl acetate	No	2 layers: both the bottom and top layers are clear	All the excipients in the IL have been extracted into ethyl acetate except water.

5.4.2.1. Method validation

5.4.2.1.1. Linearity

The calibration plot of extracted IL samples gave a linear response with a correlation coefficient of 0.9999 (y = 0.0358x +0.0215).

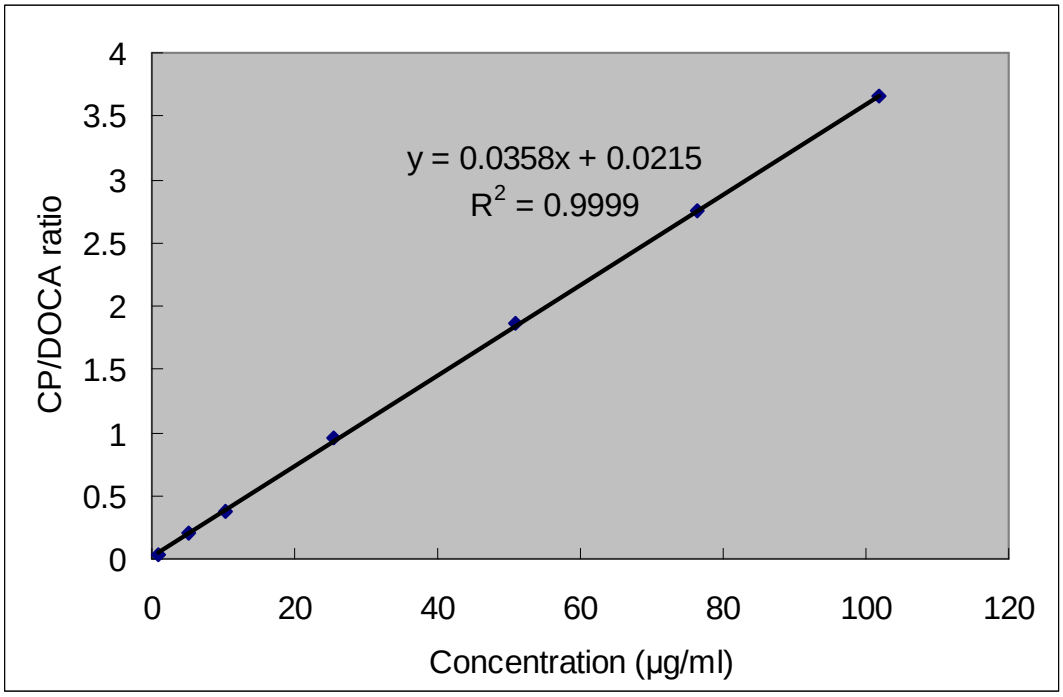

Figure 5.16. Calibration plot of extracted IL samples

5.4.2.1.2. Accuracy and precision

The accuracy of the method was found to be in the range of 89.64 – 101.75% with a RSD of <5.5%, and intra- and inter-day precision is shown in Tables 5.9 and 5.10, respectively.

Table 5.9. Accuracy of MD samples

QC samples (n=5)	Day	Actual conc. (µg/ml)	Calculated conc. (µg/ml)	Accuracy	Inter-day RSD (%)
Low	1	2.51	2.25	89.64	3.25
	2	2.56	2.49	97.27	5.00
	3	2.60	2.37	91.15	4.28
Medium	1	50.20	49.97	99.54	1.77
	2	51.20	51.63	99.29	4.12
	3	52.10	52.78	101.31	2.31
High	1	75.30	76.62	101.75	2.02
	2	76.80	74.65	97.20	2.47
	3	78.30	79.55	101.60	3.84

Table 5.10. Precision study of MD samples

QC samples (n=5)	Day 1 Mean conc. ± SD (µg/ml) (RSD%)	Day 2 Mean conc. ± SD (µg/ml) (RSD%)	Day 3 Mean conc. ± SD (µg/ml) (RSD%)	Inter-day Mean conc. ± SD (µg/ml) (RSD%)
Low	2.32 ± 0.08 (3.48)	2.17 ± 0.08 (3.63)	2.40 ± 0.09 (3.60)	2.29 ± 0.12 (5.08)
Medium	48.62 ± 1.21 (2.49)	51.63 ± 2.13 (4.12)	48.90 ± 0.51 (1.05)	49.72 ± 1.60 (3.34)
High	72.92 ± 2.26 (3.09)	74.65 ± 1.84 (2.47)	74.03 ± 2.58 (3.49)	73.87 ± 0.88 (1.19)

5.4.2.1.3. Limits of quantification and detection

The LOQ and LOD of extracted IL samples were 0.5 µg/ml and 0.25 µg/ml, respectively,

with an injection volume of 20 µl. An injection volume of 10 µl yielded LOQ and LOD values of 1.0 µg/ml and 0.5 µg/ml, respectively.

5.4.2.1.4. Specificity

The extracted blank IL samples showed two peaks relating to constituents of the IL emulsion (Figure 5.17). However, these peaks did not interfere with either CP or the internal standard, DOCA (Figure 5.18). The extraction method was thus found to be acceptable for the analysis of CP in IL.

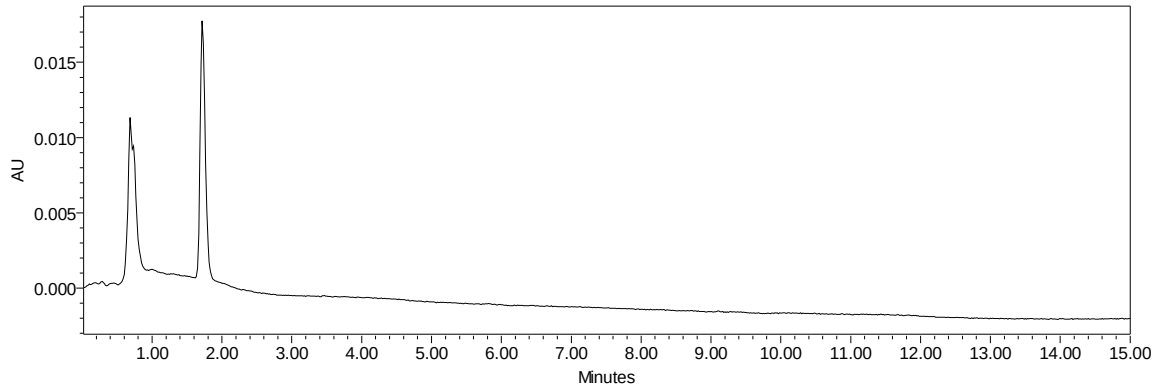

Figure 5.17. Extraction of blank IL

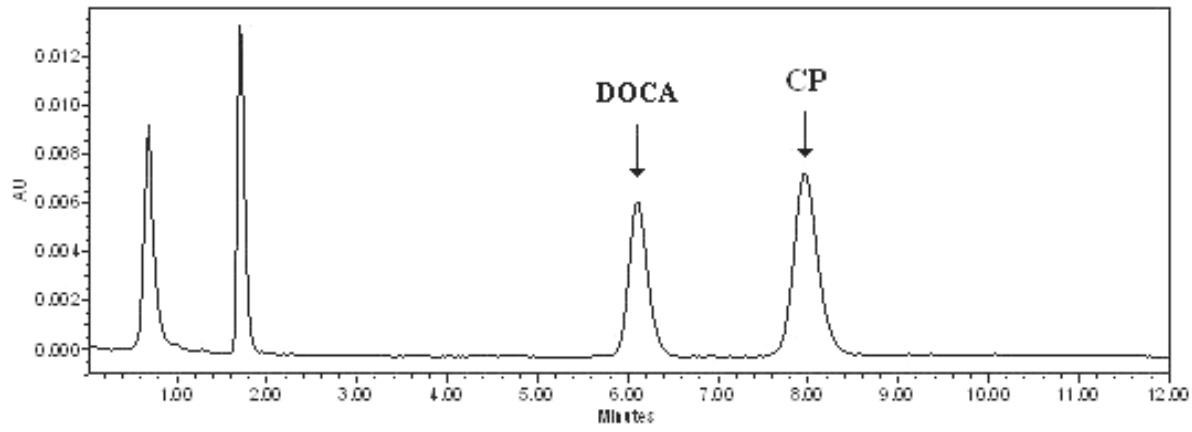

Figure 5.18 Extraction of blank IL spiked with DOCA and CP.

5.4.2.1.5. Recovery

The recovery of CP from extracted IL samples was 79.08 ± 1.43 %, as shown in Table 5.11.

Table 5.11. Extraction efficiency of CP from IL at three different concentrations.

QC samples	Concentration of extracted sample (µg/ml)	Concentration of acetonitrile sample (µg/ml)	Recovery (%)
High	57.24	73.21	78.19
Medium	39.52	50.46	78.31
Low	2.03	2.52	80.73

5.4.2.1.6. Stability

CP is stable in IL after 24 hours storage in the dark at room temperature.

5.5. CONCLUSIONS

The foregoing describes quantitative methods for the determination of CP in 3 different media i.e. mobile phase, tape strips and MD samples consisting of CP in IL. The methods were found to have the necessary specificity, accuracy and precision and were therefore suitable for the quantitative analysis of CP from the various matrices. These analytical methods were successfully applied to assess the BA/BE of CP from topical formulations containing CP using TS and for the assessment of the permeation of CP through human skin using MD.

6. CHAPTER 6

6.1. TAPE STRIPPING

As previously mentioned, in Chapter 4, BE assessment of orally administered products intended for the systemic circulation is carried out by measuring plasma drug concentrations following administration of a test and reference dosage form to human subjects. However, in the case of topical preparations intended for local action (i.e. not intended to be absorbed into the systemic circulation), the general BE procedure used for products where the active ingredient is intended to be absorbed into the systemic circulation, cannot be used.

Several methods have been developed to study the penetration of active ingredients from topical formulations as well as their BA and BE. With the exception of topical corticosteroid preparations, BE assessment of topical products usually requires that clinical studies in patients be undertaken to compare a new formulation (test) versus an approved product (reference). However, clinical efficacy trials are time consuming and expensive [132].

Currently, the HSBA is the only acceptable BE method approved by the USA FDA [158] and also by many other international regulatory bodies [118,232-234] for the BE assessment of topical corticosteroid products. This method was originally developed by McKenzie and Stoughton [159], but it is only applicable for assessing topical corticosteroid products which produce skin blanching following application to the skin. Hence, alternative methods for the BE assessment of other topical dosage forms are needed.

TS is one of the many techniques that can be applied to study the permeation of a topically applied drug into the different layers of the epidermis. TS studies of human and animal SC have been used to calculate percutaneous absorption profiles and the retention time of the drug in the skin. Studies where skin has been stripped to disrupt the SC barrier have also been done to assess barrier homeostasis under different conditions. [235-238].

TS has been investigated by several researchers as a surrogate measure for BA and BE assessment of topical products [174,239-242]. This is based on the concept of determining drug permeation through the SC following application of the product to the skin of human subjects. This technique utilizes adhesive tape strips which consecutively remove layers of

corneocytes, after which drug content is quantitatively measured in each layer of stripped skin or calculated as the cumulative amount present in all the skin strips (Figure 6.1). This method is considered to be relatively non-invasive due to the homeostatic nature of the skin. The SC for example has been shown to restore itself relatively quickly after disruption [243].

In 1998, a draft TS guidance was issued by the USA FDA to assess BE of topical dermatological drug products [244]. This draft was later withdrawn due to flaws found in the recommended procedures of TS [245]. Data from inter-laboratory comparative studies on the same products were found to have conflicting and opposite results [173]. However, in spite of the withdrawal of the FDA's draft guidance [245], TS remains a promising tool and is still being investigated and optimized by a number of researchers [174,246,247]. Previously published reports have indicated the potential of the TS technique for use in assessing the BE of topical preparations. One study showed correlations between the amount of corticosteroid in 10 tape strips versus the 90% CI obtained from the HSBA of creams and ointments [248] whereas another study [160,249] showed a correlation between the mean amount of corticosteroid in 10 tape strips versus the AUEC results obtained from visual HSBA data. The above studies, however, did not use the data to assess BE.

Although the total amount of drug found in the stripped skin layers was determined in the abovementioned studies, the removed SC thickness was not taken into account. Since the total thickness of the SC removed from different sites using the same number of tape strips for each site may and usually does vary, a valid comparison between each subject and even between the tested sites within a subject cannot be made. Hence, normalization of subject skin thickness is necessary and can be undertaken using transepidermal water loss (TEWL) [250,251].

The main objective of the current study was to explore the applicability of standardized TS methodology as a viable option for BE assessment of topical dosage forms. The study design takes into account skin thickness normalization using TEWL data as well as refinements involving dosage application and duration of contact, special attention to removal of excess formulation from the skin after dosing as well as control of relative humidity and temperature of the study environment.

A pilot study was undertaken to establish the dose duration to be used to assess the BE of CP

formulations using TS. This was deemed necessary to ensure the requisite discriminatory capability of the method. Subsequently, another pilot study, using the established dose duration, was undertaken to estimate the number of subjects required for subsequent pivotal studies based on inter-individual variability obtained from area under the curve (AUC) of test/reference ratios. Dermovate® cream was used as both the test and reference product.

Subsequently, a pivotal study was conducted to determine whether TS was able to establish BE between 2 different topical cream formulations, a test product (Dovate® cream) and a reference product (Dermovate® cream). A concurrent pivotal study was also undertaken between an ointment formulation (Dermovate® ointment) and the same reference formulation previously used (Dermovate® cream). All of the formulations contained 0.05 % CP.

According to the USA FDA [118] and most regulatory authorities, the declaration of BE between a test and reference product using the 2 one-sided t-test, requires that the 90% CI should fall within the range of 80-125%. Hence, these criteria were used to assess BE for our studies.

6.1.1. Application of TS

Studies have demonstrated that TS methodology is a promising tool for use in the selection and comparison of vehicles for topical drugs [132,172,241,252]. The TS technique has been used to analyze biological activity, taking into account binding, decomposition, and metabolism of a given drug. Pershing *et al* [160,249,253] and Tsai *et al* [248] compared skin blanching with the drug content in human SC following topical application of commercial corticosteroid products. It was found that there was good correlation between the detected penetration behaviour and the biological response of the corticosteroids. This is a good indication that TS may be used for determining BA and BE of topical formulations.

Comparative drug uptake by the skin can be assessed by applying test and reference products to an anatomical site on a study subject. The SC samples can be obtained at various intervals of time following application of the formulation. This facilitates the observation of the drug's absorption and elimination profiles [174]. However, this approach uses different dose

durations for each application site which may result in different discriminatory capability at each site. This will likely have important implications when attempting to assess BE between formulations.

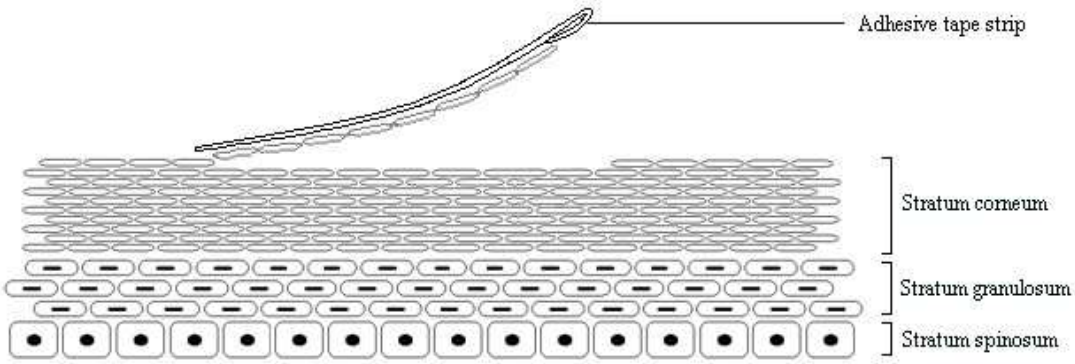

Figure 6.1. Removal of a layer of SC with an adhesive tape strip

Apart from the potential to use TS to assess BA/BE, Surber *et al* [254] postulated that the data obtained from TS experiments can also provide the following useful information:

1) Chemical penetration into the skin
2) Chemical permeation through the skin
3) Chemical elimination from the skin
4) Pharmacodynamic parameters
5) Clinical parameters

6.1.2. Factors affecting TS

6.1.2.1. Seasonal

It has been found that the time of the year affects the adhesiveness of tapes used to strip skin [255]. That particular study surmised that accumulation of fluid beneath the applied tape was significantly higher during summer than in the winter, thus the dermal peeling force was lower in summer than in winter. However, the amounts of corneocytes stripped were larger in summer than in winter. This was probably caused by the deterioration of the strength of the inter-corneocyte cohesion because of skin hydration

6.1.2.2. Individual

The variations in barrier disruption in tape stripped individuals were found to be significant. The number of tape strips used was seen to vary between subjects to yield the same TEWL content per site [256]. In addition, different anatomical sites have demonstrated that the SC physiology is not consistent on each part of the body [257,258].

6.1.2.3. Tape

Various kinds of adhesive tapes have been compared for use in TS. Commercial adhesive tapes that are commonly utilized include Tesa® tapes, D-squame®, Transpore™, Micropore®, and Scotch® tapes. Results showed that a majority of the tapes successfully stripped the SC and induced SC disruption. The tapes did not significantly influence the mass of SC removed, but a difference in the degree of barrier disruption to TEWL was observed between some of the tape types [258-260]. It has been demonstrated that the choice of tape can alter the thickness value (H) in the equation used to determine water diffusivity across the SC (equation 6.1 shown in section 6.1.4.2). Stronger adhesive tapes have a higher dermal peeling force which cause an increase in destruction of the skin surface and TEWL followed by a more noticeable skin irritation [261,262].

6.1.3. Side effects

The occurrence of inflammation in the dermis can be associated with the tape stripping procedure. Inflammation is caused by trauma induced by stripping of the outer layer of the skin during tape removal which releases the promotors of inflammation from the keratinocytes. The irritation was not so great in the skin under the inner part of the tapes, but was found to be more severe on the skin under the edge portion of the applied tape [263]. Dermal reaction was proven to be greater in summer than in winter [255].

6.1.4. Transepidermal water loss

6.1.4.1. Determination of SC thickness using transepidermal water loss

The SC is not absolutely waterproof i.e. it is a semi-permeable membrane [58]. It allows a continuous diffusion of water out of the body, known as TEWL. This is a passive process and must be differentiated from active sweating which occurs in response to thermal stress or emotion [61]. TEWL is used to estimate SC water barrier function, an increase in TEWL reflects impairment in the barrier function of the SC.

6.1.4.2. Rationale for the use of transepidermal water loss

The principle function of the human SC is to restrict the loss of water to the external environment. Typical basal values of TEWL in healthy, adult, human skin are in the range of 5 – 10 $g.m^{-2}.h^{-1}$. Disruption of the barrier can compromise the role of the SC. The measurement of TEWL is an effective marker to evaluate the degree of perturbation by physical or chemical attacks, or even the cause of the disease [264].

The following equation is a linearized form of Fick's first law used to estimate water diffusivity across the SC and the membrane thickness [265].

$$1/TEWL_x = [H/(K\Delta C.D)] - [x/(K\Delta C.D)] \qquad \text{(equation 6.1)}$$

Where $TEWL_x$ = transepidermal water flux when x μm of SC has been removed by tape-stripping.

K = the SC-viable tissue partition coefficient of water.

D = the average apparent diffusivity of drug molecule in SC of thickness H (μm).

ΔC = the drug concentration difference across the membrane

It has been shown that, despite the various SC thicknesses, a very consistent barrier to TEWL resides in the population of healthy human subjects. The relative efficiency of this barrier is a constant i.e. removing a certain fraction of SC thickness from a thick skin has the same impact on TEWL as removing the same fraction of thin membrane [250].

6.1.4.3. Methods of measuring TEWL

The gravimetric method [61] employs sensitive scales to determine body weight changes. TEWL is calculated by subtracting respiratory water loss, and taking into account the weight loss that occurs when oxygen is consumed and carbon dioxide is excreted. There are many sources of error within the gravimetric method which makes this approach unreliable.

The chamber methods [61] measure TEWL directly by enclosing an area of the skin and collecting the water given off. The disadvantage is that the relative humidity in the chamber changes during the period of observation and alters passive diffusion. Ventilating the chamber can reduce this error, but an extra variation will be introduced which is the drying effect of the draught.

The evaporimeter [266] is the most innovative and versatile technique used to measure TEWL. This method uses the principle of estimating the vapour-pressure gradient adjacent to the surface of the skin. Minimal influence on ambient humidity and temperature surrounding the skin is achieved. Water loss is measured electronically with a small probe placed above an area of the skin. The instrument is intended for use in the temperature range of 15-40°C and the atmospheric pressure range of 98-104 kPa [266]. This instrument measures water loss from the skin by relying on the observation that as water evaporates from the skin there is a linear decline in water vapour pressure which is directly proportional to the rate of evaporation. The TEWL can be measured accurately when concentrated on one site, but when assessed over a number of sites it only yields an approximate value [61]. Although TEWL may be influenced by a number of variables, experiments have shown that the evaporimeter yields repeatable results *in vitro* and *in vivo*.

6.1.4.4. Factors affecting TEWL

6.1.4.4.1. Body temperature

Skin permeability and surface evaporation is increased by heat. TEWL is greater from warm

than cold skin. Increasing the ambient temperature also increases skin water loss, although some of this effect is probably secondary to a concurrent increase in body temperature and fall in ambient relative humidity [61].

6.1.4.4.2. Ambient relative humidity

An increase in ambient humidity leads to a linear fall in skin water loss [267]. This is indicative of the passive nature of TEWL.

6.1.4.4.3. Radiant heat

Radiators, when used as a means of thermal control can increase the TEWL by over 50%. This is due to an increase in skin surface evaporation. Any instruments, such as phototherapy devices which radiate heat can cause a small increase in water loss [61].

6.2. COMPARISON BETWEEN TRANSPORE™ AND SCOTCH® TAPES

6.2.1. Experimental

6.2.1.1. Instrumentation

TEWL was assessed by a vapometer (Delfin Technologies Ltd., Kuopio, Finland). Tape strips were pre-weighed with a Mettler® Toledo balance (model AG135, Columbus, USA). Analysis of tape strips were carried out as shown in section 5.3

6.2.1.2. Materials

TS was performed using Transpore™ dressing tape (3M, Isando, South Africa) and Scotch® Magic™ tape (3M, Pymble, Australia) of approximately 2.4 x 2.4 cm dimensions.

6.2.1.3. Formulations

Biocort® cream, containing 1% hydrocortisone acetate, (Adcock Ingram Ltd., Johannesburg, South Africa) was applied once daily to the stripped area at the end of each TS experiment to assist with the healing process and prevent any possible scarring or hyperpigmentation. Application of Biocort® was continued daily until the disappearance of any resulting pigmentation at the tape stripped site.

6.2.1.4. Subjects

In order to assess the applicability and utility of the different tapes, 3 healthy volunteers (1 female and 2 males) who met the selection criteria were enrolled after providing informed consent. Subjects were required to have no skin disorders and were instructed not to apply medicated topical products to the test sites for 2 months before and during the study. The subjects were also informed not to apply any emollient products on their forearms 24 hours prior to the start of the study.

6.2.1.5. Study design

The volar aspect of the forearm of each subject was washed 1 hour prior to tape stripping. Four 2 x 2 cm^2 square application sites were demarcated using an adhesive label (Tower®, South Africa, Cape Town) on the forearms of the subjects. The labels were cut to the appropriate size with a blade prior to the study. The sites were placed 1-2 cm apart and 6cm away from the wrist and elbow.

Fifteen individual ~2.4 x 2.4 cm^2 squares of Scotch® tape (Scotch® Magic™ Tape, no. 810, 24 mm x 50 m, 3M, Pymble, Australia) were utilized to sequentially tape strip the SC of the exposed square skin sites. The demarcation label remained on the skin during the TS procedure and was held intact by Scotch® tape such that all skin stripping was confined to the demarcated site only. Each tape strip was weighed on a precision balance (Mettler® Toledo, model AG135, Columbus, USA) prior to the study and immediately after stripping

to quantitatively determine the weight of the SC removed and to minimize weight loss of the stripped skin due to possible changes in water content. The tape strips were removed with a rapid movement. The stripping process involved removing consecutive tape strips in directions changing in order of a clockwise rotation as shown in Figure 6.2. A pair of forceps was used to apply pressure onto the tape and was rubbed backwards and forwards 10 times to ensure that each tape strip adhered evenly to the skin site prior to stripping. Fifteen tape strips were used per site.

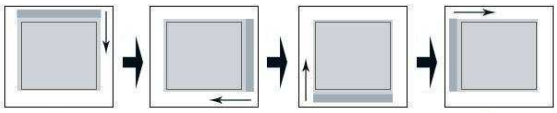

Figure 6.2. Direction of tape strip placement and removal.

All sites were used for the determination of individual SC thickness. TEWL and stripped SC weight was used to calculate individual SC thicknesses [250]. A vapometer was used to obtain TEWL readings recorded as $g/m^2/h$.

Room temperature (22 ± 1 °C) and humidity (45 ± 2 %) were controlled throughout the studies.

6.2.1.6. Data and statistical analysis

The two-tailed t-test (95% CI) was used to assess differences in mass of SC removed between the two different tapes.

6.2.2. Results and discussion

The stripped sites showed progressive inflammation from continuous stripping with the adhesive tapes. The study subjects reported slight discomfort on the stripped sites after the 10^{th} strip, especially with the use of Transpore™ tape.

Figure 6.3 shows the pooled data from 3 subjects which compares the total mass of SC

removed from 15 tape strips on a single site per subject. The average mass of SC removed using Scotch® tape was 3.71 ± 0.74 mg, and for Transpore™, an average mass of 5.17 ± 1.28 mg. The p-value of 0.20 (>0.05) indicated no significant difference in the mass of SC removed between the two different tapes.

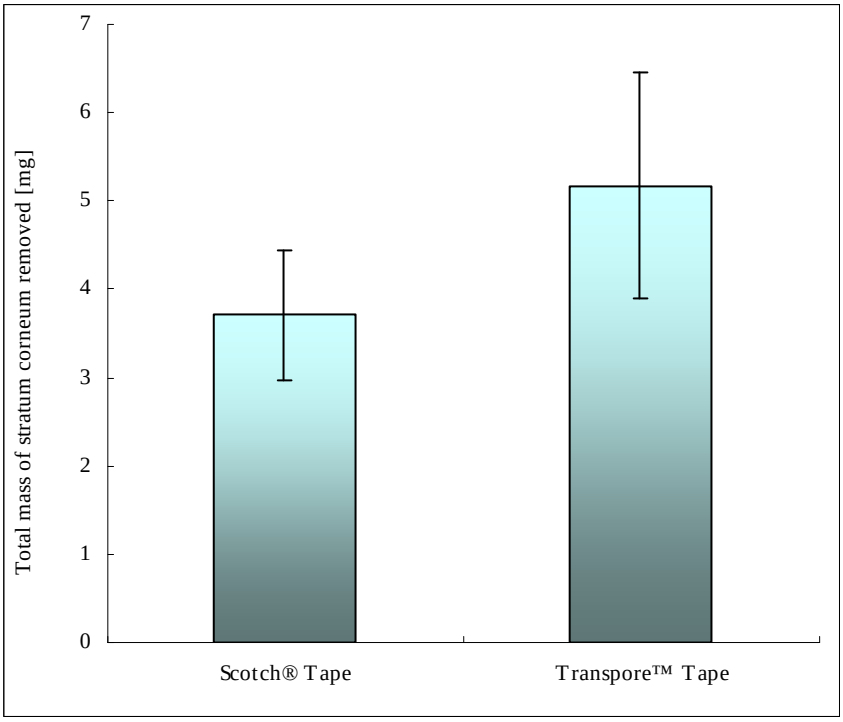

Figure 6.3. The comparison of SC removed from the two different adhesive tapes.

Although the Transpore™ tape appears to be slightly more superior in removing and disrupting SC compared to the Scotch® tape, components in the Transpore™ tape interfered with the HPLC assay for CP (see chapter 5 section 5.3.2.1.4).

Table 6.1 shows the amount of CP removed per tape strip using 15 tape strips of Scotch® tape in total. It is therefore evident from the amount of CP found in the strips from 10 onwards that the use of 15 strips is adequate to remove most of the SC which contains CP and hence no more than 15 strips were considered necessary for use in all the TS studies.

Table 6.1. Mean amount of CP removed using Scotch® Magic tape (n = 3 subjects)

Tape strip no.	Amount of CP ± SD (µg)	%RSD
1	0.26 ± 0.09	35.56
2	0.18 ± 0.06	34.82
3	0.12 ± 0.06	46.00
4	0.10 ± 0.08	72.44
5	0.06 ± 0.05	98.68
6	0.08 ± 0.07	93.02
7	0.05 ± 0.04	80.00
8	0.05 ± 0.05	96.76
9	0.04 ± 0.06	152.43
10	0.03 ± 0.04	137.01
11	0.01 ± 0.03	185.75
12	0.02 ± 0.04	194.05
13	0.01 ± 0.02	264.58
14	0.00 ± 0.01	264.58
15	0.00	0.00

6.3. PILOT STUDY FOR DOSE DURATION DETERMINATION

6.3.1. Experimental

6.3.1.1. Instrumentation

As described in the above section 6.2.1.1

6.3.1.2. Materials

Scotch® Magic™ tape (3M, Pymble, Australia) of 2.4 x 2.4 cm was used in the study.

6.3.1.3. Formulations

A commercially available cream, Dermovate® cream (Glaxo Wellcome, South Africa),

containing 0.05% m/m CP was used as the reference product in this study and in subsequent studies.

6.3.1.4. Subjects

6 healthy human subjects (2 males and 4 females) who met the same criteria as the subjects in section 6.2.1.4 (comparison of tapes) were utilized in this study.

6.3.1.5. Study design

The same tape stripping procedure was used as described in section 6.2.1.5 above.

All formulations were tested on the volar aspect of one of the forearms of each subject. The treated sites were placed closer to the mid-section of the volar aspect of the forearm due to variations in percutaneous absorption which may occur from different areas on the arm [268]. Nine 2 x 2 cm square skin sites on the volar aspect of the forearm were demarcated for the application of Dermovate® cream. One site was reserved as a blank to determine each subject's SC thickness and the remaining eight sites were each assigned a dose duration of 0.5, 1, 2, 4, 6, 8, 10 and 12 hours (Figure 6.4). A dose of ~ 5.5 mg/cm^2 of Dermovate® cream was applied using a previously calibrated Eppendorf pipette (Eppendorf Ag, Hamburg, Germany) (see HSBA chapter 4, section 4.2.1.5) and the cream was spread evenly over the demarcated site using a glass rod. The amount applied was chosen by taking into consideration a previous FDA guidance [244] which suggested the use of a predetermined amount of 5 mg/cm^2. Although different amounts of various formulations have been used by other researchers, no explanation has been given as to how those amounts were predetermined. To facilitate the quantitative measurement of CP, each individual tape strip was analysed separately for CP content, this is in contrast to a number of other studies [174,242,246,248] in which groups of tape strips were combined before analysis

The forearm of each subject was protected after application of the relevant product, using a non-occlusive armguard to prevent the spreading of the applied topical formulation. The armguard was a clear perspex custom-made mould which fitted around the forearm and

included holes for ventilation. The arm guard was raised slightly from the skin using adhesive strips of 1cm thick sponge. Treated and untreated sites were randomized between each subject. The measured doses of cream were applied onto each application site at the same time. At the end of the particular dose duration period the excess formulation was removed followed by tape stripping of the application site. Prior to stripping the treated sites (these sites were not measured for TEWL), cotton wool buds were used to remove the excess formulation from each site and the sites were allowed to equilibrate to room conditions for 5 minutes. The blank site was tape stripped first and the treated sites were then tape stripped at the end of each dose duration.

The blank site was used for the determination of individual SC thickness. TEWL and stripped SC weight were used for the calculation of individual SC thickness [250]. A vapometer was used to take TEWL readings. TEWL measurements were taken prior to the stripping procedure and immediately after each stripping.

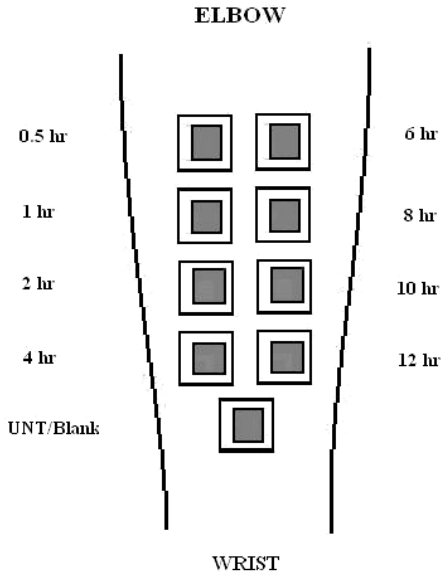

Figure 6.4. Scheme of application of the different dose durations on the volar aspect of the forearm

6.3.1.6. Data and statistical analysis

The first tape strip was analyzed but not included in the data analysis since the first tape strip may still contain some formulation residue remaining on the skin surface and not removed

from the skin by swabbing [174]. The CP content of the first tape strip and cotton swabs were used to perform a mass balance but were not included in the data analysis. It was generally found that when the amounts of CP in the first tape strip plus the amount in the cotton swabs were added to the amount of CP recovered at the end of the stripping process, the total amount of CP was approximately equal to the applied dose (22 μg/site). For example, recovery of CP from Dermovate® cream, Dovate® cream and Dermovate® ointment following application of the respective formulations to 30 subjects resulted in a mean amount of 20.71 μg CP (%RSD = 17.21), 19.21 μg CP (%RSD = 14.81), and 22.09 μg CP (%RSD = 26.00), respectively (See Appendix III for raw data).

SC thickness (H) was determined from the following equation [250]:

$$1/TEWL_x = H\text{-}x / K.D.\Delta C \qquad \text{(equation 6.2)}$$

where $TEWL_x$ is the transepidermal water flux of x μm of SC removed by a tape strip; H is the total SC thickness; x is the SC thickness removed by a tape strip i.e. partial of H; K is the partition coefficient of water from the SC to viable tissue; D is the average apparent diffusivity of water in the SC of thickness, H, and ΔC is the difference of water concentration across the membrane.

The SC stripping data were expressed as amount of CP per normalized fraction of SC (x/H) removed, based on TEWL determinations. The normalized fraction of SC allows for the comparison of data between subjects with varying SC thickness. The area under the curve of a plot of amount of CP versus normalized SC fraction was determined. The AUC found was then plotted against the dose duration for a dose-sigmoidal response fit to determine ED_{50}. Graphpad Prism®, version 4.03, was used to determine the AUCs and ED_{50}.

6.3.2. Results and discussion

Currently, there have been no reports where an appropriate dose duration has been determined for use in a BE assessment of topical preparations using tape stripping. Apart from a single report [248], the majority of TS studies have generally utilized very long dose durations where steady state may have been reached resulting in a lack of discriminatory capability to assess similarity or differences between the bioavailability of the active

ingredients in the formulations studied [174,242,247,269]. Whereas Tsai *et al* [248] considered the use of a predetermined dose duration, the dose duration used was determined from a HSBA study and not from a TS pilot study. We have shown that the appropriate dose duration for the same drug may differ between that required for HSBA and TS investigations, i.e. 0.5 hrs for HSBA and 2 hrs for TS using CP as the investigative drug. Hence, in the absence of appropriate dose duration for a topical drug product being assessed for BE, resulting data may be questionable. It is thus considered extremely important to establish the requisite dose duration required to provide the necessary sensitivity to confirm BE between formulations. This is readily accomplished using the sigmoidal dose-response model [34] where the application exposure time or dose duration (ED_{50}) can be established. The approach followed is in line with the requirements of the HSBA where a pilot study is undertaken in accordance with the FDA guidelines for the assessment of BE of topical corticosteroid formulations [158]. The use of an ED_{50} dose duration has been discussed in the HSBA Chapter 4.

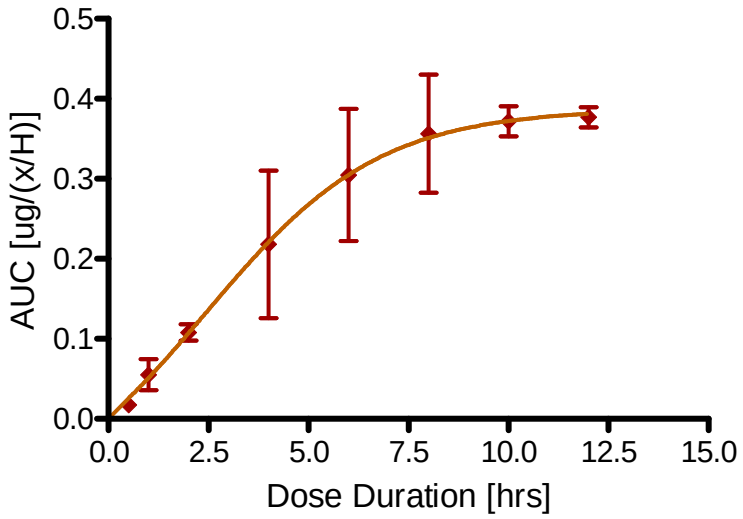

Figure 6.5. A sigmoidal dose-response curve for the determination of ED_{50}

Figure 6.5 illustrates the graph where AUCs from TS data are plotted against dose durations. The ED_{50} was found to be 2.4 hours for CP in Dermovate® cream. Hence, 2 hours was chosen as the dose duration used to compare test and reference products in the subsequent BE studies using the tape stripping technique.

6.4. PILOT STUDY TO [178,270]:

6.4.1. VALIDATE TAPE STRIPPING FOR USE IN BIOEQUIVALENCE ASSESSMENT

6.4.2. DETERMINE NUMBER OF SUBJECTS REQUIRED FOR A PIVOTAL TAPE STRIPPING STUDY

6.4.2.1. Experimental

6.4.2.1.1. Instrumentation

Vide infra section 6.2.1.1.

6.4.2.1.2. Materials

Vide infra section 6.3.1.2.

6.4.2.1.3. Formulations

Dermovate® cream was used as the test and reference product.

6.4.2.1.4. Subjects

7 subjects (2 males and 5 females) who met the previously described inclusion criteria (section 6.2.1.4) were enrolled into the study and written informed consent was obtained.

6.4.2.1.5. Study design

The TS procedure previously described (section 6.2.1.5) was followed, with the exception that one site was reserved as the blank for the determination of individual SC thickness, two sites were used for the test product (Dermovate® cream) and one site was used for the

reference product which was also Dermovate® cream (Figure 6.6.). The sites for test and reference product application were randomized amongst the subjects. A weighed dose of 5.5 mg/cm^2 was applied onto the assigned skin site with a previously calibrated Eppendorf pipette. The preparations on each demarcated skin site were carefully spread using a glass rod. The forearm of each subject was protected after application of the relevant product, using a non-occlusive armguard to prevent the spreading of the applied topical formulation. The preparations were left in contact with the skin for 2 hours before removal. The excess formulation was removed by swabbing the application sites using two dry cotton buds per treated site. The skin sites were allowed to equilibrate thereafter for 5 min prior to tape stripping.

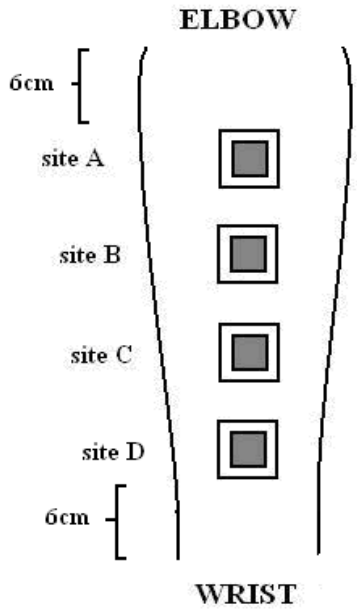

Figure 6.6. Scheme of application on the volar aspect of the forearm

6.4.2.1.6. Data and statistical analysis

AUC was determined as described in section 6.3.1.6. BE was assessed using untransformed AUC data (Locke's method - as described in the FDAs HSBA guidance [158]) as well as log-transformed data to calculate the 90% CI for the AUC_{test}/AUC_{ref} ratios. The two statistical analysis methods were compared, since no method has yet been approved for assessing TS data for BE.

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Inter-individual variability (CV%) of the log-transformed AUC_{test}/AUC_{ref} ratios was determined using the following equation:

$$CV\% = 100*(\sqrt{e^{MSE}}-1) \quad \text{(equation 6.3)}$$

For Locke's method, inter-individual variability of the untransformed AUC_{test}/AUC_{ref} ratios was calculated from the equation:

$$CV\% = 100*\sqrt{(MSE/mean)} \quad \text{(equation 6.4)}$$

Statistical data were processed using SAS® (version 9.1.3).

6.4.3. Results and discussion

6.4.3.1. Validate tape stripping for use in bioequivalence assessment

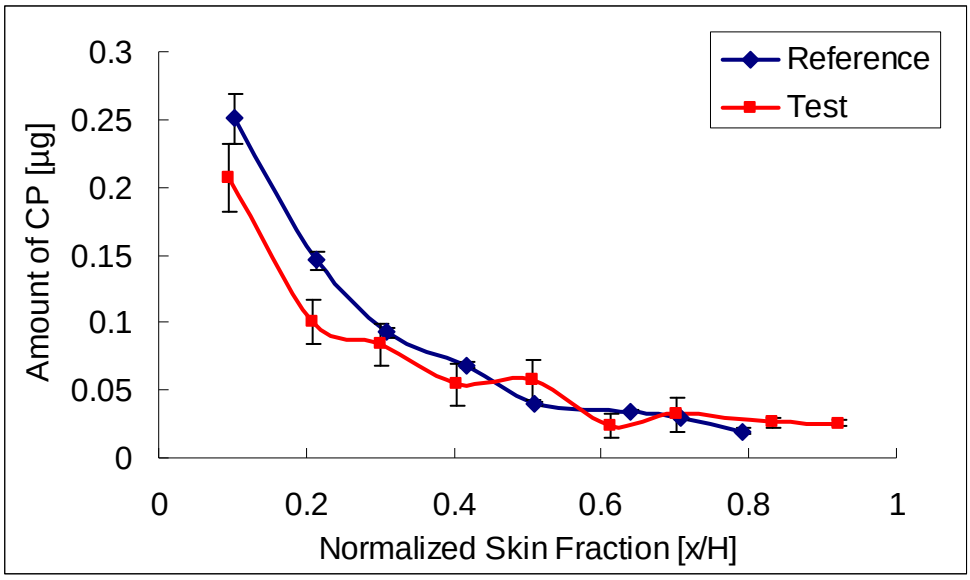


Figure 6.7. Mean TS profiles of the test and reference applications of Dermovate® cream. Penetration of CP from Dermovate® cream into the SC for all volunteers (n = 7)

The permeation profile from the TS study comparing the penetration of CP from Dermovate® cream used as both test and reference product, is shown in Figure 6.7 where the amount of CP found in each tape strip was plotted against the normalized skin fraction. The above tape stripping profiles (Figure 6.7) showed fairly similar permeation profiles of CP into the SC

To achieve BE between an orally administered test and reference product, the USA FDA recommends that the 90% CI should fall within the range of 80-125% [118] using log-transformed data. The specific guidance for topical dermatologic corticosteroids requires the use of untransformed data [158]. The 90% CIs found for the TS pilot study were within the recommended range of 80-125% thus indicating that the test product (Dermovate® cream) is bioequivalent to the reference product (Dermovate® cream). In a previously conducted pivotal HSBA study, using Dermovate® cream as both the test and reference products (*vide infra* section 4.3), BE between these two products was confirmed, as expected. Interestingly, statistical analysis of this pilot TS data provided the same outcomes as that of the pivotal HSBA study (Table 6.2). Hence, TS has been shown to be a feasible tool for application in the BE assessment of topical products containing CP.

In this study, two different statistical methods were used where both log-transformed and untransformed data were analysed using either AUC data and are shown in Table 6.2. The use of Locke's or the 2 one-sided t-test statistical methods achieved very similar BE results. Therefore, the use of either statistical method is applicable for the determination of the 90% CI for BE assessment of topical products using TS.

Table 6.2. BE assessment of identical products (test product – Dermovate® cream, reference product – Dermovate® cream) [178]

	Mean T/R ratio (%)		90% CI (%)	
	Untransformed	Log-transformed	Untransformed	Log-transformed
HSBA				
Chromameter	104.3	-	90.2 – 120.7	-
Visual	102.9	-	97.9 – 109.2	-
TS				
Pilot study	101.8	101.4	88.0-118.3	87.4-117.7

6.4.3.2. Determine the number of subjects required for a pivotal tape stripping study

The inter-individual variability (CV%) values for log-transformed and untransformed

AUC_{test}/AUC_{ref} ratios in the pilot study were found to be 14.4% and 13.9%, respectively, This indicated that approximately 32 subjects would be required to achieve a power of 80 % [271]. On the basis of these data, pivotal TS studies were undertaken using 30 subjects to provide a power of at least 80 %.

6.5. BIOEQUIVALENCE PIVOTAL STUDIES USING TAPE STRIPPING [178,270]

6.5.1. Experimental

6.5.1.1. Instrumentation

Vide infra section 6.2.1.1

6.5.1.2. Materials

Vide infra section 6.3.1.2

6.5.1.3. Formulations

In this TS study, the test products Dovate® cream and Dermovate® ointment were each compared against the reference product Dermovate® cream (*vide infra* appendix IV).

6.5.1.4. Subjects

The subsequent pivotal TS studies were conducted on 30 healthy human volunteers (15 males and 15 females, aged 20-36) with skin phototype of II-VI [272] who also met the same inclusion/exclusion criteria as in the initial TS study. Written informed consent was obtained from each volunteer before the study. The research with human subjects followed the recommended guidelines as set out in the Declaration of Helsinki (1964) and associated amendments. The study protocol (appendix IV) was approved by the Ethical Standards Committee of Rhodes University (Grahamstown, South Africa).

6.5.1.5. Study design

The study design used in this pivotal study was the same as that described in section 6.4.2.1.5 where one site was reserved as the blank for TEWL measurements, two sites for the test products (Dovate® cream and Dermovate® ointment) and one site for the application of the reference product, Dermovate® cream. The blank and application sites were randomized between subjects.

6.5.1.6. Data and statistical analysis

The AUC of a plot of amount of CP versus normalized SC fraction was determined (AUC_{corr}) as previously described in section 6.3.1.6. For comparison purposes, the uncorrected AUC (AUC_{uncorr}) as used by previous researchers [174,246] was also determined for each formulation. This approach using AUC_{uncorr} involves computing the total amount of drug penetrated into the skin per unit area and makes no adjustment for differences in SC thickness.

The BE range was determined using untransformed AUC data (Locke's method - as described in the FDAs HSBA guidance [158]) and also log-transformed data to calculate the 90% CI for the AUC_{test}/AUC_{ref} ratios.

6.5.2. Results and discussion

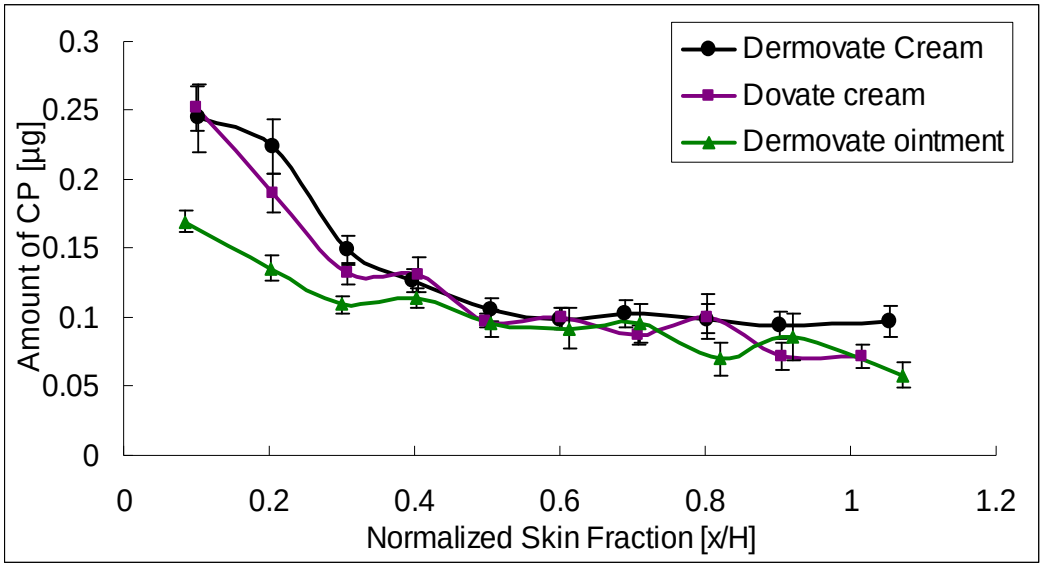


Figure 6.8. Mean TS profiles for the three topical products. Penetration of CP from Dovate® cream (purple line), Dermovate® ointment (green line) and Dermovate® cream (black line) into the SC for all volunteers (n = 30) where the error bars show the SEMs.

The mean permeation profiles for the CP creams and ointment are depicted in Figure 6.8. The AUC_{test}/AUC_{ref} ratios were obtained from the data used to generate these TS profiles and used to determine BE of the test product (Dovate® cream) vs. the reference product (Dermovate® cream), and Dermovate® ointment vs. the same reference product (Dermovate® cream), all containing 0.05% CP. The mean TS profiles of Dovate® and Dermovate® creams from the pivotal study, normalized for skin thickness, were found to be similar.

BE analysis of Dovate® cream vs. Dermovate® cream indicated that the products were bioequivalent where the 90% CIs found were within the acceptance limits of 80-125% (Table 6.3) using both untransformed (Locke's method) and log-transformed (2 one-sided t-test) data. Dermovate® ointment showed bio-inequivalence, as expected, when compared to Dermovate® cream as the reference where the 90% CIs using both untransformed and log-transformed data were outside the acceptance criteria of 80-125 % as shown in Table 6.3.

Table 6.3. Pivotal TS studies of CP creams and ointment products using AUC_{corr} data [178]

	Mean T/R ratio (%)		90 % CI (%)	
Pivotal TS Studies	Untransformed	Log-transformed	Untransformed	Log-transformed
Dovate® Cream vs. Dermovate® Cream	93.8	92.8	84.7-103.6	82.9-103.9
Dermovate® Ointment vs. Dermovate® Cream	66.3	55.2	48.8-82.2	46.1-66.1

Although creams and ointments are not pharmaceutically equivalent and BE assessment between such products is generally not done, a comparison was undertaken between two different types of formulation, Dermovate® ointment (test) and Dermovate® cream (reference), to determine whether the TS method was able to discriminate differences between these formulations. Dermovate® ointment showed a lower permeation of CP into the SC than the creams (Figure 6.8). As expected, Dermovate® ointment was shown to be bio-inequivalent to Dermovate® cream using both log-transformed and untransformed AUC_{corr} data. This provided a useful model to show that TS was able to determine that the ointment was indeed not bioequivalent when compared to the cream (Table 6.3).

The use of AUC_{corr} data takes into account the normalized thickness of the SC into which the drug has penetrated. The thickness of intact *SC* on the forearm varies from 5 – 20 µm in healthy adults. As a result, normalization of the data is necessary to allow for comparison between sites and subjects as demonstrated in a previously published report [250]. TS data corrected for skin thickness using TEWL measurements (AUC_{corr}) and also the uncorrected AUC data (AUC_{uncorr}) were investigated and compared for use in the assessment of the BE between the topical products.

Figure 6.9 depicts both mean AUC_{uncorr} (Figure 6.9a) and AUC_{corr} (Figure 6.9b) histograms for the different formulations. The AUC_{uncorr} ± SEM from Dovate® cream, Dermovate® ointment and Dermovate® Cream were 0.36 ± 0.03 µg/cm^2, 0.37 ± 0.03 µg/cm^2, and 0.39 ±

0.03 µg/cm^2, respectively (Figure 6.9a). The AUC_{corr} ± SEM found for Dovate® cream, Dermovate® ointment and Dermovate® Cream were 0.051 ± 0.09 µg/(x/H), 0.036 ± 0.07 µg/(x/H) and 0.055 ± 0.09 µg/(x/H) respectively (Figure 6.9b), where x/H is the fraction of SC removed. In Figure 6.9, using AUC_{uncorr}, all three products appear similar, whereas using AUC_{corr} data, the creams appear similar but the ointment quite different.

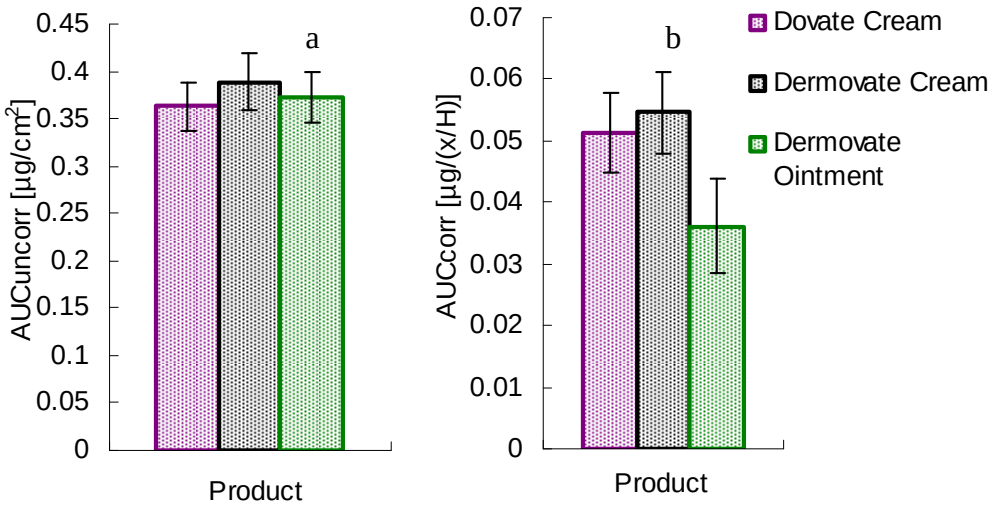


Figure 6.9. A comparison between the use of the AUC_{uncorr} and AUC_{corr} values of the different formulations. Pharmacokinetics obtained from TS, showing the (a) mean AUC_{uncorr} values with SEM and (b) mean AUC_{corr} values with SEM of Dovate® cream (purple), Dermovate® ointment (green) and Dermovate® cream (black) for all subjects (n = 30) [178].

Interestingly, the pivotal studies using AUC_{uncorr} data (log-transformed) resulted in the 90% CIs for both studies falling within the acceptance range of 80-125% for the declaration of BE for Dovate® Cream vs. Dermovate® cream, and for Dermovate® ointment vs. Dermovate® cream. However, when the AUC_{corr} data were used, more realistic results were obtained showing that Dovate® cream was bioequivalent to Dermovate® cream whereas Dermovate® ointment was bio-inequivalent to Dermovate® cream.

Table 6.4. Pivotal TS studies of CP creams and ointment products using AUC_{uncorr} data [178]

	Mean T/R ratio (%)		90% CI (%)	
Pivotal TS Studies	Untransformed	Log-transformed	Untransformed	Log-transformed
Dovate® Cream vs. Dermovate® Cream	93.4	93.6	86.3 – 101.2	86.22.-101.5
Dermovate® Ointment vs. Dermovate® Cream	95.9	96.3	86.8 – 106.1	86.6 – 107.1

When comparing the two statistical analytical methods, log-transformed AUC_{uncorr} values were used and BE was demonstrated for Dovate® cream versus Dermovate® cream. The same result was found for these creams using Locke's method (untransformed data). However, Dermovate® ointment (test) was surprisingly shown to be bioequivalent to Dermovate® cream (reference) using both statistical methods (Table 6.4) when AUC_{uncorr} data was used. It should be noted, that previous studies using TS for BE assessment only used log-transformed AUC_{uncorr} data [174,246]. This clearly demonstrates the value of using AUC_{corr} values to enhance the discriminatory capability using the TS method. Additionally, the use of either of the two statistical analytical methods are applicable in demonstrating BE between products.

6.6. CONCLUSIONS

To date, no statistical methods have been officially recommended for the BE assessment of topical products other than for topical corticosteroid products using the HSBA [158]. In light of this, 2 different approaches to determine the CIs for the AUC_{test}/AUC_{ref} were used, *viz*: the classical approach using the 2 one-sided t-test [273] with log-transformed data and Locke's method [209] which uses untransformed data. Application of either method provided similar results (Tables 6.2, 6.3 and 6.4).

The TS method was successfully used to assess formulations using either log-transformed or

untransformed AUC_{corr} data. The use of AUC_{corr} data by normalization of the skin thickness appears to provide better discriminatory power and should be considered when using the TS method for BE assessment. Whilst the TS method has clearly been shown to be a viable alternative approach for BE assessment of CP topical products, it is important to optimize the TS method to control sources of variability such as:

- the use of an appropriate dose duration
- careful removal of residual application prior to skin stripping
- controlled systematic stripping orientation of each site
- normalization of individual skin thickness
- careful control of the dose and application of doses to demarcated skin sites
- avoidance of areas on the volar aspect of the forearm where increased variability in uptake may exist such as areas near the wrist and elbows
- control of temperature and humidity of the environment where the study is being conducted.

The results of these studies illustrate the potential for TS as an alternative method for the BE assessment of topical products not intended to be absorbed into the systemic circulation.

7. CHAPTER 7

7.1. MICRODIALYSIS

The determination of *in vivo* BA of topical drug products has always been a challenge. As a result, dermal MD has been developed as one of the several promising *in vivo* methods which could be used to assess cutaneous drug penetration. It facilitates continuous sampling of the unbound drug fraction in the extracellular fluid in the skin after topical or systemic delivery [274]. This technique first emerged from the neuroscience field [275] where it was developed for neurotransmitter sampling in the rat brain. Subsequently, it has been used to investigate endogenous substances in the extracellular fluid in the skin [276,277], and has progressed for use to study absorption of exogenous substances as well [278,279].

The application of dermal MD to study drug penetration through the skin is based on the use of a micro-fibre consisting of a thin semi-permeable membrane which can be introduced into the skin and which will imitate the function of a capillary blood vessel as shown in Figure 7.1 [280,281,281]. The fibre only allows for the passive diffusion of certain molecules across the membrane, based on their molecular size. Different MD membranes are available with a range of molecular weight cut-off values. MD membranes typically used for the assessment of unbound drug fraction (i.e. free drug) are in the range of 6 – 100 kDa [282], whereas the assessment of larger molecules such as proteins require membrane molecular weight cut-off values of 100 – 3000 kDa [283]. The dialysis membrane is continuously perfused with a suitable physiological solution (perfusate) throughout the experiment. A concentration gradient is created between the extracellular fluid in the skin and the perfusate which allows for passive diffusion of endogenous and exogenous substances across the membrane and into the perfusion medium (microdialysate) [282,284]. Samples of the microdialysate can then be collected at specific time intervals and analyzed for the solutes of interest.

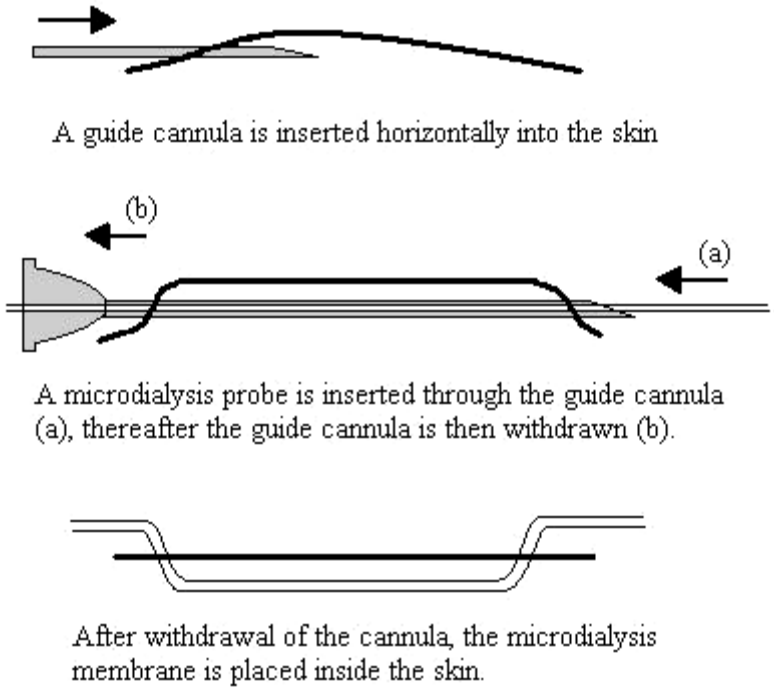


Figure 7.1. Insertion of the MD probe in the skin.

7.1.1. Recovery and loss

Recovery of a substance partitioning between the perfusate and the surrounding medium of the MD membrane depends on the composition of the perfusate and the hydrophilic/lipophilic nature of the surrounding medium. The fraction of drug collected in the dialysate relative to the surrounding medium of unbound drug is termed "relative recovery" (RR) [280] and is calculated using the following equation:

$$\text{Relative recovery (RR)} = \frac{C_{dialysate} - C_{perfusate}}{C_{medium/tissue} - C_{perfusate}} \qquad \text{(equation 7.1)}$$

Initially, the perfusion medium does not contain any drug substances (investigative drug or marker, etc.) before being pumped through the MD probe in the skin. The initial concentration of the drug in the perfusate is therefore zero, i.e. the value of $C_{perfusate}$ in Equation 7.1 is zero and the equation can be simplified to:

$$\text{Relative recovery (RR)} = \frac{C_{dialysate}}{C_{medium/tissue}}$$

(equation 7.2)

The determination of RR is necessary in order to estimate the true unbound extracellular concentration of any drug substance. A low RR of drug could lead to unacceptably long sampling periods or the need of a very sensitive analytical assay to quantitatively measure very low drug concentrations. MD can be used for collecting a substance in the dialysate or to deliver it into the surrounding fluid. The latter MD setup is known as retrodialysis. RR can be determined *in vitro* by both MD and retrodialysis to assess the % of drug lost or gained through the membrane [282].

RR can be influenced by the following factors [280,282,285,286]:

- The physicochemical characteristics of the compound
- MD instrumentation such as the type of probes, probe material, probe surface area, perfusate medium, perfusate flow rate
- Sampling in biological tissue *in vivo* may reduce or increase the transport of drug substances across the MD membrane depending on the affinity of the substances for the perfusate or for extracellular fluid.
- Effects of blood flow and metabolism in the tissue, as well as the phenomenon of tissue dependent recovery as explained by Ståhle [287] can alter the *in vivo* recovery of the substance.
- Effect of diffusion and temperature

Most of these factors must be optimized to yield the best possible RR *in vitro* prior to commencing a MD experiment.

However, a main variable associated with *in vivo* MD of the skin that can not be altered is the partition coefficient between tissues and perfusate which is influenced by the physicochemical properties of the drug

These properties can influence inter- and intra-individual results.

7.1.2. Invasiveness of cutaneous microdialysis

Insertion trauma induced by the introduction of a needle into the skin has been studied and found to be variable between species [288,289], type of probe and guide cannula [280]. The trauma induced by the implantation of the MD fibre can affect drug recovery and elimination. This trauma can cause an increase in blood flow and erythema of the skin at the site of needle insertion, as well as histamine release [289-291]. Hence, it is essential for the insertion trauma and cutaneous blood flow to stabilize before commencing the MD study. Anderson *et al*. found that the increased blood flow stabilized around 60 min after insertion of a probe in humans [290] and histamine levels were normalized after 40 min [277]. The use of a local anaesthetic has been shown to reduce the intensity of vascular effects but does not alter the time needed for the vascular reaction to return to normal as shown by Groth and Serup [289]. Hodges *et al* [292] investigated the effect of MD needle trauma on cutaneous vascular responses in humans with the use of ice or local anaesthesia and has also shown that the use of ice/local anaesthesia did not alter the equilibration time.

7.1.3. Drug analysis

The quantitative analysis of drugs in MD samples has always been a challenge. The small volume samples collected in MD, often accompanied with low concentration of the drug substances of interest, presents a challenge. It is necessary to use a highly sensitive detection method with optimal separation technique for these studies. The most commonly used separation techniques include liquid chromatography and capillary electrophoresis. Ultraviolet, fluorescence, electrochemical and mass spectrometric detection accompanied by HPLC have often been utilized to improve the sensitivity and selectivity of the analytical method [282,293].

7.1.4. Perfusate composition

The perfusate medium should resemble the tissue/solution surrounding the MD probe as much as possible. Hence, the most commonly used perfusate media for MD studies is saline,

phosphate buffers and Ringer's solution [246,294-296]. These perfusates are mostly suitable for the study of hydrophilic compounds. However, lipophilic compounds bind to the MD membrane material when using hydrophilic perfusates due to the low affinity of these compounds for the aqueous perfusion medium [293,297,298]. This in turn leads to a low RR of these compounds from the dialysate. The use of lipid emulsions [299-301], or the inclusion of albumin [299,302] or cyclodextrins [301,303,304] into the perfusate to prevent adhesion of these compounds to the membrane and other MD components have been used to improve RR.

7.1.5. Probe depth

Probes are usually inserted over a narrow range of depth in the dermis (0.6 – 1.0 mm) when conducting a dermal MD experiment. At the end of the MD experiment, probe depth can be measured by a non-invasive technique such as ultra-sound scanning using an instrument, e.g., the Dermascan C® (Cortex, Hadsund, Denmark) as shown in Figure 7.2. It is recommended that skin thickness and probe depth be measured in three separate scans along the length of the probe *in situ* (i.e. the middle of the probe, and near the entrance and exit points of the probe) [281].

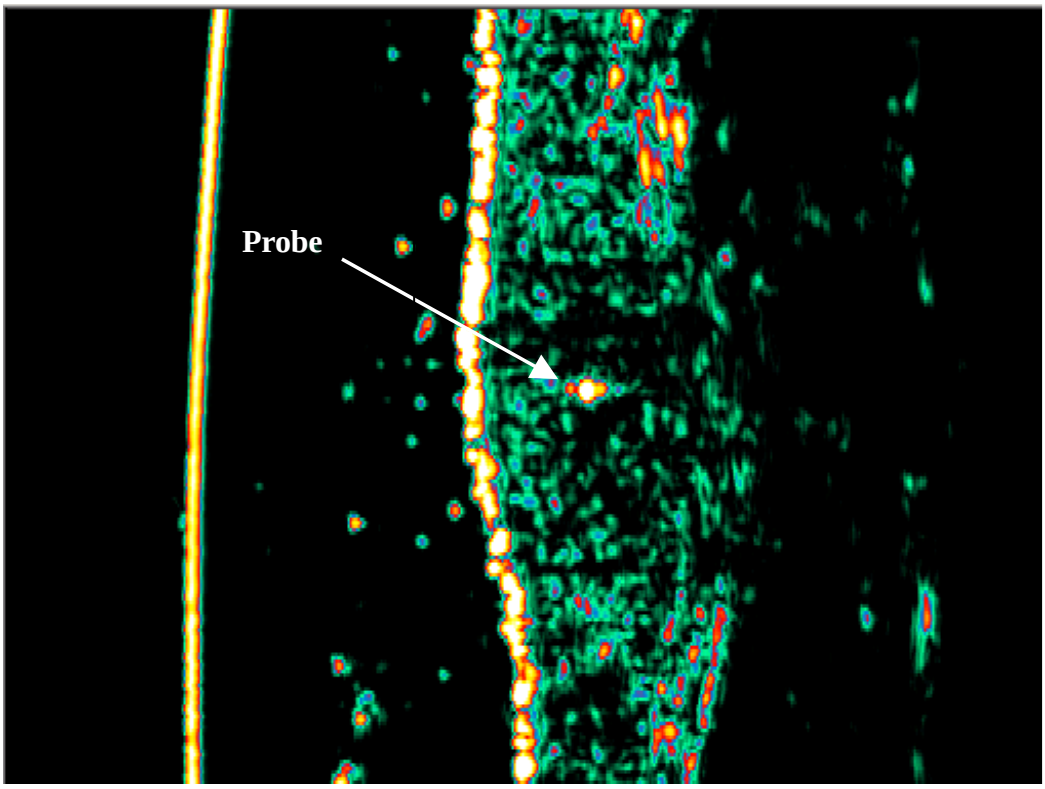


Figure 7.2. Cross-sectional scan of the skin showing the position of the MD probe

7.1.6. Bioequivalence/bioavailability studies

The MD technique can be used to assess the permeation of topically applied drugs across both healthy and diseased or perturbed skin [274,305,306]. MD is therefore potentially a useful technique to address the issue of bioequivalence of topically applied formulations. Although some researchers have investigated the usefulness of MD as a technique to assess the BE of topical formulations [246,294], at present there are no "officially" recognized/approved MD methods for BE assessment.

In this chapter, dermal MD was investigated as a possible tool to assess the *in vivo* BA of a topical formulation containing a corticosteroid, CP, and to investigate this method for use in BE studies.

7.2. *IN VITRO* MICRODIALYSIS

7.2.1. Relative recovery study

7.2.1.1. Experimental

7.2.1.1.1. Instrumentation

A MD syringe pump system (CMA, model 400, Chromatography Sciences Company, Quebec, Canada) was used to perfuse vehicle from 2.5 ml Exmire microsyringes (Aurora Borealis Control BV, Schoonebeek, The Netherlands). Blue tubing adapters (CMA MD AB, Stockholm, Sweden) were used to connect the syringes to the MD probes.

A custom-designed perspex cell was developed in–house for use in the RR studies. The cell (Figure 7.3) comprised of 3 components, i.e. 2 identical inlet and outlet blocks and the main block, which is situated in the middle of the cell, and accommodates an enclosed circular chamber into which the surrounding medium was filled. Both inlet and outlet blocks had a small circular opening in the centre through which the linear MD probe was introduced. The inlet and outlet tubes were connected to the main chamber to permit filling and removal of the surrounding medium. Four holes were drilled into each block to accommodate the 4 bolts which secure the inlet and outlet blocks to the main cell. Leakages were prevented from the chamber by positioning rubber o-rings at each end of the circular chamber. The cell was placed on top of a shaker (The Chemical Rubber Company, Cleveland, Ohio, USA) during the relevant experiments.

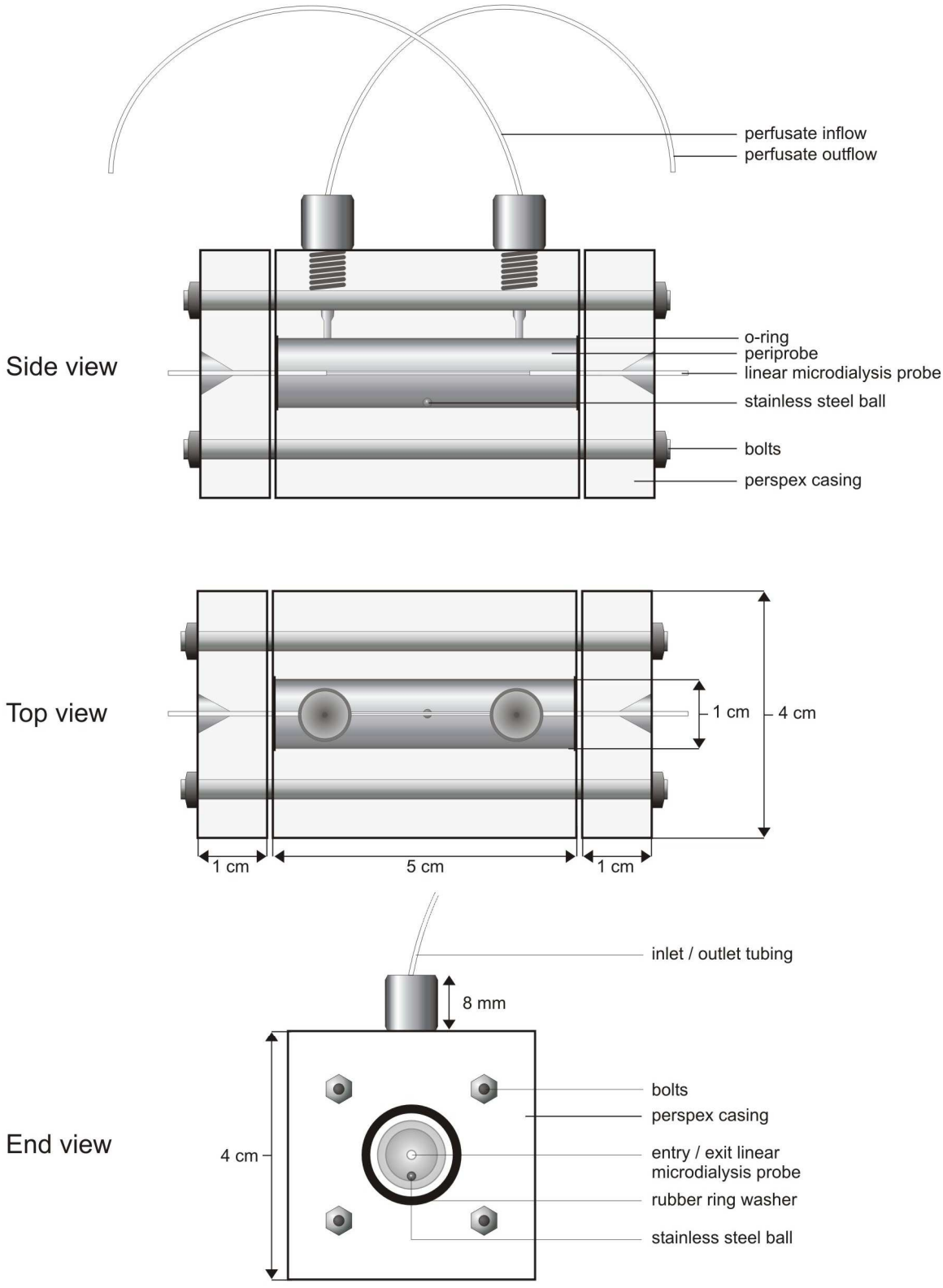


Figure 7.3. Cell for *in vitro* MD studies [307]

7.2.1.1.2. Reagents, chemicals and materials

CP was purchased from Symbiotec Pharmalab P.V.T. Ltd. (Pigdamber, Maharastra, India). Saline solution was prepared by dissolving 9 g of NaCl (Rochelle Chemicals, Johannesburg, South Africa) in one litre of HPLC-grade water (filtered by Milli-Q® system, Millipore Co., Bedford, MA, USA) using a 1 L volumetric flask. The NaCl used to make the saline was of analytical reagent grade.

One litre of sterile lipid emulsion, IL, consisting of purified soybean oil (200 g), purified egg phospholipids (12 g) glycerol anhydrous (22 g), sodium hydroxide (used to adjust the pH of the emulsion to 8) and water for injection was purchased from Fresenius Kabi (Midrand, South Africa) and used before the expiration date.

Linear MD probes (Figure 7.4) were manufactured in-house no more than 24 hours prior to the study. The MD probes were all composed of portex tubing (inner diameter 0.5 mm and outer diameter 0.63 mm, Scientific Laboratory Suppliers Ltd, UK, Nottingham), with a stainless steel guidewire included (outer diameter 100 µm, Metalann, Belgium, Meslin-I-'Eveque) and a dialysis membrane (Haemophan fibre dialysis cartridges, Al wall GFS plus 12, Gambro, Leuven, Belgium). The membrane's internal diameter was 210µm with a molecular weight cut-off value of 5 kDa. The components of the MD probes were assembled with the use of cyanoacrylate glue (Bostik® Ltd, Swindon, England).

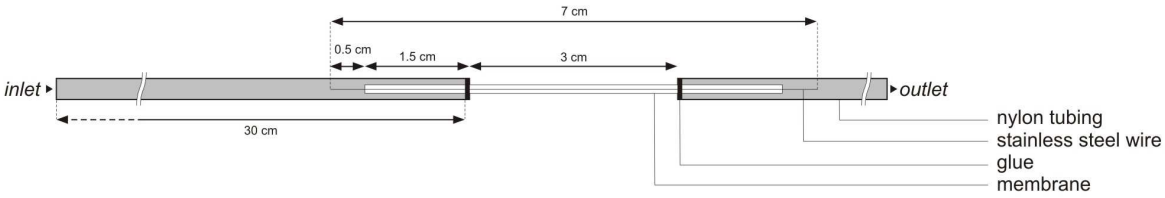


Figure 7.4. A linear MD probe [307]

The MD probes were connected to the MD glass microsyringes with blue tubing adapters and a CMA 400 precision MD pump was used to deliver the perfusate through the MD probes.

7.2.1.1.3. Study design

Lipophilic drugs tend to adhere to various components of a MD system [293,297,298], hence a retrodialysis experiment was done to determine possible CP interactions with components. Standard CP solutions of 1 μg/ml in IL and 3 μg/ml in saline were perfused through the MD system with air as the surrounding medium (n = 4).

Standard CP solutions of 3 μg/ml, 1.5 μg/ml, 0.75μg/ml and 0.5μg/ml in saline were used as the surrounding medium in the perspex cell, which was mounted on a shaker. IL was used as the perfusate (n=4).

A similar experiment was also carried out where the standard solutions were made up in IL and used to replace the saline solution as the surrounding medium in the cell at concentrations of 1, 30, 50 and 80 μg/ml.

The perfusate (IL) was pumped at a flow rate of 1.0 μL/min. Dialysate samples were collected every hour over a period of 5 hours. The samples were collected, extracted from the IL and analysed using a validated HPLC system as previously described in section 5.4. When saline was used as the perfusate, the dialysate samples were analysed by direct injection into the HPLC system without extraction. All the experiments were performed at room temperature.

7.2.1.1.4. Data and statistical analysis

RR of CP was analysed and the mean, standard deviation and CV% were determined.

7.2.1.2. Results and discussion

The retrodialysis experiment where a saline solution containing CP (3 μg/ml) was perfused through the MD probe with air as the surrounding medium resulted in no drug being found in the dialysate over a period of 5 hours (n = 4). This indicated that CP, an extremely lipophilic

drug, adhered aggressively to the MD probe as shown by others [293,297,298]. To overcome this adherence by CP to the MD probe, IL with CP was investigated as an alternative medium and perfused through the MD probe with air as the surrounding medium. In this case 102.6 % of the drug was recovered in the dialysates (%RSD = 2.9; n=4). This clearly indicated the preference of CP for IL compared to its affinity for either the membrane or other system components such as the tubing, etc.

A MD system similar to Kurosaki *et al* [300] and Carneheim [299] was constructed where IL was the perfusate and the surrounding medium was a solution of CP in saline. The RR was found to be in the range of 210.66 – 215.05 % (Table 7.1). The RR is very high because the drug has a much higher affinity for the IL emulsion (perfusate) than for the aqueous saline solution. This indicated that IL would be a suitable perfusate for use in *in vivo* MD studies.

Table 7.1 RR of CP using i*n vitro* MD (perfusate = IL, surrounding medium = saline solution of CP)

Concentrations (μg/ml)	**RR (%)**	**CV (%)**
0.5	209.23	5.96
0.75	215.06	1.38
1.5	214.42	3.66
3.0	210.66	6.73

CP recovery using IL (containing 1 μg/ml of CP) as the surrounding medium and perfusing with IL (drug-free) did not show the presence of any CP in the dialysate. This was presumably due to a relatively low concentration of drug being transported across the membrane during the sampling intervals. On the other hand, the average RR of CP using 30, 50 and 80 μg/ml was ~ 2.05 %, whereas the *in vitro* retrodialysis study recovered ~ 97.27 % of CP (as shown in Table 7.2). Mass balance calculations resulted in 99.32 % indicating the suitable solubility of CP in IL and the feasibility of using that medium as a vehicle for MD studies. The low recovery from the MD experiments may be due to a slow release of CP from the components of IL. The results of this study are similar to those obtained by Rojas *et al* [308] when investigating the RR of triamcinolone acetonide using albumin solution as the perfusate. The low RR of triamcinolone was attributed to the drug being highly bound to

protein in the perfusate.

Table 7.2. RR of CP using *in vitro* retrodialysis and MD where IL was the vehicle for both the perfusing and surrounding medium.

Concentrations (µg/ml)	Microdialysis (%)	Retrodialysis (%)
30	2.18	96.80
50	2.07	97.46
80	1.90	97.55

7.2.2. Release of CP from commercial formulations

Whereas dissolution testing is a standard technique to assess drug release from solid oral dosage forms, various methods have been reported for the assessment of the release of drugs from semi-solid dosage forms such as creams, ointments, gels, etc. [309-311]. However, with the exception of Chamboko [230] and Mandimika [312], the use of MD to evaluate drug release from these formulations has not previously been published.

The use of *in vitro* MD to assess the release of CP from various commercial formulations was investigated in this research project.

7.2.2.1. Experimental

7.2.2.1.1. Instrumentation

Vide infra section 7.2.1.1.2. In these experiments the perspex cell and shaker were replaced with a hollow glass cylinder surrounding the MD membrane. The cylinder had a length of 6 cm and an internal diameter of 4 mm (as shown in Figure 7.5). The inner opening of the cylinder was filled with the formulation. This alternative arrangement facilitated the introduction and positioning of the formulation which was not possible using the perspex cell due to the viscosity of the CP formulations and the relatively narrow opening in the perspex cell.

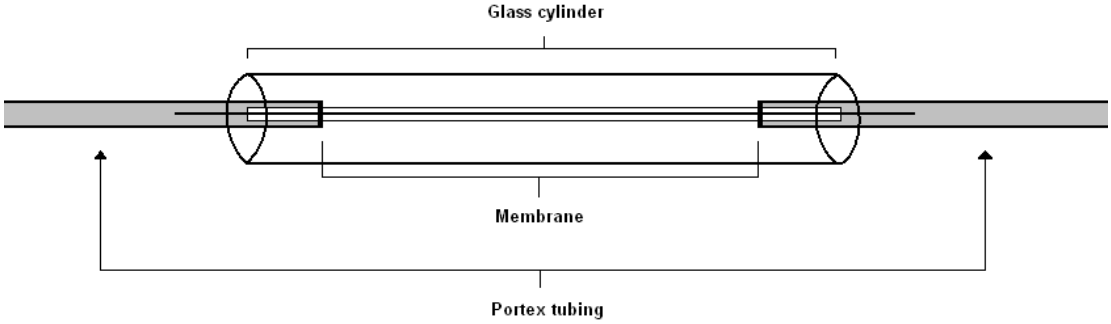


Figure 7.5. Setup of MD probe in the glass cylinder.

7.2.2.1.2. Reagents, chemicals and materials

IL 20%, a lipid emulsion, was purchased from Fresenius Kabi (Midrand, South Africa) and used as the perfusate. The MD probes were constructed in-house as previously mentioned above in section 7.2.1.1.2

7.2.2.1.3. Formulations

Dermovate® cream (Glaxo Wellcome, Midrand, South Africa), Dovate® cream (Aspen Pharmacare, Port Elizabeth, South Africa) and Dermovate® ointment (Sekpharma, Johannesburg, South Africa,) each containing 0.05% CP were purchased from a local pharmacy (Wallaces Pharmacy, Grahamstown, South Africa). *In vitro* release of CP from these three formulations was assessed using MD.

7.2.2.1.4. Study design`

The relevant commercial formulation (~0.45g of cream or ointment) was placed into the previously described glass cylinders used as a holding cell. The dosage forms comprised the surrounding medium around the membrane of the MD probe and IL was used as the perfusate. The membrane was thus in contact with the relevant formulation inside the “cell”.

The evaluation of the release of CP from each commercial formulation was assessed using four replicates (n = 4).

The perfusate was pumped at a flow rate of 1.0 μL/min. Dialysate samples were collected hourly over a period of 5 hours. The samples were collected and analysed using a validated HPLC system as described in Chapter 5 section 5.4. All experiments were carried out at room temperature (22 ± 1 °C)

7.2.2.1.5. Methods used to compare release profile data

7.2.2.1.5.1. Model-dependent methods

In vitro drug release/dissolution from a pharmaceutical dosage form has been recognized as an important element in drug development. When a new dosage form is developed, it is important to ensure that drug dissolution/release from the dosage form is appropriate for its intended therapeutic use. As a result, drug release from a dosage form can be described by various kinetic models which allow for comparison between dissolution profiles between products. To investigate the mechanism of drug release from the commercially available topical formulations, the release data were analyzed using zero-order kinetic, first-order kinetic, and Higuchi models [313]. The various mathematical models and equations that describe the release profiles in this study are summarized in Table 7.3.

Table 7.3. Mathematical representation of models used to describe the release profiles from the topical formulations.

Model	**Equation**
Zero-order	$Q_t = k_0 t$
First-order	$\ln Q_t = \ln Q_0 - k_1 t$
Higuchi	$Q_t = k_H t^{1/2}$

The abovementioned kinetic models were used to assess the release of CP from the various dosage forms. The best fit to a relevant equation was identified by evaluating the coefficient of determination (R^2). The closer the R^2 value is to 1, the better the fit.

7.2.2.1.5.2. Statistical methods - ANOVA

Statistical methods take into account both the variability and correlation structure of the release profile during comparison of products.

The one-way analysis of variance (ANOVA) compares the mean release data at each time point. This method takes into account the variability in the profile data and ignores the correlation between the time points. It is a risk which may incorrectly conclude that the mean profiles are different (where the nominal 5% level for comparison can be higher). This problem can be resolved to some extent by employing a Bonferroni-type adjustment to ensure that the over-all significance level of comparison remains at the 5% level. Comparison may only be statistically significant at some time points and it is difficult to conclude whether the mean profiles are actually different. Even though the use of one-way ANOVA is straightforward, it is also rather inefficient, tedious to perform and ambiguous in interpretation [314]. The one-way ANOVA method with Tukey's *post hoc* test was used to assess the differences in *in vitro* drug release from the formulations even though it is not recommended due to the limitations mentioned above. Graphpad® prism version 4 was used to obtain the ANOVA results.

The alternative to the one-way ANOVA method is the two-way ANOVA model which involves the use of formulation and time data as class variables. However, the assumption of independence in the ANOVA model is violated because issues with multiple comparisons and correlation between dissolution time points arise. Furthermore, the addition of the interaction effect between time and formulation variables fitted into the ANOVA model makes it difficult to interpret the outcome [314].

7.2.2.1.5.3. Mathematical comparison methods

To determine similarity and dissimilarity between the reference and test formulations, the profile data were analysed using the difference factor (f_1) and similarity factor (f_2) described by Moore and Flanner [315]. These factors are usually used in the case of drug dissolution

studies.

The difference factor (f_1) measures the percent error between the curves over all time points using the following equation:

$$f_1 = \{[\Sigma_{t=1}^{n} |R_t - T_t|]/[\Sigma_{t=1}^{n} R_t]\} \cdot 100 \qquad \text{(equation 7.3)}$$

where:

n = number of sampling points

R_t = percentage dissolved of the reference product at time t

T_t = percentage dissolved of the test product at time t

In order to show similarity in the dissolution profiles, the f_1 values should be close to 0 and lower than 15 (0 - 15). f_1 is zero when the test and reference drug release profiles are identical and increase proportionally [313].

The similarity factor (f_2) can be obtained from the following equation:

$$f_2 = 50 \cdot \log \{[1 + (1/n) \Sigma_{t=1}^{n} w_t(R_t - T_t)^2]^{-0.5} \cdot 100\} \qquad \text{(equation 7.4)}$$

where w_t is an optional weight factor. The similarity factor value range is 0-100 and when the value of f_2 is > 50, the test and reference profiles are considered to be similar whereas at f_2 values < 50, dissimilarity between the profiles increases. This method is only applicable to dissolution profiles comparing three or more time points. However, not more than one measurement should be used after 85 % release of the active ingredient from both products [313].

The similarity factor (f_2) has been adopted by the FDA [316-319] and EMEA [320] as a criterion for the assessment of the similarity between two *in vitro* dissolution profiles of immediate release solid oral dosage forms. The equation differs slightly from the one proposed by Moore and Flanner in the weight factor as shown below:

$$f_2 = 50 \cdot \log \{[1 + (1/n) \Sigma_{t=1}^{n} (R_t - T_t)^2]^{-0.5} \cdot 100\} \qquad \text{(equation 7.5)}$$

Equations 7.3 and 7.5 were utilized to determine the similarity in drug release profiles of the

three topical formulations.

7.2.2.2. Results and discussion

Dissolution testing is extremely useful for the assessment of drug release from a dosage form and serves as a prerequisite for understanding the BA of the drug *in vivo*. It is also used as a quality assurance tool during regular production and to meet regulatory requirements before marketing the product.[321].

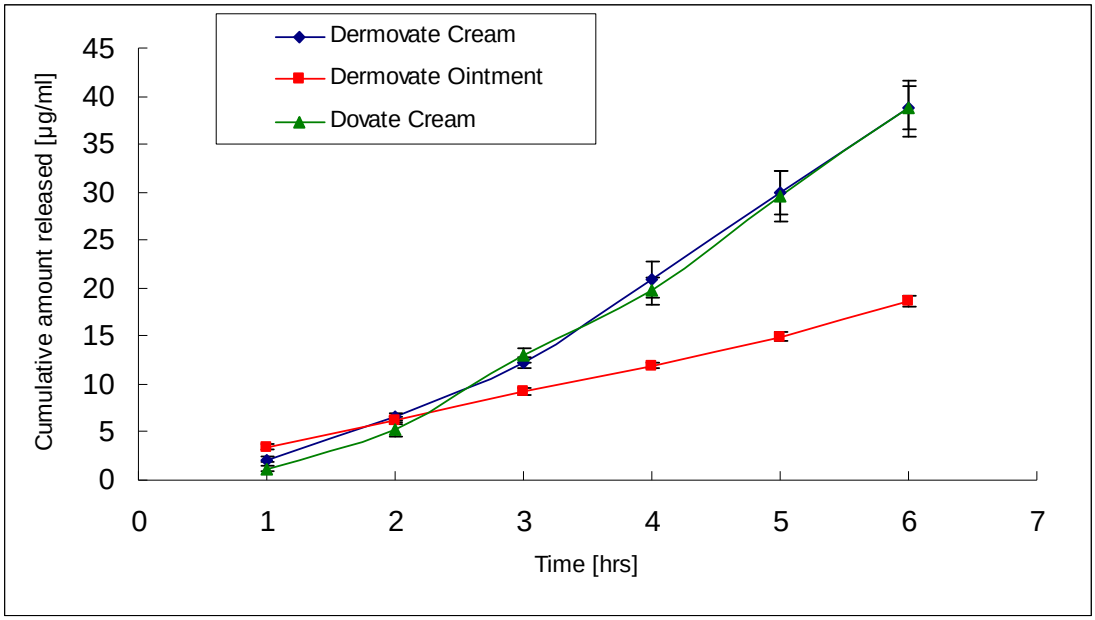


Figure 7.6. Drug release profiles of the three commercially available topical formulations each containing 0.05% of CP (n=4 for each profile).

The release of CP from the three commercially available topical products (Dermovate® cream, Dermovate® ointment and Dovate® cream) are depicted in Figure 7.6. Visual comparison of the profiles show that Dovate® cream has a similar release pattern as the innovator product Dermovate® cream, whereas the ointment formulation appears to release CP quite differently to the cream formulations.

ANOVA results comparing the different formulations are shown in Table 7.4. No differences were found in drug release at the different time points between Dovate® cream and

Dermovate® cream (p-value > 0 .05). Some differences were found in the release pattern at time points 4, 5 and 6 hours when comparing Dermovate® ointment to Dermovate® cream (p-value < 0.05). Dermovate® ointment vs. Dovate® cream showed differences at time 3, 4, 5 and 6 hours. The results indicate that the release of CP from Dermovate® cream is equivalent to Dovate® cream, and confirm that the release of CP from the cream formulations are different to the ointment formulation after 3 hours.

Table 7.4. One-way ANOVA results comparing the release profiles of the three formulations (T – test product, R – reference product).

Comparison between formulations	**Sampling Time**	**Mean Difference**	**P-value**	**95% Confidence Interval**		**Summary**
				Lower limit	**Upper limit**	
Dovate®	1	-0.08855	P > 0.05	-4.776	3.005	Not significant
cream (T)	2	-1.203	P > 0.05	-4.683	2.277	Not significant
vs.	3	0.8150	P > 0.05	-2.665	4.295	Not significant
Dermovate®	4	-0.9728	P > 0.05	-4.453	2.507	Not significant
cream (R)	5	0.2995	P > 0.05	-3.180	3.779	Not significant
	6	0.6908	P > 0.05	-2.789	4.171	Not significant
Dermovate®	1	-1.347	P > 0.05	-4.827	2.133	Not significant
ointment (T)	2	0.3770	P > 0.05	-3.103	3.857	Not significant
vs.	3	3.056	P > 0.05	-0.4240	6.536	Not significant
Dermovate®	4	8.967	P < 0.001	5.487	12.45	Significant
cream (R)	5	14.97	P < 0.001	11.50	18.45	Significant
	6	20.13	P < 0.001	16.65	23.61	Significant
Dermovate®	1	-2.232	P > 0.05	-5.712	1.247	Not significant
ointment (T)	2	-0.826	P > 0.05	-4.306	2.654	Not significant
vs.	3	3.871	P < 0.05	0.3901	7.350	Significant
Dovate®	4	7.994	P< 0.001	4.514	11.47	Significant
cream (R)	5	15.27	P < 0.001	11.79	18.75	Significant
	6	20.82	P < 0.001	17.34	24.30	Significant

Table 7.5 compares the similarities and release profile data using the various mathematical models i.e. a difference factor (f_1) and a similarity factor (f_2). CP release from Dovate® cream

is similar to Dermovate® cream where the f_1 value is near zero (4.2) and the f_2 value tending towards 100 (93.6).When comparing the cream formulation to the ointment formulation, (B) and (C), differences in CP release between the two formulations are shown in Table 7.5. It is seen that the f_1 values indicated a difference between the formulations (f_1 >15), whereas the f_2 values showed dissimilarity in their release profiles (f_2 < 50). The use of f_2 values are recommended since they have been shown to be more sensitive for profile dissimilarity compared to differences shown by f_1 values [322]. Hence, the f_2 results are more reliable indicators for drug release profile comparison.

Table 7.5. Comparison of the release profiles pertaining to the three commercially available formulations using difference (f_1) and similarity factors (f_2) (T = test product, R= reference product).

	Comparison between release profiles	**f_1 value**	**f_2 value**
A	Dovate® cream (T) vs. Dermovate® cream (R)	4.2	93.6
B	Dermovate® ointment (T) vs. Dermovate® cream (R)	44.2	47.9
C	Dermovate® ointment (T) vs. Dovate® cream (R)	46.0	48.3

The release of CP from the cream and ointment bases were assessed by fitting the release data with a regression line pertaining to zero-order, first-order, and the Higuchi drug release model. As mentioned earlier, the closer the R^2 value is to 1 the better the data fits that particular model. Table 7.6 depicts the drug release data of all three CP formulations and suggests that CP follows zero order drug release kinetics from each of the formulations.

Table 7.6. Comparison of the release kinetics of CP using the various mathematical models.

	R^2-values		
Formulation	**Zero-order**	**First-order**	**Higuchi**
Dermovate® Cream	0.984	0.937	0.938
Dermovate® Ointment	0.997	0.958	0.973
Dovate® Cream	0.985	0.901	0.941

The release rates of CP from the cream base formulations were 1.12 μg/cm^2.hr and 1.14 μg/cm^2.hr for Dermovate® cream and Dovate® cream, respectively. The release of CP from the ointment base was considerably slower at 0.45 μg/cm^2.hr.

The comparison between the cream formulations shows that CP release from these two commercially available creams is very similar. ANOVA results, difference factors (f_1) and the similarity factors (f_2) between the cream formulations all confirm the similarity between the products. The aforementioned comparison parameters also showed significant differences in the release of CP from the cream formulations when compared to the ointment formulation.

Furthermore, comparison of the release of CP from the three commercial formulations using i*n vitro* MD correlated well with the TS study. This indicates that an *in-vitro-in-vivo* correlation (IVIVC) may be possible using i*n vitro* MD to assess release rates from topical formulations and to correlate the results with *in vivo* BA data from TS studies. *In vitro* MD may therefore be a useful tool to assess the release of CP from different formulation bases.

7.3. *IN VIVO* MICRODIALYSIS

7.3.1. Experimental

7.3.1.1. Equipment

The same MD equipment as previously described was used (section 7.2.1.1.1).

PVC custom-made chambers Figure 7.7 were used (3.5 x 2.3 x 2.5 cm) as reservoirs to apply liquid preparations containing CP to the skin.

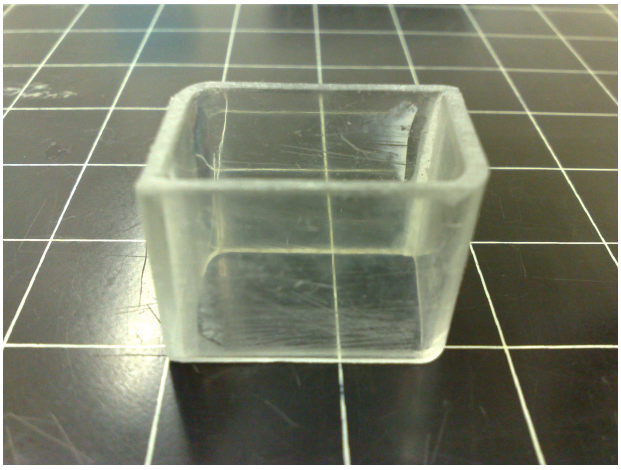


Figure 7.7. PVC custom-made chamber

7.3.1.2. Reagents, chemicals and materials

Ethanol (96 %, v/v)(Merck, Wadeville, South Africa) was used to sterilize the probes and all associated surgical equipment used in these *in vivo* MD studies. IL, a lipid emulsion, was used as the perfusate.

Linear MD probes were fabricated in-house as previously described with the exception that only 1 side of the membrane was connected with a length of Portex tubing (30 cm).and prepared one day prior to the study (see Figure 7.8) .

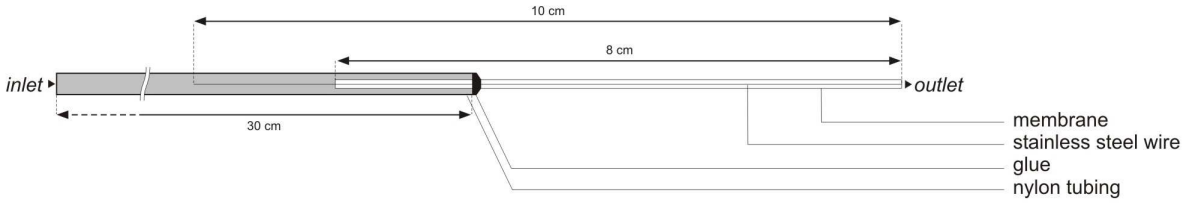


Figure 7.8. Linear MD probes used for *in vivo* MD studies [307]

7.3.1.3. Formulations

CP (Symbiotec Pharmalab P.V.T. Ltd., Pigdamber, Maharastra, India) was dissolved in ethanol to achieve 1, 2 and 4 % m/v ethanolic solutions. An extemporaneous 1% m/m

Dermovate cream was prepared by incorporating a weighed amount of CP into the Dermovate® cream. The cream was assayed prior to use. In addition a 2% m/v solution of CP made up in a 50/50 mixture of propylene glycol/ethanol was also prepared.

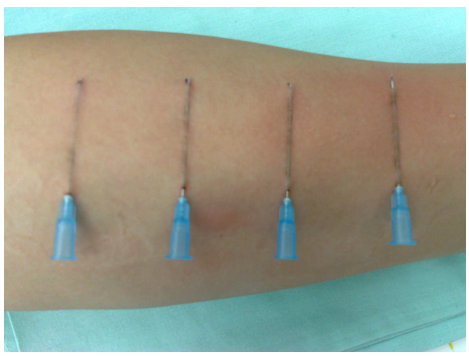
7.3.1.4. Subjects

An *in vivo* MD study using 10 healthy human volunteers (4 males and 6 females, aged 18-26) who met the necessary inclusion/exclusion criteria (appendix V) with skin phototype I-III [323] was undertaken .Written informed consent was obtained from each volunteer before the study. The research with human subjects followed the recommended guidelines as set out in the Declaration of Helsinki (1964). The study protocol was approved by the Ethical Standards Committee of Rhodes University (Grahamstown, South Africa).

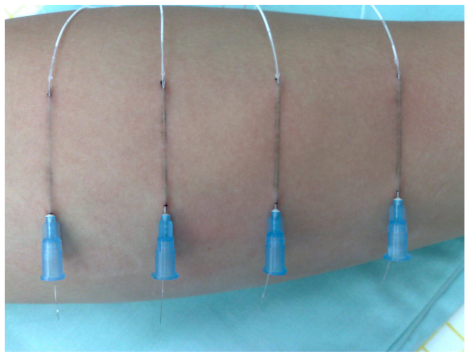
7.3.1.5. Study design

7.3.1.5.1. *In vivo* microdialysis method

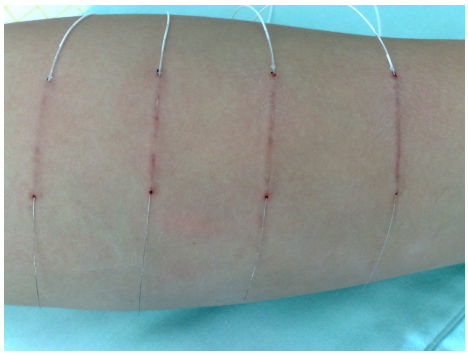
A total of four sites were used on the arm and one linear MD probe was inserted into each site in the skin using a 23G cannula (Crown®, Pretoria, South Africa) as a guide. Entry and exit points were marked ensuring that a length of 30 mm of the membrane of the MD probe was placed intra-dermally. An ice pack (Medac (Pty) Ltd, Cape Town, South Africa) was placed directly on the skin for a few minutes to induce a local anaesthetic effect at the needle insertion site and subsequently removed prior to inserting the needle guide. The probes which were previously sterilized in ethanol (70% v/v) were inserted through the cannula. Once the MD probes were in place, the cannulae were removed. The IL, used as the perfusate, was perfused at 1.0 µl/min for approximately 1 hour to allow for equilibration. During the equilibration period, the probes were secured in place with an epoxy-based glue gel (Super Glue, Genkem®, Johannesburg, South Africa) and custom-made PVC reservoirs (Figure 7.7 and step 5 of Figure 7.9) were glued to the application sites on the skin using clear adhesive glue (Bostik®, Cape Town, South Africa). The sequences of the procedure are shown in Figure 7.9 below.



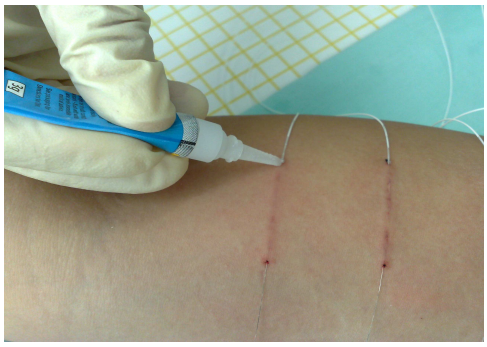
1) Insertion of needles into the skin



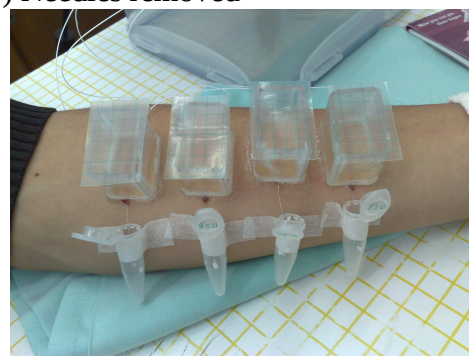
2) Insertion of MD probes through the needles



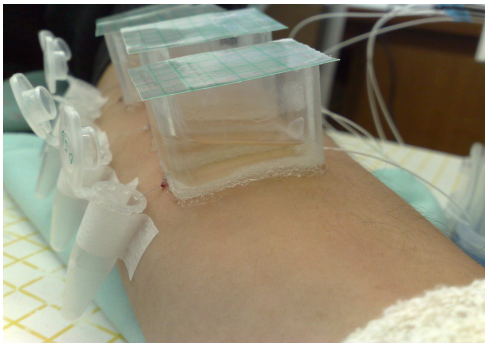
3) Needles removed



4) Securing MD probes into place



5) Positioning of sample vials and PVC chambers



6) Initiation of the MD study

Figure 7.9. Procedure of *in vivo* MD setup.

After the equilibration period, the study was initiated by filling the PVC chambers with ~4 ml of 4% m/v CP ethanolic solution. IL was perfused through the MD probes at a rate of 1.0 µl/min. Dialysate samples were collected every 30 minutes for 4 hours. The samples were immediately extracted and analysed using a validated HPLC system (section 5.4).

At the end of the sampling period, the probes were detached from the pump but not removed from the skin and the excess ethanolic solution along with the PVC reservoirs were carefully removed. The skin sites were then gently wiped with alcohol swabs. Prior to the removal of

the MD probes from the skin, the depth of the probes were measured in triplicate by ultrasound scanning at 20MHz using the A-mode of the Dermascan C® ultrasound scanner (Cortex Technologies, Hadsund, Denmark). The probes were then carefully removed from the skin and the sites were gently wiped with alcohol swabs and cotton pads to remove any remaining residue. Biocort® cream (1% m/m hydrocortisone acetate, Adcock Ingram Ltd, Bryanston, South Africa) was provided to each subject to treat the application sites on the arm as a prophylactic measure against post-traumatic skin inflammation. A post-study medical follow-up examination of the sites of each subject was performed twice weekly for a month.

7.3.1.5.2. Choice of delivery system and CP concentration for topical application

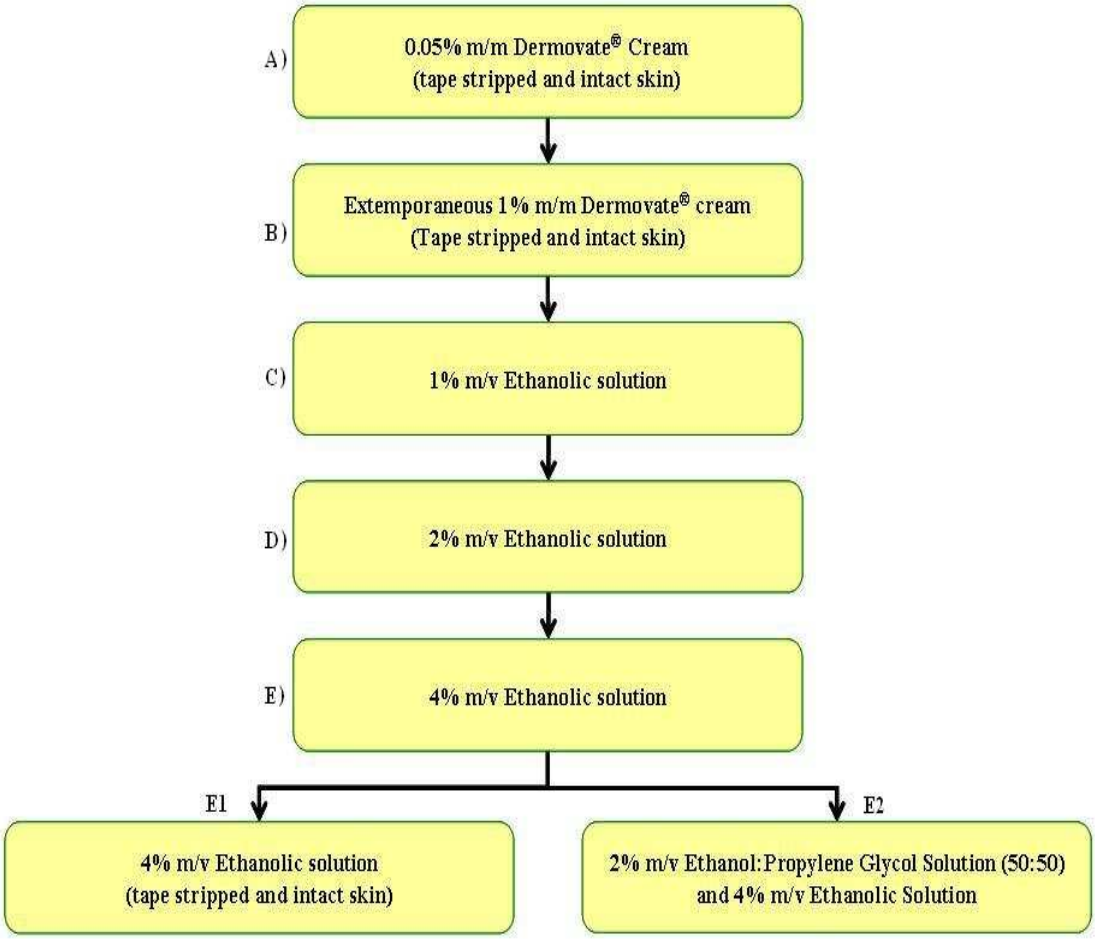


Figure 7.10. Flow diagram of *in vivo* MD experiments conducted

The above experiments (Figure 7.10) were carried out to determine the minimun concentration of CP that must be applied to the skin in order for a detectable amount of CP to be present in the dialysate. A further objective was to investigate the effect of formulation on the penetration of CP into the skin. Dermovate® cream (0.05%) and the 1% m/m re-enforced Dermovate cream, were initially tested by applying sufficient of the respective cream to cover the application site (3 cm x 1 cm) and the MD procedure described above was used. However, the presence of CP was not found in the dialysate samples following application onto either stripped or intact skin.The 1 % and 2 % CP ethanolic solutions were also assessed by MD using the PVC chambers filled with ~ 4 ml of respective solution and applied to the skin as described previously. Once again, the presence of CP could not be detected in the dialysate samples. The concentration of CP was subsequently increased to 4% m/v (Formulation E) which resulted in the appearance of CP in the dialysate samples.

As a result of these findings, the 4% m/v CP ethanolic solution was subsequently chosen to compare the penetration of CP into the skin following application to tape stripped and also to intact skin. Scotch® Magic tape was used to tape strip the relevant application sites until the TEWL reached ~60 g/m^2hr in order to remove a substantial amount of the SC at the intended site of CP application.

A further study using a 2% m/v solution of CP in 50/50 propylene glycol/ethanol applied to the skin was undertaken concurrently with the application of a 4% m/v ethnanolic solution of CP.

7.3.1.5.3. Methods to increase detection of CP following MD

Initially, one of the objectives was to assess BE using MD to compare the bioavailability of CP from three commercially available dosage forms (Dermovate® cream, Dovate® cream and Dermovate® ointment). However, the concentration of CP in these products was too low to allow detection of CP release into the skin. The concentration of CP in the dialysate following application of the above formulations was below the LOQ (0.5 μg/ml) of the HPLC method, this was despite the fact that the same HPLC method was sensitive enough for use during the TS studies and also to assay the various formulations and preparations

which were investigated. Various approaches to enhance detection of CP following application of CP preparations to the skin were considered, such as:

- ✧ Increasing the length of the MD membrane under the skin in order to increase the area for more uptake of the drug into the perfusate.
- ✧ Introducing two MD probes into one test site to increase the volume of sample collected.

7.3.1.5.4. Assessment of the presence of CP reservoirs in the skin using the HSBA

A separate study was performed on three volunteers to determine whether a reservoir containing CP exists after application of the 4% m/v ethanolic solution of CP. The objective of this study was to investigate whether the very low concentrations of CP following MD were due to the formation of reservoirs in the SC. The volunteers were tested for skin blanching prior to commencing this study. Dermovate® cream was applied to a small area of the forearm of each subject and inspected for blanching 6 hours after application, this was used as the inclusion criterion.

Following *in vivo* MD using the 4% m/v ethanolic solution of CP (Study E), a plastic film was wrapped around the volar aspect of the forearm of each subject, covering the application sites, This was done12 hours after termination of the MD experiment and was left on for 2 hours.

7.3.1.5.5. The assessment of the bioavailability of a 4% m/v CP ethanolic solution

Finally, the 4% m/v ethanolic solution was used to assess the BA of CP into the skin using MD. The uptake of CP by the skin was assessed on the volar aspect of one forearm in each of 10 healthy human subjects. Ethanol was chosen because of its pharmaceutical acceptability gleaned from its ubiquitous use in pharmaceutical products and also in cosmetic preparations [106].

7.3.1.6. Statistical analysis

The mean, standard deviation and intra- and inter-individual variability following the *in vivo* MD study using 4% m/v ethanolic solution of CP in 10 subjects, were determined. Differences in penetration of CP between subjects, gender and application sites on the forearm were assessed using one-way ANOVA and the two-tailed t-test (95% confidence interval) where relevant. These two statistical analyses and AUC from 0 – 4 hrs were determined using GraphPad® Prism version 4.03.

7.3.2. Results and discussion

7.3.2.1. Choice of delivery system and CP concentration for topical application

7.3.2.1.1. Applications A to E

The presence of CP in dialysate samples was not detected in any of the samples following the application of any of the following preparations: Dermovate® cream (0.05 %), 1% m/m extemporaneously re-enforced Dermovate cream, 1% and 2% m/v CP ethanolic solutions to either stripped or intact skin. Although CP may have penetrated the skin following application of the various preparations described above, this could not be confirmed using the developed HPLC method in view of the lack of sensitivity to detect very low concentrations of CP. The HPLC method used has an LOQ of 0.5 μg/ml and coupled with the fact that very low sample volumes are collected in the MD samples, it is clear that much higher concentrations of CP would need to be applied to the skin in order to detect its presence following MD.

It has been shown that partial removal of the SC (tape stripped skin) allows more penetration of applied drug into the skin [274], therefore studies using the applications denoted in Figure 7.10 A, B and E1 were conducted on stripped as well as on intact skin to investigate whether the removal of SC will facilitate the detection of CP by enhancing penetration. However, partial removal of the SC did not appear to enhance the penetration of CP from any of the preparations. Since CP is a lipophilic drug, the removal of the SC will most probably not enhance penetration into the hydrophilic epidermis. Similarly, the use of a penetration

enhancer, ethanol, alters the SC which essentially also removes the barrier (or some of it) and no enhancing effects were seen there as well..

Approaches to enhance/increase the amount of drug penetration into the skin include the use of a penetration enhancer into the formulation or increasing the concentration of drug in the formulation [91]. Hence, both approaches were investigated by using increased concentrations of CP such as a 1% m/m cream and 1%, 2% and 4% m/v CP ethanolic solutions of the corticosteroid and also a 2 % CP preparation containing propylene glycol in addition to ethanol as a penetration enhancer.

The 4% m/v CP ethanolic solution was investigated together with the 2% m/v CP solution containing both ethanol and propylene glycol in the same human subject. MD was carried out as previously described and the results are depicted in Figure 7.11. As can be seen from the MD profile below and the associated AUCs, the amount of CP which diffused through the skin from the 4% m/v ethanolic solution (AUC = 2.39 µg/ml.hr) was greater which diffused into the skin following application of the 2% m/v propylene glycol/ethanol solution (AUC = 1.41µg/ml.hr) up to 2 hours. This is in agreement with the hypothesis that suggests that increasing the concentration of the active in the preparation will increase the amount of drug that diffuses into the the skin [91]. It has also been shown that the use of propylene glycol enhances the penetration of corticosteroids into the skin after topical application [92,93]. However, in this case, the use of propylene glycol/ethanol solution had a similar penetration enhancing effect when compared to using ethanol only.

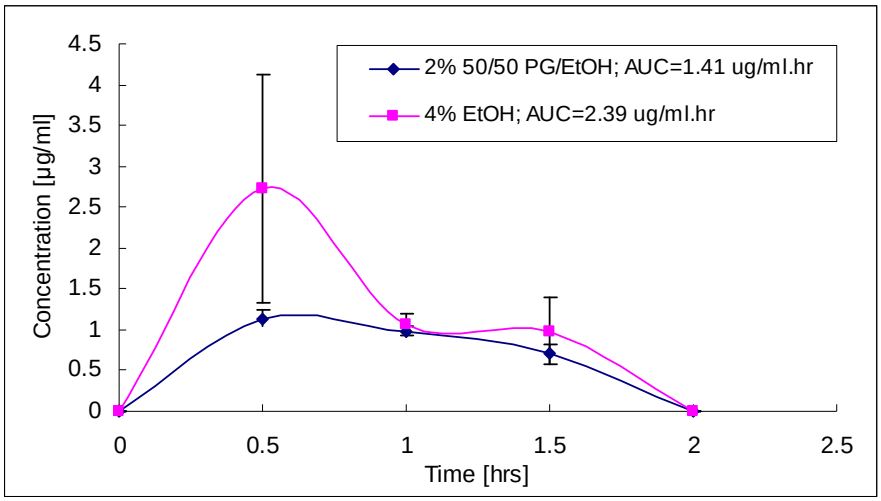


Figure 7.11. Comparison between the 2% m/v CP in 50/50 PG/EtOH solution and the 4% m/v CP ethanolic solution using *in vivo* MD.

7.3.2.2. Methods to increase detection of CP following MD

Although considerations were given to increasing the length of the probes for insertion under the skin, due to practical limitations such as using a considerably longer needle as a guide, the above was not pursued.

The use of 2 probes inserted into each site was also considered as a method to improve detection of CP in the skin following MD. Although the volume collected using 2 probes will be twice that of a single probe maintained at the same flow rate, the concentration of CP will remain the same. Whereas, a larger dialysate volume would be collected using 2 probes and could facilitate and improve the detection limit by pooling and concentrating the combined samples, concentrating the IL extract also resulted in an increase in interference from the other extracted components in the lipid emulsion (perfusate) and the detection limit was not improved.

7.3.2.3. Assessment of the presence of a CP reservoir in the skin using the HSBA

When a plastic film was used to wrap around the volar aspect of the forearm of each subject, 12 hours after an MD investigation following the application of a 4% m/v CP ethanolic solution to the skin for 4 hours, no blanching was seen 2 hours after removal of the occluding film. Hence, the presence of a CP reservoir could not be established. Although, corticosteroid reservoirs in the skin can be readily seen following occlusion of the application sites when semi-solid formulations of the corticosteroid are used [324-327], it may be concluded that following application of an ethanolic solution, unlike the application of semi-solid formulations, the corticosteroid appears to penetrate beyond the SC. Hence, the low recovery of CP in the dialysate is unlikely to be influenced by a reservoir effect.

7.3.2.4. The assessment of the bioavailability of a 4% m/v CP ethanolic solution

Following application of a 4% m/v CP ethanolic solution to the skin of 10 volunteers, concentrations of CP were detected in the MD dialysate samples at various times. Figures

7.12 and 7.13 below depict the penetration of CP in the forearms of human subjects. After the topical application of the solution (t = 0hrs), concentrations of CP, although relatively low, were rapidly detected in the dialysate samples. Figure 7.13 illustrates that CP penetrates the skin from ethanol at a fairly constant rate. It was interesting to note that soon after (~ 15 min) applying the 4% m/v ethanolic solution onto the skin sites, the erythemic response from the needle insertion trauma faded and the skin began to whiten indicating that skin blanching was occurring. This is consistent with the skin blanching response observed following application of Dermovate® cream used in the HSBA studies described in Chapter 4. This is a clear indication that CP penetrated the skin at a relatively rapid rate from the ethanolic solution, although at low concentrations.

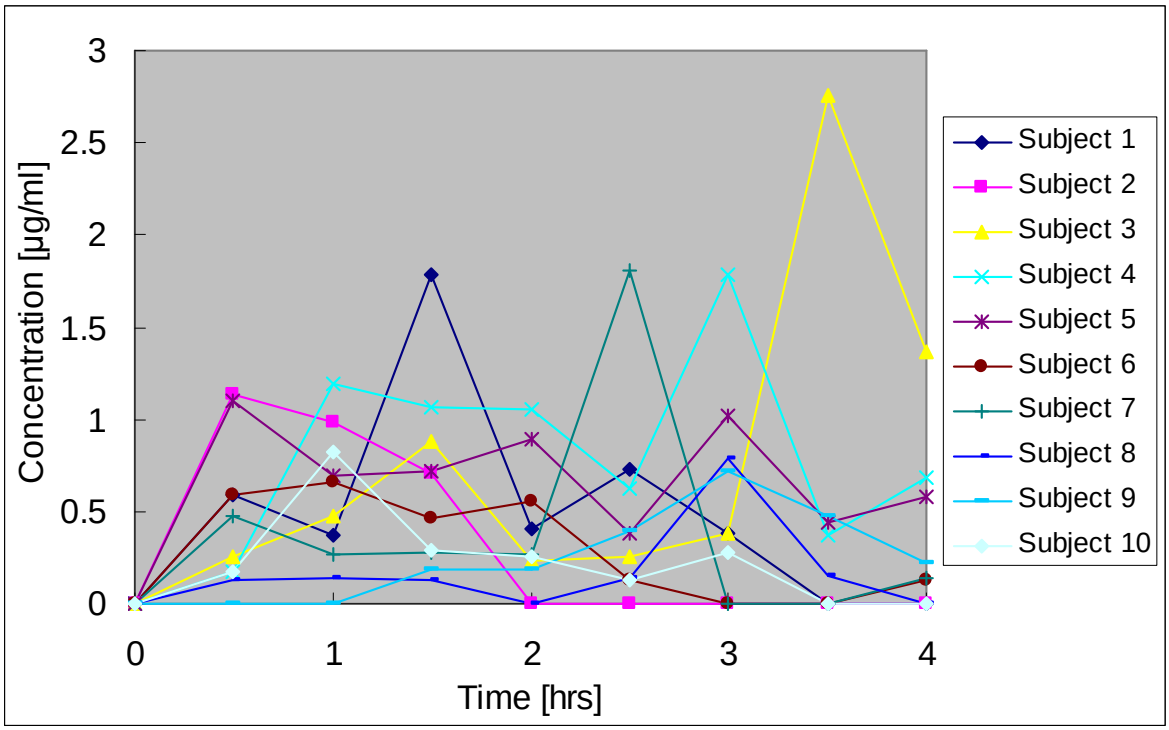


Figure 7.12. Penetration profiles of CP into the skin for each subject.

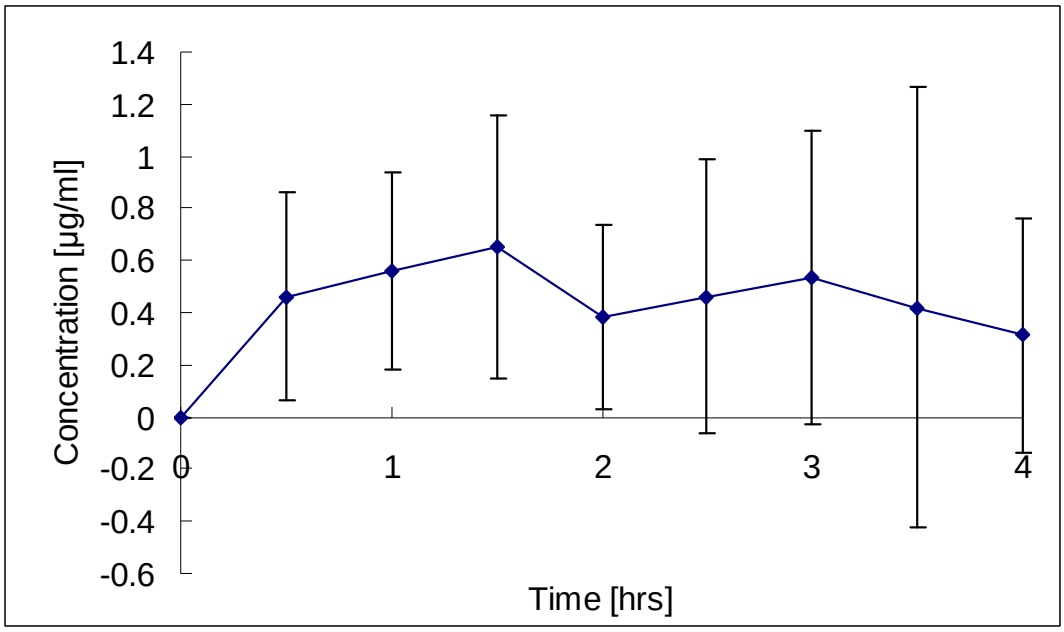


Figure 7.13. Mean concentration of CP penetrated through the skin of 10 subjects

Although a relatively high concentration of CP was applied to the MD sites, the percentage of CP recovered from the skin was relatively low. The reasons for this low recovery could be due to a number of factors such as protein binding [308], very slow diffusion of the bulk amount entering the SC and the exertion of a "barrier" effect within the SC. From Fig 1.12, it is seen that the average concentration of CP appears to be quite steady up to 4 hours and in some cases even beyond. This supports the appearance of relatively low concentrations of CP from the relatively high CP concentration applied as a result of somewhat restricted diffusion within the SC

As shown in Table 7.7, the intra-individual variation (CV%) ranged between 10.17 – 121.65% and the inter-individual variation was found to be 50.56 %. The inter-individual $AUCs_{0\text{-}4}$ ranged from 0.74 ± 0.90 µg/ml.hr to 3.30 ± 1.24 µg/ml.hr. It is therefore apparent that even within the same subject and using a confined area of the body (volar aspect of the forearm) the penetration of CP varies. In spite of this, when the data were statistically analysed according to the one-way ANOVA, no significant differences were found when comparing the penetration profile of CP between subjects (p-value > 0.05).

Table 7.7. Intra- and inter-individual MD data of 10 subjects

Subject no.	AUC ± SD (μg/ml.hr)	CV (%)
1	2.13 ± 1.52	71.34
2	1.41 ± 0.14	10.17
3	2.96 ± 1.64	55.43
4	3.30 ± 1.24	37.60
5	2.78 ± 0.37	13.20
6	1.23 ± 0.29	23.67
7	1.58 ± 1.62	102.90
8	0.74 ± 0.90	121.65
9	1.04 ± 0.94	90.39
10	0.97 ± 1.07	109.46
Mean data of 10 subjects	1.81 ± 0.92	50.56

When the data were re-visited, close inspection of the profiles suggested the possibilities of "outliers". For example, the following Table 7.8 lists the resulting data which appear to be anomalous and may be considered as "outliers".

Table 7.8. "Outliers" data

Subject no.	Site	Time (hrs)	Concentration (μg/ml)
1	C	1.5	4.80
2	-	-	-
3	A	3.5	5.83
4	D	3.0	4.31
5	A	3.0	2.45
6	D	4.0	0.51
7	C	2.5	6.13
8	D	3.0	3.14
9	C	3.0	1.30
10	D	1.0	2.78

The following profile (Figure 7.14) shows the profile obtained after the removal of "outlier" data superimposed on the original profile where all data were included. It is therefore apparent that the removal of the so-called anomalous data had no significant effect on the profile. Furthermore, the variability was only slightly reduced from a CV% of 50.56 to 48.52.

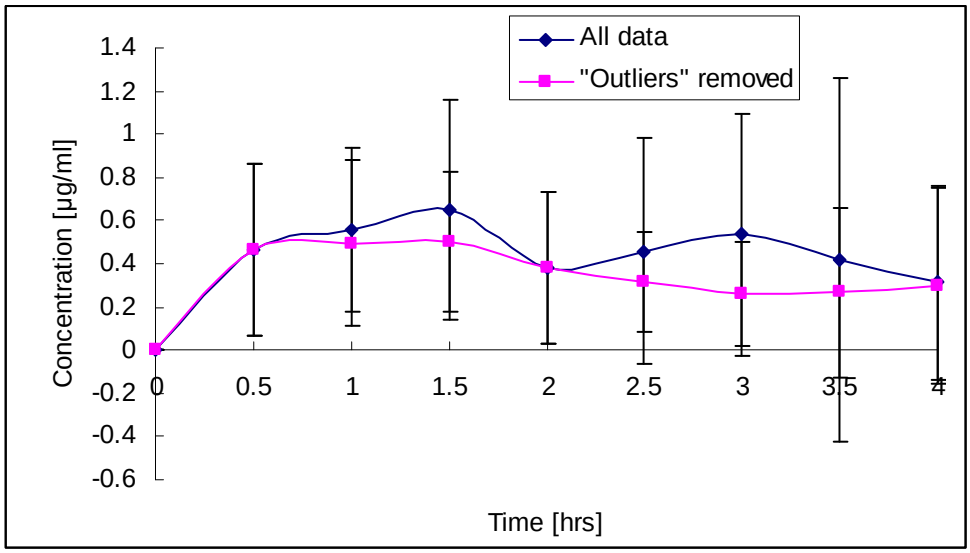


Figure 7.14. Comparison between profiles containing all data and data in which "outliers" were removed.

Figure 7.15 illustrates the penetration of CP into the skin when comparing male and female subjects. No differences were observed in the dermal penetration of CP between genders (p-value = 0.1058)

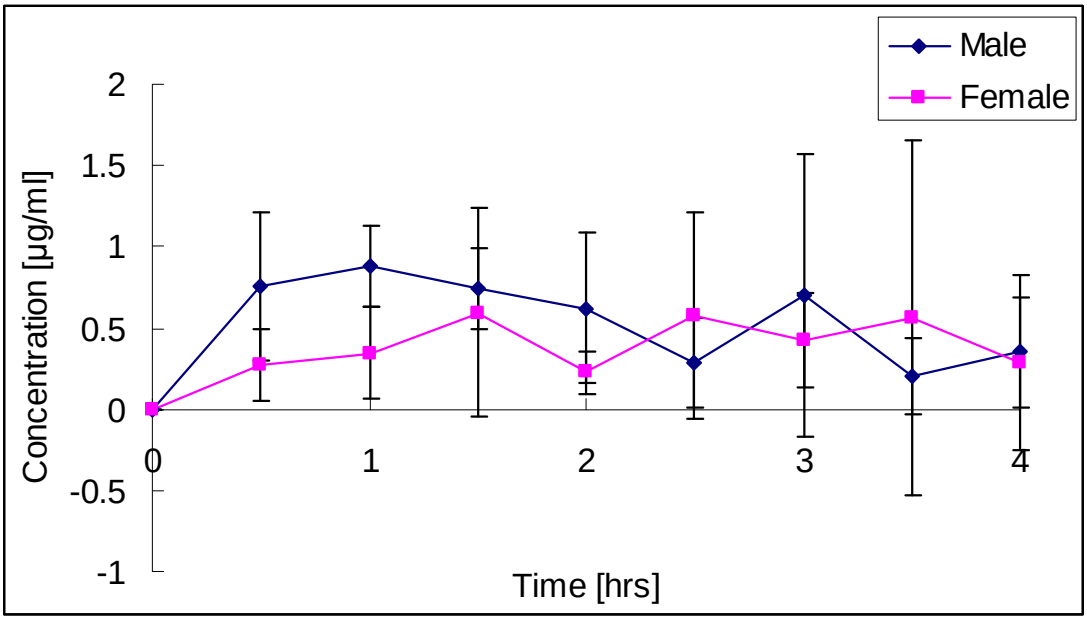


Figure 7.15. Comparison between male and female mean concentration of CP penetrated through the skin

However, when the "outliers" were removed as described above, and the profiles (Figure

7.16) of mean male vs. mean female without the "outliers" were compared, a significant difference was seen with a p-value of 0.0016 using the two-tailed t-test. The corresponding profiles are shown below. However, it should be noted that amongst the ten subjects used, six were females and four were males. Data removed to obtain the profile below involved a single data point removed per subject in 3 of the 4 males, whereas a single data point was removed in each of the six females. The resulting data had the effect of lowering the female profiles. Processing data in this way is clearly disproportionate and the significance of the findings showing differences between male and female subjects is questionable.

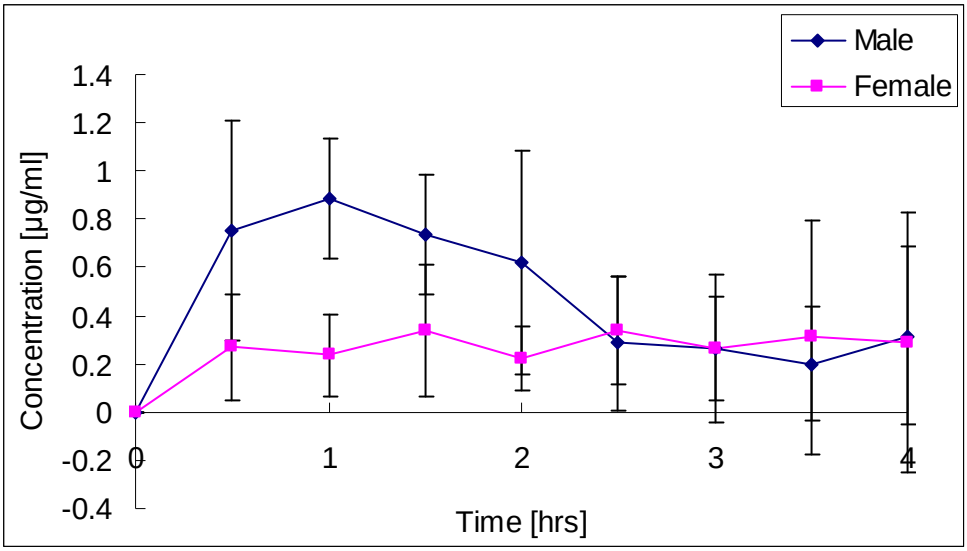


Figure 7.16. Comparison between male and female mean concentration of CP penetrated through the skin after the removal of "outlier" data.

The following profiles (Figure 7.17) were obtained when comparisons were made between sites A to D i.e sites arranged from the elbow to the wrist. No differences were observed (p-value = 0.8159). Removal of the "outlier" data had no effect on the results (p-value = 0.1880).

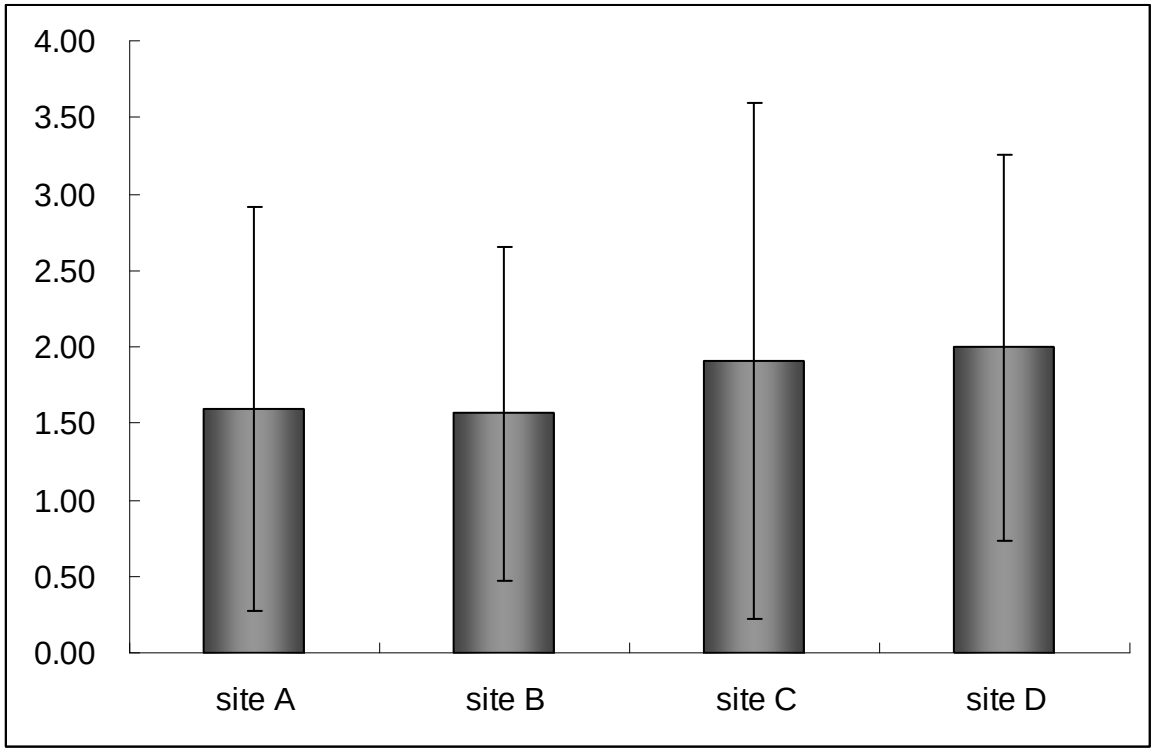


Figure 7.17. Comparison of CP penetration between the four sites.

Figure 7.18 depicts the flux of CP from the extracellular fluid of the skin through the membrane of the MD probe into the perfusate. To determine the flux of CP from the surrounding medium into the perfusate, the slope of the plot (Figure 7.18 was determined. The flux of CP was found to be 0.15 μg/cm^2.hr. The permeation coefficient was calculated from equation $P = J/C_0$ and found to be 3.68 x 10^{-6} cm/hr where P is the permeation coefficient, J is the flux and C_0, the starting concentration which was based on the concentration of CP applied at the site. However, CP recovery following MD indicated that considerably less than the total amount applied had been collected up to the end of the MD sampling time of 4 hours, implying that not all the CP had penetrated through the skin in that time. Hence, the value of the permeation coefficient is probably considerably lower than that calculated above which is based on complete recovery (i.e. penetration of the entire amount of CP applied).

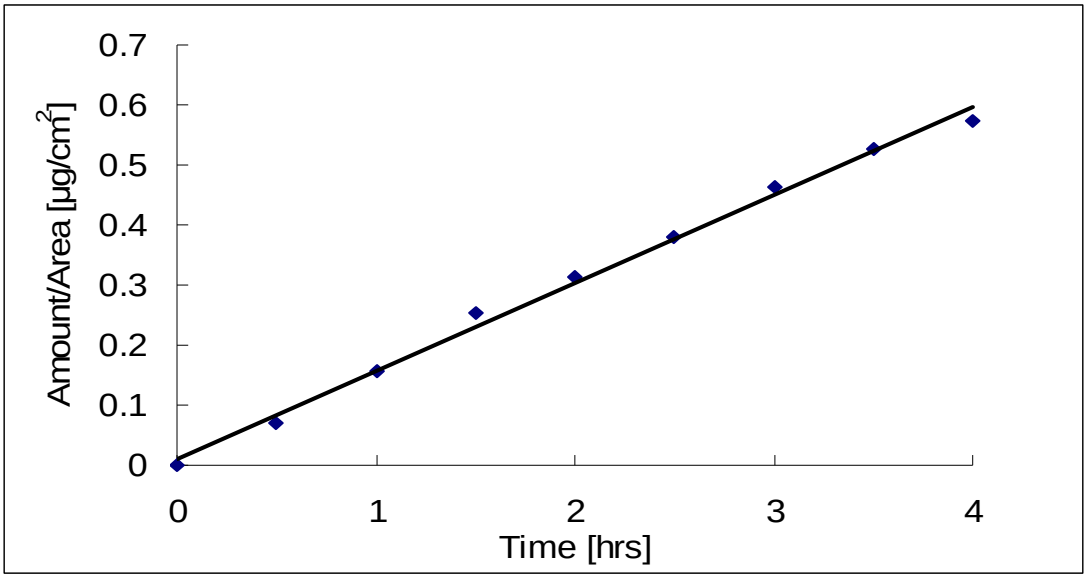


Figure 7.18. Flux of CP through the membrane of the MD probe *in vivo*.

The depth of each MD membrane was measured using the Dermascan®. The MD probes were inserted at a mean depth of 0.692 ± 0.128 mm from the surface of the skin as depicted in Table 7.9 below. No correlation between AUCs and probe depth were observed.

Table 7.9. Probe depth measurement (n = 4 for each subject)

Subject	Mean (mm)	SD (mm)	CV (%)
1	0.81	0.051	6.27
2	0.67	0.034	4.96
3	0.95	0.17	18.01
4	0.66	0.12	18.76
5	0.79	0.040	4.99
6	0.63	0.046	7.23
7	0.63	0.066	10.34
8	0.59	0.098	16.63
9	0.64	0.038	5.94
10	0.518	0.024	4.609
All subjects	0.692	0.128	18.422

7.4. CONCLUSIONS

The *in vitro* retrodialysis experiments indicated that the CP adhered to the MD probe when saline was used as the perfusate, but when IL replaced saline as the perfusate, CP was completely recovered in the dialysate. Since lipophilic substances are known to have high affinity to bind to membranes and other components, the use of IL therefore has the additional advantage of overcoming those limitations. Further *in vitro* experiments to investigate CP recovery confirmed that IL was an acceptable perfusate for use in MD studies on CP as shown in the mass balance reconciliation calculations.

In vitro MD indicated the potential of this technique for use to assess the release of CP from various formulations where it was shown that the release from two cream preparations, Dermovate® cream and Dovate® was similar whereas the release from Dermovate® ointment was different. These findings corresponded well with those data obtained from the previously conducted TS bioequivalence studies, which suggests the possible use of *in vitro* MD as a tool for the assessment of BA/BE of topical products.

Various mathematical models and ANOVA as well as the evaluation of f_1 and f_2 used in dissolution studies were investigated to compare the release profiles from the various formulations. ANOVA, f_1 and f_2 showed their applicability in determining similarities and differences in CP release between products. Application of mathematical models to describe the release kinetics of CP from the formulations provided useful information on the possible release mechanisms.

In vivo MD was used to assess the BA of CP penetration into the skin from various preparations. Previous reports have described the use of *vivo* MD to assess BE of topical dosage forms [246,294]. However, one of the important challenges when using *in vivo* MD is the requirement to have a sufficiently sensitive analytical method to quantitatively measure extremely low concentrations of the investigative drug. The findings in this research on CP penetration into the skin has clearly indicated that BE assessment of CP formulations, whilst feasible in the light of the current findings, will be highly dependent on the availability of a sufficiently sensitive analytical method to detect and measure concentrations of CP in the low ng/ml and even pg/ml ranges. The concentration of current CP formulations are 0.05%

and CP released from the commercial formulation, Dermovate® cream, could not be detected using MD. However, it was shown that provided the concentration of CP was much higher, such as the 4% application used, MD studies would be feasible to assess BE. A further challenge is to find a suitable physiologically compatible perfusate in which the investigative drug dissolves. The usual perfusates consisting of saline solutions or similar physiologically acceptable electrolyte/buffer solutions etc. could not be used due to the insolubility of CP in such media. Intralipid®, a lipid emulsion which is used in Total Parenteral Nutrition (TPN) and is a sterile preparation which is physiologically acceptable was chosen as the perfusate since the solubility of CP was found to be in the order required.

8. CHAPTER 8

8.1. CONCLUDING REMARKS

The HSBA/VCA was once again shown to be a reliable tool for the BA/BE assessment of a topical corticosteroid formulation containing CP when either visual or chromametric assessment was used. The findings indicated that either of the assessment methods is equally suitable for skin blanching assessment. However, for visual assessment, observers must be well-trained, and for chromameter assessment, the optimized handling of the instrument must be assured. Furthermore, an optimized screening procedure involving the application of the reference formulation for 2 dose durations, D_1 and D_2, was used to screen subjects. Profiles from both the D_1 and D_2 applications were subsequently evaluated to establish whether the AUECs of $D_2/D_1 > 1.25$ in order to identify potential "detectors". This screening procedure differs from that recommended in the FDA guidance where only a simple qualitative response is used to include subjects based on the appearance or non-appearance of a blanching response. This *a priori* identification of potential "detectors" has the advantage of using less subjects in the pivotal study and therefore obviates the need to unnecessarily include subjects, who in spite of responding in the initial screening, may not comply with the inclusion requirements for "detectors".

The development and validation of an HPLC-UV analytical method for the quantitative determination of CP in the various matrices (i.e. from mobile phase and extractions from creams, ointment, tape strips and IL samples) was in compliance with the FDA requirements as per guidelines. The same analytical method, apart from some minor differences in sample preparation, was successfully applied to assess samples obtained from TS studies, *in vitro* and also for the *in vivo* MD samples. However, a more sensitive analytical method will be required for the detection of CP when assessing the BA/BE of commercial CP formulations in view of the very low content (0.05 % m/m) of CP in those products. The method developed, however, had the necessary sensitivity to detect and monitor the appearance of CP by *in vivo* MD following the application of a 4 % m/v ethanolic solution of the corticosteroid.

TS proved to be a useful technique for the assessment of BA/BE of clobetasol propionate

products and was shown to have the potential for use to assess other topical products for local use apart from only those containing topical corticosteroids. It is recommended that it is necessary to conduct a dose-response pilot study in order to establish a relevant discriminatory dose duration for the product under investigation for use in a subsequent BE assessment. Normalization of skin thickness using TEWL is important and shown to be necessary to compare TS data between subjects. Furthermore, to reduce variability of the method, several other factors have been identified and described for the optimization of the TS procedure. It is also important to use AUC_{corr} data (correction of TS data using skin fraction to normalize SC thickness between subjects) as this was shown to have a significant influence and affect the resultant data for BE assessment. The statistical analysis of TS data for BE indicated that either log-transformed or untransformed data could be used since similar results were obtained using either method.

To date, reports in the literature have described the application of microdialysis for the assessment of the BA/BE of topically applied hydrophilic drugs. Furthermore only a few reports have been published where MD has been used to monitor lipophilic drugs in dialysates, either *in vitro* or *in vivo* in animals. Since CP is highly insoluble in aqueous media, an alternate perfusate (IL) was investigated for use in the MD investigations. It was found that CP could be detected in dialysate samples obtained from healthy human subjects using *in vivo* MD following the application of a 4 % m/v ethanolic CP solution and using IL as perfusate. IL proved to be an appropriate perfusate for the recovery of CP with the advantage of reducing adsorption/adherence to the membrane and/or other components of the MD system.

A novel approach to monitor and assess the *in vitro* release of CP from commercially available topical dosage forms was investigated using MD. Similarities and differences were evaluated using a number of mathematical models to establish the respective release mechanisms, and the f_1 and f_2 factors used in the comparison of dissolution profiles were applied to the *in vitro* MD release data comparing the various formulations. The *in vitro* MD results concurred with previously obtained data from the BE studies using TS. This suggests that *in vitro* MD systems may be useful to investigate BA/BE of topical formulations.

APPENDIX I

RESEARCH PROTOCOL

A PILOT DOSE DURATION-RESPONSE STUDY OF CLOBETASOL 17-PROPIONATE 0.05% CREAM IN HEALTHY VOLUNTEERS

STUDY NO. WL1

Protocol version 2

2006 – 02 – 21

Reference Product	Dermovate® Cream (Clobetasol 17-Propionate 0.05%) Glaxo Wellcome, South Africa

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Conducted by	Wai Ling Au
Supervised by	Prof. I. Kanfer Rhodes University Grahamstown, 6140 South Africa

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_____ _____

Investigator — Date (yyyy-mm-dd)

Wai Ling Au

_____ _____

Supervisor — Date (yyyy-mm-dd)

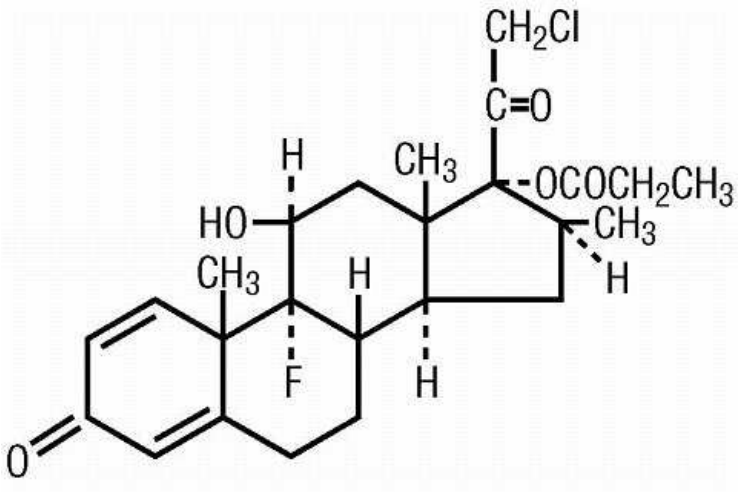
Prof. I. Kanfer

CONTENTS

1. BACKGROUND INFORMATION

1.1 Molecular Structure

The molecular structure of clobetasol 17-propionate is represented below.



clobetasol 17-propionate

1.2 Mode of Action and Uses

Clobetasol 17-propionate is a highly potent synthetic corticosteroid that has anti-inflammatory, antipruritic, and vasoconstrictive properties. Its anti-inflammatory effect is thought to be due to its ability to control biosynthesis of potent mediators of inflammation. It stimulates phospholipase A(2) inhibitory proteins and subsequently blocks the release of arachidonic acid, which is a common precursor to leukotrienes and prostaglandins [1].

Clobetasol 17-propionate is used in the topical treatment of various skin disorders such as eczema, some forms of psoriasis and seborrhoeic dermatitis [2].

1.3 Pharmacokinetics

To achieve a therapeutic effect, it is necessary that the topically applied drug enters the epidermis or the dermis of the skin. Skin penetration and permeation of the drug after topical administration depend on the physicochemical properties of the drug molecule, the function

of the skin as a transport barrier and the composition of the delivery vehicle [4, 5, 6]. Topical use particularly under an occlusive dressing or when the skin is broken, systemic effects may occur if sufficient clobetasol 17-propionate is absorbed. Clobetasol 17-propionate is rapidly distributed to all body tissues. It can cross the placenta to varying degrees and may be excreted in small amounts in breast milk. The synthetic corticosteroids exhibits less extensive protein binding and tend to have longer half-lives when compared to the other corticosteroids. Metabolism of clobetasol 17-propionate occurs in the liver and is excreted via the urine and bile [2].

1.4 Adverse Effects

Upon topical application of clobetasol 17-propionate, one may experience burning sensation, pruritus and stinging of the skin. Headache and other irritation symptom(s) may occur [1].

1.5 Contraindications

Administration should be avoided in persons who exhibit hypersensitivity to clobetasol 17-propionate or to any ingredient in the preparation or to other corticosteroids. It is contraindicated in the presence of acute dermatological infections e.g. scalp infection [1].

2. STUDY PRODUCTS

2.1 Description

	Reference Product
Generic Name	-
Commercial Name	Dermovate® Cream
Dosage Form	Cream
Strength	0.05% m/m
Manufacturer	GlaxoWellcome, South Africa
Batch/Lot No.	255573
Expiry Date	01/2009
Description	White cream

Dosage	30μL (±11mg) per application site

2.2 Supply, Storage and Use

Sufficient products should be timeously supplied to the Biopharmaceutics Research Institute prior to study. Clobetasol 17-propionate is available in cream, ointment or lotion dosage forms. It should be used externally only. The product should be kept in the container it came in, tightly closed and stored at room temperature away from excess heat and moisture [3].

3. OBJECTIVES

The objective of this pilot study is to measure the skin blanching efficacy for the determination of the dose duration-response relationship of clobetasol 17-propionate. The assessment of the blanching will be done with the use of a chromameter and visually. Data from this study is required for the application times in the pivotal bioequivalence study. This study is equivalent to producing a standard curve in the assay. The outcome of this pilot study provides information to determine the parameters of ED_{50}, D_1 and D_2 that will be used in the pivotal study.

The study will be conducted according to the FDA Guidance for Industry: Topical Dermatologic Corticosteroids, dated February 1997.

4. STUDY POPULATION AND MEDICAL ASSESSMENT

4.1 Inclusion Criteria

Only the subjects that meet the following criteria will be included in the study:

1. Mentally competent subjects who are available for the entire study period and willing to adhere to the protocol requirements and able to give informed consent by signing the informed consent form, which is also signed by a witness.
2. Subjects demonstrating adequate vasoconstriction to topical corticosteroids, i.e. ‘responders’.

3. Male or female between 18 and 50 years of age.
4. Normal in terms of dermatological medical history at the pre-study screening medical, or in the case of an abnormality, the medical practitioner considers the abnormality to be clinically insignificant.
5. Normal in terms of dermatological examination at the pre-study screening medical, or in the case of an abnormality, the medical practitioner considers the abnormality to be clinically insignificant.

4.2 Exclusion Criteria

Subjects that meet one or more of the following criteria will be excluded from the study:

1. Individuals smoking within one week of study.
2. Caffeine intake greater than 500mg per day prior to or during the study. (A cup of coffee contains about 85mg of caffeine)
3. Clinically significant history of alcoholism or drug abuse.
4. Use of topical dermatologic drug therapy on ventral forearms, including prior dosing of topical corticosteroid in pharmacodynamic study to a particular skin site, within one month prior to study.
5. Adverse reactions to topical or systemic corticosteroids.
6. Any current or past medical condition, including active dermatitis or any other dermatologic condition, which might significantly affect pharmacodynamic response to the administered drug.
7. Persons who would require shaving ventral forearms to insure consistent dose on skin surface.
8. Use of any vasoactive (constrictor or dilator) medication, prescription or OTC, that could modulate blood flow. Examples of such drugs include nitroglycerin, antihypertensives, antihistamines, NSAID's, aspirin, and OTC cough/cold products containing antihistamines and/or either phenypropanolamine or phentolamine.
9. Any obvious difference in skin color between arms.
10. Any mental deficiency or handicap.
11. Any contact allergy to the label adhesive on labels used to demarcate application sites.
12. Consumption of alcohol or other enzyme inducing agents within 24 hours of the start of the study (all barbiturates, corticosteroids, phenylhydantoins, etc.)

13. Participation in natural or artificial sunbathing within 6 weeks of the study start date.

4.3 Subject restrictions

1. No exercise with either arm and no strenuous exercise overall, for study duration.
2. No bathing or showering during the periods of drug application and assessment of skin blanching.
3. No use of creams, emollients, or similar products on forearms for 24 hours prior to and throughout the study.
4. No alcohol may be taken by subjects from 24 hours prior to product application until the last visual assessment has been completed.
5. No caffeine intake greater than 500mg per day during the study. (A cup of coffee contains about 85mg of caffeine)
6. No prescription medication and OTC medication (e.g. cold preparations, vitamins, natural products used for therapeutic benefits, antacids) will be allowed for at least one week prior to the study.

   With the exception of study products no concomitant medication will be taken by subjects during the study.
7. Smoking will not be permitted during the course of the study.

Subjects will be informed of the above restrictions and each subject will be specifically questioned on these points prior to product application. Any deviations from the above restrictions which are made known to the investigators either voluntarily or on questioning will be recorded on the appropriate case report form. A decision as to whether the affected subject continues with the study will be taken by the study investigators.

4.4 Criteria for Removal from the Study

Any subject may be withdrawn from the study at any time due to the following:

1. Voluntary withdrawal by the subject due to any reason.
2. Illness or injury during the study if regarded as clinically important by the study physician, Principal or Co-investigator.
3. Any adverse reactions or signs of toxicity if regarded as clinically important by the

study physician, Principal or Co-investigator.

4. Failure of the subject to comply with or be uncooperative towards any study requirements or restrictions if regarded as clinically important by the study physician, Principal or Co-investigator.

5. STUDY PROCEDURE

5.1 Pre- and Post-Study Medical Screening

Pre-study and post-study evaluations will be conducted as listed in the following table:

Note: Pre-study screening will be conducted not more than 14 days prior to start of the study. Post-study follow-up will be conducted within 48 hours of the final blanching assessment, termination or withdrawal from the study.

Screening Tests	**Pre-study**	**Post-study**
Medical History	✓[1]	✓[5]
Physical Examination	×	✓[6]
Dermatological Assessment	✓[2]	×
Blanching Response	✓[3]	×
Adhesive sensitivity	✓[4]	×

1. Medical History: Demographic data (date of birth, sex, age), emotional (psychiatric), skin, alcohol consumption, smoking habits, dietary habits, medications, allergies, illnesses (hypertension, circulatory diseases) and sporting commitments.
2. Dermatological: General assessment of the skin and any dermatological condition(s) which may influence the barrier function of the skin and the impact on the absorption of the clobetasol 17-propionate. Assessment of skin colour difference between the two arms which may influence the measurement of the blanching efficacy.
3. Blanching response: Assessment of subject's ability to blanch after topical application of clobetasol 17-propionate 0.05% cream.
4. Adhesive sensitivity: Assessment of subject's sensitivity to adhesive on application site demarcation tape.
5. Medical History: Since start of study.
6. Physical Examination: Examination of forearms and applications sites.

If any study related abnormalities are observed at the post-study medical, appropriate follow-up action will be taken and re-examination conducted until the abnormality returns to normal or until the study physician considers the abnormality to be clinically insignificant.

5.2 Blanching Response and Adhesive Label Sensitivity Screening

Screening for blanching response and adhesion label sensitivity will be conducted no less than 24 hours prior to the start of the study. Volunteers will be assessed on their ability to blanch in response to the topical application of the corticosteroid. They will also be assessed on their tolerance to the adhesive on medical tape used to demarcate the application site.

About 11mg of clobetasol 17-propionate cream 0.05% will be applied to a site (11 X 11 mm) on the upper arm demarcated by an adhesive label. Six hours after application, the medical tape will be removed slowly and residual cream removed by gently washing with soap and water. Application sites will be assessed for blanching four hours later.

Volunteers who exhibit blanching will be categorized as poor, good or excellent responders on the basis of visual observations and will qualify as candidates for the study. Volunteers who do not blanch will not qualify. The majority of subjects enrolled into the study will be good responders and the remaining subjects will be made up of similar numbers of poor and excellent responders.

5.3 Check-in and Confinement

Subjects will check-in at the Biopharmaceutics Research Institute (BRI) at 07:00 hours on the morning of the study day when they undergo a brief medical examination, inclusion and exclusion criteria check and study restriction check.

Subjects accepted into the study will then be prepared for application of the study product. Following application, subjects will stay in the BRI clinic for the course of the study. On the last day, the subjects will be allowed to leave the BRI clinic after the first assessment of the day and they will be instructed to return at specified times for the rest of the assessments until completion of the study. However, if a subject is experiencing adverse reaction(s) the affected subject will need to remain at the BRI until alternative supervision outside the clinic

has been arranged.

5.4 Posture and Physical Activity

Subjects will not be restricted with respect to posture during the study. However, the application sites on the ventral forearm will be protected by custom made guards to avoid contact with any material or bodily contact, until the subjects are told by the study observers that they are permitted to remove them. Strenuous physical exercise will not be allowed particularly on the forearms.

5.5 Food and fluids

Subjects will not be restricted with respect to food and fluid intake except for those listed in the restricted terms.

5.6 Subject Monitoring

1. The study physician will be contactable by phone for the duration of the study.
2. Subjects will be asked open-ended questions about their health at the time of each assessment and any adverse reactions will be recorded.
3. Subjects will be reminded of study restrictions at the time of each assessment.

6. PILOT STUDY

6.1 Study design

1. Dose duration-response study is based solely on the reference product, with randomization of dose duration skin sites.
2. Dose durations of 0.25 to 6 hours will be used for the application of the product onto the specified sites.
3. Untreated control sites on each arm will also be utilised to enable correction of active drug skin sites for color changes during the study unrelated to drug exposure.
4. Chromameter and visual measurement of the pharmacodynamic response to the topical corticosteroid will be measured at various time periods.

5. A minimum of twelve subjects will be enrolled.

6.2 Method of Application and Removal

The method of a staggered application with synchronized removal of the topical corticosteroid will be used. The product will be applied to skin sites at different times and removed simultaneously.

Just prior to the first application time of the product, sufficient eppendorf (0.2ml) pipettes will be filled with the product. The settings (3 on the dial of the eppendorf) for the correct amount (11 mg) of the product required for application on each site will be adjusted. The Epperdorf pipettes will be used to ensure that an accurate amount of product is applied to each application site for each subject.

Eight application sites will be demarcated on the ventral surface of each forearm using adhesive labels with 1.1cm x 1.1cm squares punched out at appropriate intervals (2.5cm). The eight application sites will be aligned in a straight line on the ventral-side of the forearm from the elbow joint to the wrist. The product will be applied to the eight sites on each forearm according to the appropriate application template (See appendix 1). The twelve subjects will be evenly divided (four subjects per group) and will be allocated a certain template per group of subjects.

The product will be applied to the first subject at 08:00 and to subsequent subjects at five minutes intervals thereafter. Approximately 11mg of the product will be applied to each site from the preloaded eppendorf pipettes at the start of each appropriate dose durations. Individual doses of the product will be spread evenly over the application site using a glass rod. The product will be applied according to the three different application templates to ensure that no discernable pattern in blanching can be perceived by observers during the course of the study.

Removal of the product will be done by gently washing all the skin sites with soap and water then blotting dry with a non-abrasive towel, thereafter allowing the forearm to air-dry for at least five minutes prior to assessment

6.3 Visual and Chromameter Assessment of Blanching

Skin blanching will be assessed at time 0, 2, 4, 6, 8, 10, 12, 14, 18, 22 and 26 hours after product removal. Time 0 equals time of product removal. After the removal of the product and the adhesive label, the forearms will be air- dried for 5 minutes to allow equilibration with the room environment prior to the time zero reading.

Visual assessment of blanching over the whole application site will be based on a subjective 0 – 4 intensity scale as given below:

0 = No discernible blanching

1 = Faint blanching

2 = Faint – to – moderate blanching

3 = Moderate – to – strong blanching

4 = Strong – to – intense (maximal) blanching

Each subject will be assessed and assigned a blanching score to each site at each assessment time by comparing skin at the site of application with adjacent skin unaffected by blanching.

A mechanically mounted chromameter will be used for the assessment of blanching over the whole application site. The chromameter will be aligned into position directly over and covering the application site with the measuring head. The same orientation of the chromameter will be maintained to get accurate readings for each assessment time for each site. Three consecutive readings will be taken on the each site without moving the chromameter. The three readings will be automatically averaged by the chromameter and the mean value reported.

Visual and chromameter assessment will be made with the forearm placed horizontally in front of the observer with the medial surface facing upwards and illuminated by normal fluorescent lighting. A total of ten assessments, including pre-dose baseline assessment, will be made for each subject over the duration of the study.

The exact clock times for assessments will be calculated according to the time of product application (see appendix 2). The actual clock time of assessments will be recorded for each subject. Any deviation from the assessment schedule of more than 10 minutes will be

reported as a protocol deviation.

6.4 Study Day Activities and Procedures

1. Study sessions will begin at approximately the same time each study day (within one hour).
2. Verification by history of adequate washout of excluded drugs.
3. The forearm should be free of any dirt or particulate matter before the study, thus it will be necessary for the forearms to be clean. Subjects will be instructed to use soap and water to clean their forearms and then blot them dry with non-abrasive towel, not less than 2 hours prior to the application of the product.
4. Eight application sites of identical surface area (1.44 cm^2) will be demarcated with an adhesive label on the ventral side of each forearm. The application sites will be spaced as close as 2.5cm and will not be closer than 3 – 4cm to the wrist and elbow joint.
5. The product will be applied to the sites according to the pre-determined application templates (see appendix 1) designed for each group of subjects. The three different application templates consist of six dose durations that will be equally divided between the two arms i.e. 0.25, 0.5, 1, 2, 4 and 6 hours.
6. The application templates include two randomly assigned, untreated control sites that will be used per arm for the studies based on chromameter readings. The use of untreated control sites will enable the correction of active drug skin sites for any color changes during the study unrelated to drug exposure.
7. Application of the product to the first subject will be at the following times: 08:00, 10:00, 12:00, 13:00, 13:30, 13:45 and 14:00. Subsequent subjects will be done at 5 minute intervals.
8. A protective, non-occlusive guard will be used for each forearm to cover the application sites to prevent smearing or removal of the topical product from the skin sites.
9. Prior to the measurement of the pharmacodynamic response at the end of the application period, the non-occlusive guards, and the adhesive labels, and the remaining topical corticosteroid will be removed. Removal of the product will be done by gently washing all the skin sites with soap and water then blotting dry with a non-abrasive towel, thereafter allowing the forearm to air-dry for at least five minutes prior to assessment at time zero.
10. Opposite sides of the demarcated application sites and the corresponding sites on the

chromameter base will be marked with reference marks to ensure the exact placement of the chromameter over the site for successive readings.

11. The site closest to the wrist will be labeled as site 1 and the site closest to the elbow joint will be site 8.
12. Measurements will be done using the mechanically mounted chromameter and visually by the observers, which will start at site 1 to site 8 on the left arm before proceeding to the right arm.
13. Assessment will be done according to the pre-determined assessment times as shown in appendix 2. Assessment will begin for subject 1 at the following times: On day 1 - 14:05, 16:00, 18:00, 20:00, 22:00, and on day 2 – 09:00, 12:00, 14:00, 16:00. The subsequent subjects will be assessed at 5 minute intervals.
14. The results of the blanching assessment will be recorded on the forms shown in appendix 2 for each subject.

Note: At least one reading scheduled between 5pm and midnight.

6.5 Data Analysis

The chromameter raw data of each skin blanching response vs. time profile will be adjusted with the baseline value and the untreated control site at that site. Adjustment with the baseline value will be the difference between the measurement after product removal and the baseline measurement for all treated and untreated sites on that arm. Adjustment with the untreated control site will be calculated by taking the treated baseline-adjusted site reading and subtracting it from the average of the untreated control site reading on that arm. The area under the effect curve (AUEC) for each baseline-adjusted, untreated control site-corrected dose duration will be calculated using the trapezoidal rule.

AUEC vs. dose duration data will be fitted based on the assumption of a nonlinear mixed effect model using suitable software. The pharmacokinetic parameters (ED_{50}, D_1 and D_2) will be obtained from this model. D_1 and D_2 (33% and 67% respectively of the maximal response) will represent the sensitive portion of the dose duration-response curve.

The AUEC for each vasoconstriction time profile will be calculated using the visual data as well. The dose duration-response data will also be fitted based on the assumption of a nonlinear mixed effect model using suitable software, thus ED_{50}, D_1 and D_2 will also be

determined.

From the skin blanching response vs. time profiles, the following pharmacokinetic parameters will be derived as described in the FDA Guidance:

1. E_{max}: The maximum blanching effect observed in a subject's skin blanching vs. time profile following the application of the product.
2. T_{max}: The skin blanching assessment time at which the E_{max} was attained. If this occurs at more than one time point, it is defined as the first time point with this value (hour).
3. $AUEC_{(0-26)}$: This is the area under the effect curve for time 0 – 24 hours. This can be obtained using the trapezoidal rule.
4. ED_{50}: Dose duration at which 50% of the maximum effect is obtained.
5. D_1: The shorter dose duration calibrator i.e. equal approximately 0.5 times ED_{50}.
6. D_2: The longer dose duration calibrator i.e. equal approximately 2 times $ED_{50.}$

The study data and summary results will be arranged in the format as depicted in the FDA Guidance.

6.6 Statistical Analysis

The mean, standard deviation and coefficient of variation will be calculated for the following data:

1. Skin blanching response at each time point for each dose duration using individual means.
2. E_{max}, T_{max} and $AUEC_{(0-26)}$

7. REFERENCES

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APPENDIX 1

Application Template

TEMPLATE 1

Subject's Right Arm		Subject's Left Arm	
	Ventral	**Ventral**	
Proximal	8 – 0.25	8 – 0.25	**Proximal**
(elbow)	7 – 0.5	7 – 0.5	**(elbow)**
	6 – UT	6 – UT	
	5 – 1	5 – 1	
	4 – 2	4 – 2	
	3 – UT	3 – UT	
Distal	2 – 4	2 – 4	**Distal**
(wrist)	1 – 6	1 – 6	**(wrist)**

TEMPLATE 2

Subject's Right Arm		Subject's Left Arm	
	Ventral	**Ventral**	
Proximal	8 – UT	8 – UT	**Proximal**
(elbow)	7 – 2	7 – 2	**(elbow)**
	6 – 4	6 – 4	
	5 – UT	5 – UT	
	4 – 6	4 – 6	
	3 – 1	3 – 1	
Distal	2 – 0.5	2 – 0.5	**Distal**
(wrist)	1 – 0.25	1 – 0.25	**(wrist)**

TEMPLATE 3

Subject's Right Arm		**Subject's Left Arm**	
	Ventral	**Ventral**	
Proximal	8 – 2	8 – 2	**Proximal**
(elbow)	7 – 0.5	7 – 0.5	**(elbow)**
	6 – 1	6 – 1	
	5 – 0.25	5 – 0.25	
	4 – UT	4 – UT	
	3 – 6	3 – 6	
Distal	2 – 4	2 – 4	**Distal**
(wrist)	1 – UT	1 – UT	**(wrist)**

APPENDIX 2

Product Application Times

APPLICATION TIMES

Subject 1: **Template 1**

Application Times (hours before drug removal)	6	4	2	1	0.5	0.25	Removal
Clock Time	08:00	10:00	12:00	13:00	13:30	13:45	14:00
Actual Time							

Subject 2: **Template 2**

Application Times (hours before drug removal)	6	4	2	1	0.5	0.25	Removal
Clock Time	08:05	10:05	12:05	13:05	13:35	13:50	14:05
Actual Time							

Subject 3: **Template 3**

Application Times (hours before drug removal)	6	4	2	1	0.5	0.25	Removal
Clock Time	08:10	10:10	12:10	13:10	13:40	13:55	14:10
Actual Time							

Subject 4: **Template 1**

Application Times (hours before drug removal)	6	4	2	1	0.5	0.25	Removal
Clock Time	08:15	10:15	12:15	13:15	13:45	14:00	14:15
Actual Time							

Subject 5: **Template 2**

Application Times (hours before drug removal)	**6**	**4**	**2**	**1**	**0.5**	**0.25**	**Removal**
Clock Time	**08:20**	**10:20**	**12:20**	**13:20**	**13:50**	**14:05**	**14:20**
Actual Time							

Subject 6: **Template 3**

Application Times (hours before drug removal)	**6**	**4**	**2**	**1**	**0.5**	**0.25**	**Removal**
Clock Time	**08:25**	**10:25**	**12:25**	**13:25**	**13:55**	**14:10**	**14:25**
Actual Time							

Subject 7: **Template 1**

Application Times (hours before drug removal)	**6**	**4**	**2**	**1**	**0.5**	**0.25**	**0**
Clock Time	**08:30**	**10:30**	**12:30**	**13:30**	**14:00**	**14:15**	**14:30**
Actual Time							

Subject 8: **Template 2**

Application Times (hours before drug removal)	**6**	**4**	**2**	**1**	**0.5**	**0.25**	**0**
Clock Time	**08:35**	**10:35**	**12:35**	**13:35**	**14:05**	**14:20**	**14:35**
Actual Time							

Subject 9: **Template 3**

Application Times (hours before drug removal)	**6**	**4**	**2**	**1**	**0.5**	**0.25**	**0**
Clock Time	**08:40**	**10:40**	**12:40**	**13:40**	**14:10**	**14:25**	**14:40**
Actual Time							

Subject 10: **Template 1**

Application Times (hours before drug removal)	**6**	**4**	**2**	**1**	**0.5**	**0.25**	**0**
Clock Time	**08:45**	**10:45**	**12:45**	**14:45**	**14:15**	**14:30**	**14:45**
Actual Time							

Subject 11: **Template 2**

Application Times (hours before drug removal)	**6**	**4**	**2**	**1**	**0.5**	**0.25**	**0**
Clock Time	**08:50**	**10:50**	**12:50**	**14:50**	**14:20**	**14:35**	**14:50**
Actual Time							

Subject 12: **Template 3**

Application Times (hours before drug removal)	**6**	**4**	**2**	**1**	**0.5**	**0.25**	**0**
Clock Time	**08:55**	**10:55**	**12:55**	**14:55**	**14:25**	**14:40**	**14:55**
Actual Time							

STUDY PARTICIPATION INFORMED CONSENT FORM

INFORMED CONSENT

I ..... born on .....

Current address .....

.....

hereby give permission that the formulations stated below be administered to me during the course of this study.

Reference Product: Dermovate® Cream (Clobetasol 17-Propionate 0.05%)

My consent is given freely and I realise that it may be withdrawn at any time, without penalty to me.

I have been fully informed by ..... regarding the possible adverse effects of the drug, procedures to be used in this study and the risks thereof, as detailed in the "Patient Information Brochure".

- I undertake to comply with all the relevant conditions contained in the Patient Information Brochure and confirm that I accept any consequences arising as a result of my withholding or misrepresenting any information required of me. I agree to undergo the necessary pre- and post-study medical screenings as listed in the Patient Information Brochure.
- I undertake to inform the supervising medical personnel immediately of any symptoms - expected or unexpected - which I might experience.
- I agree to my medical records being reviewed in the event of an audit, enquiry, monitoring and/or inspection on the understanding that my anonymity will be maintained.
- I have been informed that protocol violation may result in my exclusion from the study and forfeiture of the agreed upon remuneration. I acknowledge that instructions relating to my participation in this study have been communicated to me both verbally and in writing, and that I understand them.
- I also declare that I have made the necessary arrangements regarding the attendance of lectures and other academic activities.
- I acknowledge that I will receive R200.00 for full participation in this study and that I will receive a pro-rated amount if I withdraw from the study before the end.
- Signing this consent form does not in any way waive my individual legal rights.

Sign Volunteer .....Date: .....

Sign Witness .....

Signature of person who conducted Informed Consent Discussion …….

PATIENT INFORMATION BROCHURE

Protocol No. WL1: A Pilot Dose Duration-Response Study of Clobetasol 17-Propionate 0.05% Cream in Healthy Volunteers.

Introduction

You have been invited to take part in a research study. Before you decide whether or not to participate, it is important for you to understand why the study is being done and what it will involve if you agree to participate.

Please read this document carefully. It provides information about this study to help you decide if you wish to participate. Discuss it with your friends and relatives if you wish. Please feel free to ask for explanations on anything that you do not understand. You will be given as much time as you need to decide on whether you would like to participate or not.

Your participation is completely voluntary. You do not have to give any reasons for not wanting to participate or withdrawing during the study. If you decide to take part in the study, you will be asked to confirm your agreement by signing the consent form.

Purpose of the study

The rate and extent of drug absorption from a drug formulation taken orally can be assessed indirectly from blood samples. However, this can not be applied to topical formulations that are meant for local use with no intent of entry into the systemic circulation.

The use of topical corticosteroids results in vasoconstriction which manifests as a change in skin colour and can be detected by the eye. This vasoconstriction is also known as skin blanching phenomenon. Cornell and Stoughton have shown that the intensity of the induced blanching is directly proportional to the clinical efficacy of the corticosteroid. Because this method is acceptable (to the FDA) for testing such formulations it forms a basis of comparison against other available methods such as tape stripping, suction blister formation and microdialysis to mention some.

Study Drug – Clobetasol 17-Propionate cream 0.05%

Clobetasol 17-propionate is a highly potent synthetic corticosteroid that has anti-inflammatory, antipruritic, and vasoconstrictive properties. It is used in the topical treatment of various skin conditions such as eczema, some forms of psoriasis and seborrhoeic dermatitis. Clobetasol 17-propionate is indicated for use in short courses for skin conditions which do not respond satisfactorily to less active steroids.

Duration of the study

The whole study will last for approximately two days. You will need to stay within the BRI clinic for the first day and night. On the second day, you will be allowed to leave the clinic but must return approximately 10 minutes before the next specified assessment time until completion of the study.

Blanching Response Study Procedure

- On the morning of the study you must check into the BRI clinic at 7:00am to undergo a brief medical examination, inclusion and exclusion criteria check and study restriction check. You will then be prepared for product application by having adhesive labels applied to your forearms.
- From 7.30 onwards you will have cream applied to small areas of your forearms. This cream will be left in contact with the skin for a period of 6 hours.
- After 6 hours the adhesive labels will be gently removed and your forearms washed with soap and water and then dried.
- Skin blanching will be assessed at the following time intervals: 5 minutes after washing and then again at 2, 4, 6, 8, 19, 22, 24 and 26 hours after washing. You will be given a timetable of your scheduled assessment times.
- Assessments will be done visually and by a chromameter.

You are not restricted with regard to food and drink or activities during the study period as long as you abide by the study restrictions. Professional medical assistance (registered medical practitioner and/or a registered nurse) will be available throughout the course of the study. Should any new and significant information about the study medication become

available during the course of the study, this will be communicated to you.

Side Effects and Contraindications

Upon application of the cream on the skin, you may experience burning sensation, stinging of the skin and itching.

Potent corticosteroid creams should only be used for short courses. Prolonged and intensive treatment with the corticosteroid cream may cause local atrophic changes such as stretch marks, thinning of the skin, loss of elasticity and blanching effect. However, these are more likely to occur on the face, the use of occlusive dressings e.g. plasters, or where skin folds are present.

Administration should be avoided in persons who exhibit allergic responses to clobetasol 17-propionate or to any ingredient in the preparation or to other corticosteroids. It is contraindicated in the presence of acute dermatological infections e.g. scalp infection. It is also contraindicated in pregnancy.

Subject Inclusion Criteria

6. Mentally competent subjects who are available for the entire study period and willing to adhere to the protocol requirements and able to give informed consent by signing the informed consent form, which s also signed by a witness.
7. Subjects demonstrating adequate vasoconstriction to topical corticosteroids, i.e. ‘responders’.
8. Male or female between 18 and 50 years of age.
9. Normal in terms of dermatological medical history at the pre-study screening medical, or in the case of an abnormality, the medical practitioner considers the abnormality to be clinically insignificant.
10. Normal in terms of dermatological examination at the pre-study screening medical, or in the case of an abnormality, the medical practitioner considers the abnormality to be clinically insignificant.

Subject Exclusion Criteria

14. Smoking within one week of study.
15. Caffeine intake greater than 500mg per day prior to or during the study. (A cup of coffee contains about 85mg of caffeine)
16. Clinically significant history of alcoholism or drug abuse.
17. Use of topical dermatologic drug therapy on ventral forearms, including prior dosing of topical corticosteroid in pharmacodynamic study to a particular skin site, within one month prior to study.
18. Adverse reactions to topical or systemic corticosteroids.
19. Any current or past medical condition, including active dermatitis or any other dermatologic condition, which might significantly affect pharmacodynamic response to the administered drug.
20. Persons who would require shaving ventral forearms to insure consistent dose on skin surface.
21. Use of any vasoactive (constrictor or dilator) medication, prescription or OTC, that could modulate blood flow. Examples of such drugs include nitroglycerin, antihypertensives, antihistamines, NSAID's, aspirin, and OTC cough/cold products containing antihistamines and/or either phenypropanolamine or phentolamine.
22. Any obvious difference in skin color between arms.
23. Any mental deficiency or handicap.
24. Any contact allergy to the label adhesive on labels used to demarcate application sites.
25. Consumption of alcohol or other enzyme inducing agents within 24 hours of the start of the study (all barbiturates, corticosteroids, phenylhydantoins, etc.)
26. Participation in natural or artificial sunbathing within 6 weeks of the study start date.

Restrictions during the study

8. No exercise with either arm and no strenuous exercise overall, for study duration.
9. No bathing or showering during the periods of drug application and assessment of skin blanching.
10. No use of creams, emollients, or similar products on forearms for 24 hours prior to and throughout the study.
11. No alcohol may be taken by subjects from 24 hours prior to product application until the last visual assessment has been completed.

12. No caffeine intake greater than 500mg per day during the study. (A cup of coffee contains about 85mg of caffeine)
13. No prescription medication and OTC medication (e.g. cold preparations, vitamins, natural products used for therapeutic benefits, antacids) will be allowed for at least one week prior to the study.

    With the exception of study products no concomitant medication will be taken by subjects during the study.
14. Smoking will not be permitted during the course of the study.

Criteria for Removal from the Study

5. Voluntary withdrawal by the subject due to any reason.
6. Illness or injury during the study if regarded as clinically important by the study physician, Principal or Co-investigator.
7. Any adverse reactions or signs of toxicity if regarded as clinically important by the study physician, Principal or Co-investigator.
8. Failure of the subject to comply with or be uncooperative towards any study requirements or restrictions if regarded as clinically important by the study physician, Principal or Co-investigator.

Remuneration

You will receive a gratuity of R200.00 for full participation in this study. Payment following withdrawal from the study will be calculated on a pro-rated basis.

For any questions or queries please contact the principal investigating researcher:
Genevieve Au (BPharm. R.U.)
Email: **g02a1351@campus.ru.ac.za**
Cell: **072 612 0972**

APPENDIX II

RESEARCH PROTOCOL

A DOUBLE-BLIND SKIN BLANCHING PIVOTAL STUDY OF CLOBETASOL 17-PROPIONATE 0.05% CREAMS IN HEALTHY VOLUNTEERS

STUDY NO. WL2

Protocol version 2

29-March-2007

Test Product	Clobetasol 17-Propionate 0.05% In-house batch no. WL2-02
Reference Product	Clobetasol 17-Propionate 0.05% In-house batch no. WL2-01

Conducted by	Wai Ling Au
Supervised by	Prof. I. Kanfer Rhodes University Grahamstown, 6140 South Africa

_____ _____

Investigator — Date (yyyy-mm-dd)

Wai Ling Au

_____ _____

Supervisor — Date (yyyy-mm-dd)

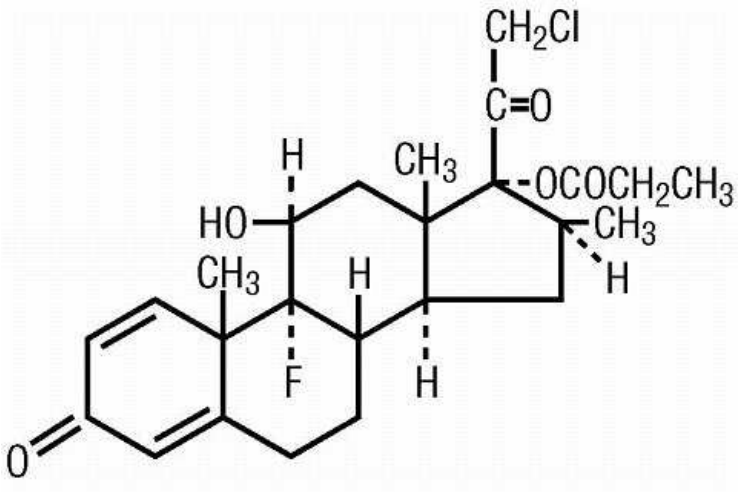
Prof. I. Kanfer

CONTENTS

1. BACKGROUND INFORMATION

1.1 Molecular Structure

The molecular structure of clobetasol 17-propionate is represented below.



clobetasol 17-propionate

1.2 Mode of Action and Uses

Clobetasol 17-propionate is a highly potent synthetic corticosteroid that has anti-inflammatory, antipruritic, and vasoconstrictive properties. Its anti-inflammatory effect is thought to be due to its ability to control biosynthesis of potent mediators of inflammation. It stimulates phospholipase A(2) inhibitory proteins and subsequently blocks the release of arachidonic acid, which is a common precursor to leukotrienes and prostaglandins [1].

Clobetasol 17-propionate is used in the topical treatment of various skin disorders such as eczema, some forms of psoriasis and seborrhoeic dermatitis [2].

1.3 Pharmacokinetics

To achieve a therapeutic effect, it is necessary that the topically applied drug enters the epidermis or the dermis of the skin. Skin penetration and permeation of the drug after topical administration depend on the physicochemical properties of the drug molecule, the function

of the skin as a transport barrier and the composition of the delivery vehicle [4, 5, 6]. Topical use particularly under an occlusive dressing or when the skin is broken, systemic effects may occur if sufficient clobetasol 17-propionate is absorbed. Clobetasol 17-propionate is rapidly distributed to all body tissues. It can cross the placenta to varying degrees and may be excreted in small amounts in breast milk. The synthetic corticosteroids exhibits less extensive protein binding and tend to have longer half-lives when compared to the other corticosteroids. Metabolism of clobetasol 17-propionate occurs in the liver and is excreted via the urine and bile [2].

1.4 Adverse Effects

Upon topical application of clobetasol 17-propionate, one may experience burning sensation, pruritus and stinging of the skin. Headache and other irritation symptom(s) may occur [1].

1.5 Contraindications

Administration should be avoided in persons who exhibit hypersensitivity to clobetasol 17-propionate or to any ingredient in the preparation or to other corticosteroids. It is contraindicated in the presence of acute dermatological infections e.g. scalp infection [1].

2. STUDY PRODUCTS

2.1 Description

	Test Product	**Reference Product**
Generic Name	Clobetasol 17-Propionate	Clobetasol 17-Propionate
Dosage Form	Cream	Cream
Strength	0.05% m/m	0.05% m/m
In-House Batch #	WL2-01	WL2-02
Expiry Date	01/2009	01/2009
Description	White cream	White cream
Dosage	12µL (±11mg) per application site	12µL (±11mg) per application site

2.2 Supply, Storage and Use

Sufficient products will be obtained by the investigator at a retail outlet prior to the study. Clobetasol 17-propionate is available in cream, ointment or lotion dosage forms. However, only the cream will be utilized in this study. It should be used externally only. The product should be kept in the container it came in, tightly closed and stored at room temperature away from excess heat and moisture [3].

3. OBJECTIVES

The objective of the pivotal study is to measure the skin blanching efficacy to determine the *in vivo* bioequivalence between the test and reference products, and for the comparison of the two assessment methods. The two assessment techniques involve measuring with a chromameter or visually by trained individuals. The data generated from the studies will be used to calculate the 90% confidence interval using Locke's method for the difference between the test and reference product. Furthermore, the data will be used for the comparison of the two assessment methods.

The study will be conducted according to the FDA Guidance for Industry: Topical Dermatologic Corticosteroids, dated February 1997.

4. STUDY POPULATION AND MEDICAL ASSESSMENT

4.1 Inclusion Criteria

Only the subjects that meet the following criteria will be included in the study:

11. Mentally competent subjects who are available for the entire study period and willing to adhere to the protocol requirements and able to give informed consent by signing the informed consent form, which is also signed by a witness.
12. Subjects demonstrating adequate vasoconstriction and qualify as a 'detector'.
13. Male or female between 18 and 50 years of age.
14. Normal in terms of dermatological medical history at the pre-study screening medical,

or in the case of an abnormality, the medical practitioner considers the abnormality to be clinically insignificant.

15. Normal in terms of dermatological examination at the pre-study screening medical, or in the case of an abnormality, the medical practitioner considers the abnormality to be clinically insignificant.

4.2 Exclusion Criteria

Subjects that meet one or more of the following criteria will be excluded from the study:

27. Individuals smoking within one week of study.
28. Caffeine intake greater than 500mg per day prior to or during the study. (A cup of coffee contains about 85mg of caffeine)
29. Clinically significant history of alcoholism or drug abuse.
30. Use of topical dermatologic drug therapy on ventral forearms, including prior dosing of topical corticosteroid in pharmacodynamic study to a particular skin site, within one month prior to study.
31. Adverse reactions to topical or systemic corticosteroids.
32. Any current or past medical condition, including active dermatitis or any other dermatologic condition, which might significantly affect pharmacodynamic response to the administered drug.
33. Persons who would require shaving ventral forearms to insure consistent dose on skin surface.
34. Use of any vasoactive (constrictor or dilator) medication, prescription or OTC, that could modulate blood flow. Examples of such drugs include nitroglycerin, antihypertensives, antihistamines, NSAID's, aspirin, and OTC cough/cold products containing antihistamines and/or either phenypropanolamine or phentolamine.
35. Any obvious difference in skin color between arms.
36. Any mental deficiency or handicap.
37. Any contact allergy to the label adhesive on labels used to demarcate application sites.
38. Consumption of alcohol or other enzyme inducing agents within 24 hours of the start of the study (all barbiturates, corticosteroids, phenylhydantoins, etc.)
39. Participation in natural or artificial sunbathing within 6 weeks of the study start date.

4.3 Subject restrictions

15. No exercise with either arm and no strenuous exercise overall, for study duration.
16. No bathing or showering during the periods of drug application and assessment of skin blanching.
17. No use of creams, emollients, or similar products on forearms for 24 hours prior to and throughout the study.
18. No alcohol may be taken by subjects from 24 hours prior to product application until the last visual assessment has been completed.
19. No caffeine intake greater than 500mg per day during the study. (A cup of coffee contains about 85mg of caffeine)
20. No prescription medication and OTC medication (e.g. cold preparations, vitamins, natural products used for therapeutic benefits, antacids) will be allowed for at least one week prior to the study.

    With the exception of study products no concomitant medication will be taken by subjects during the study.
21. Smoking will not be permitted during the course of the study.

Subjects will be informed of the above restrictions and each subject will be specifically questioned on these points prior to product application. Any deviations from the above restrictions which are made known to the investigators either voluntarily or on questioning will be recorded on the appropriate case report form. A decision as to whether the affected subject continues with the study will be taken by the study investigators.

4.4 Pre- and Post-Study Medical Screening

Pre-study and post-study evaluations will be conducted as listed in the following table:

Note: Pre-study screening will be conducted not more than 14 days prior to start of the study. Post-study follow-up will be conducted within 48 hours of the final blanching assessment, termination or withdrawal from the study.

Screening Tests	**Pre-study**	**Post-study**

Medical History	✓[1]	✓[5]
Physical Examination	×	✓[6]
Dermatological Assessment	✓[2]	×
Blanching Response	✓[3]	×
Adhesive sensitivity	✓[4]	×

1. Medical History: Demographic data (date of birth, sex, age), skin, alcohol consumption, smoking habits, dietary habits, medications, allergies, illnesses (hypertension, circulatory diseases) and sporting commitments.
2. Dermatological: General assessment of the skin and any dermatological condition(s) which may influence the barrier function of the skin and the impact on the absorption of the clobetasol 17-propionate. Assessment of skin colour difference between the two arms which may influence the measurement of the blanching efficacy.
3. Blanching response: Assessment of subject's ability to blanch after topical application of clobetasol 17-propionate 0.05% cream to determine if they meet the criteria of being a 'detector'.
4. Adhesive sensitivity: Assessment of subject's sensitivity to adhesive on application site demarcation tape.
5. Medical History: Since start of study.
6. Physical Examination: Examination of forearms and applications sites.

If any study related abnormalities are observed at the post-study medical, appropriate follow-up action will be taken and re-examination conducted until the abnormality returns to normal or until the study physician considers the abnormality to be clinically insignificant.

4.5 Blanching Response and Adhesive Label Sensitivity Screening

Screening for blanching response and adhesion label sensitivity will be conducted no less than 24 hours prior to the start of the study. Volunteers will be assessed on their ability to blanch in response to the topical application of the corticosteroid over 24 hours at a controlled room temperature of ±22°C. They will also be assessed on their tolerance to the adhesive on medical tape used to demarcate the application site.

The following screening protocol will be utilized for the selection of possible 'detectors' for the pivotal blanching studies:

1. The volunteer's arms shall be washed 1 hour prior to product application.
2. Application of the reference product will start approximately 7:00am in the morning.

3. Two application sites (1.1cm x 1.1cm) on the side of each forearm will be demarcated with a label.
4. The sites will be situated approximately 3cm away from the elbow joint and towards the side of the arm.
5. Baseline reading for each demarcated site and untreated site will be measured by the chromameter.
6. The method of a staggered application with synchronized removal of the topical corticosteroid will be used.
7. 11mg of the reference cream will be applied to the 2 application sites on each forearm.
8. Two dose durations will be used per arm. Each site will be assigned one dose duration i.e. site 1 will be assigned the dose duration of 18-minutes (D_1) and site 2 will be 72-minutes (D_2).
9. The product will be removed by gentle washing with a mild soap and water, and will then be blotted dry gently with a paper towel.
10. Chromameter and visual assessment will be carried out at 0, 6, 8, 12 and 24 hours after product removal on the treated and untreated sites.

Individual dose duration-response will be based on the D_2 /D_1 ratio of AUEC values of the reference product. The minimum value should be 1.25 in order to qualify as a 'detector'.

4.6 Criteria for Removal from the Study

Any subject may be withdrawn from the study at any time due to the following:

9. Voluntary withdrawal by the subject due to any reason.
10. Illness or injury during the study if regarded as clinically important by the study physician, Principal or Co-investigator.
11. Any adverse reactions or signs of toxicity if regarded as clinically important by the study physician, Principal or Co-investigator.
12. Failure of the subject to comply with or be uncooperative towards any study requirements or restrictions if regarded as clinically important by the study physician, Principal or Co-investigator.

5. STUDY PROCEDURE

5.1 Study design

6. The pivotal study is based on the reference product and the test product, with randomization of sites.
7. Dose durations of 18, 36 and 72 minutes will be used for the application of the products onto the specified sites.
8. Untreated control sites on each arm will also be utilised to enable correction of active drug skin sites for color changes during the study unrelated to drug exposure.
9. Chromameter and visual measurement of the pharmacodynamic response to the topical corticosteroid will be measured at various time periods.
10. Room temperature will be controlled to be at ±22°C.
11. A minimum of sixty 'detectors' will be enrolled in groups of 12.
12. The study will be conducted on each group on different days as recruitment permits.

5.2 Check-in and Confinement

Subjects will check-in at the Biopharmaceutics Research Institute (BRI) clinic at 05:30 hours on the morning of the study day when they undergo a brief medical examination, inclusion and exclusion criteria check and study restriction check.

Subjects accepted into the study will then be prepared for application of the study product. Following application, subjects will stay in the BRI clinic for the first day and night of the study. The final day of the study, the subjects will be allowed to leave the BRI clinic after the first assessment of the day and they will be instructed to return at specified times for the rest of the assessments until completion of the study. The duration of the study lasts for 36 hours. However, if a subject is experiencing adverse reaction(s) the affected subject will need to remain at the BRI clinic until alternative supervision outside the clinic has been arranged.

5.3 Product Application and Removal

The method of a staggered application with synchronized removal of the topical corticosteroid will be used i.e. the products will be applied to skin sites at different times and removed simultaneously. Application of the products will be done by trained individuals other than the visual assessors and volunteers.

Just prior to the first application time of the product, sufficient eppendorf® (0.2ml) pipettes will be filled with the product. The settings (3 on the dial of the eppendorf®) for the correct amount (12µL)of the product required for application on each site will be adjusted. The eppendorf® pipettes will be used to ensure that an accurate amount of product is dispensed to each application site for each subject.

Eight application sites will be demarcated on the ventral surface of each forearm using adhesive labels with 1.1cm x 1.1cm squares punched out at appropriate intervals (2.5cm). The eight application sites will be aligned in a straight line on the ventral-side of the forearm from the elbow joint to the wrist. The product will be applied to the sites on each forearm according to the selected application template (See appendix 1). The twelve subjects will be evenly divided (three subjects per group) and will be allocated a certain template per group of subjects.

The product will be applied to the first subject at 07:00 hours and to subsequent subjects at five minutes intervals thereafter. Approximately 11mg of the product will be applied to each site from the preloaded eppendorf pipettes at the start of each appropriate dose durations. Individual doses of the product will be spread evenly over the application site using a glass rod. The products will be applied according to the four different application templates to ensure that no discernable pattern in blanching can be perceived by observers during the course of the study.

Removal of the product will be done by gently washing all the skin sites with soap and water then blotting dry with a non-abrasive towel, thereafter allowing the forearm to air-dry for at least five minutes prior to assessment

5.4 Visual and Chromameter Assessment of Blanching

Skin blanching will be assessed at time 0, 2, 4, 6, 8, 10, 12, 15, 22, 25 and 30 hours after product removal. Time 0 equals time of product removal. After the removal of the product and the adhesive label, the forearms will be air-dried for 5 minutes to allow equilibration with the room environment prior to the time zero reading.

Visual assessment of blanching over the whole application site will be based on a subjective 0 – 4 intensity scale as given below:

0 = No discernible blanching

1 = Faint blanching

2 = Faint – to – moderate blanching

3 = Moderate – to – strong blanching

4 = Strong – to – intense (maximal) blanching

Each subject will be assessed and assigned a blanching score to each site at each assessment time by comparing skin at the site of application with adjacent skin unaffected by blanching.

A patented, mechanically mounted chromameter will be used for the assessment of blanching over the whole application site. The chromameter will be aligned into position directly over and covering the application site with the measuring head. The same orientation of the chromameter will be maintained to get accurate readings for each assessment time for each site. Three consecutive readings will be taken on the each site without moving the chromameter. The three readings will be automatically averaged by the chromameter and the mean value reported.

Visual and chromameter assessment will be made with the forearm placed horizontally in front of the observer with the medial surface facing upwards and illuminated by normal fluorescent lighting. A total of ten assessments, including pre-dose baseline assessment, will be made for each subject over the duration of the study.

The exact clock times for assessments will be calculated according to the time of product application (see appendix 2). The actual clock time of assessments will be recorded for each subject. Any deviation from the assessment schedule of more than 10 minutes will be

reported as a protocol deviation.

5.5 Study Day Activities and Procedures

15. Study sessions will begin at approximately the same time each study day (within one hour).
16. Verification by history of adequate washout of excluded drugs.
17. The forearm should be free of any dirt or particulate matter before the study, thus it will be necessary for the forearms to be clean. Subjects will be instructed to use soap and water to clean their forearms and then blot them dry with non-abrasive towel, not less than 2 hours prior to the application of the product.
18. Eight application sites of identical surface area (1.21 cm^2) will be demarcated with an adhesive label on the ventral side of each forearm. The application sites will be spaced as close as 2.5cm and will not be closer than 3 – 4cm to the wrist and elbow joint.
19. The product will be applied to the sites according to the pre-determined application templates (see appendix 1) designed for each group of subjects.
20. The application templates include two randomly assigned, untreated control sites that will be used per arm for the studies based on chromameter readings. The use of untreated control sites will enable the correction of active drug skin sites for any color changes during the study unrelated to drug exposure.
21. Application of the products to the first subject will be at the following times: 07:00, 07:36, and 07:54. Subsequent subjects will be done at 5 minute intervals.
22. A protective, non-occlusive guard will be used for each forearm to cover the application sites to prevent smearing or removal of the topical product from the skin sites.
23. Prior to the measurement of the pharmacodynamic response at the end of the application period, the non-occlusive guards, and the adhesive labels, and the remaining topical corticosteroid will be removed. Removal of the product will be done by gently washing all the skin sites with soap and water then blotting dry with a non-abrasive towel, thereafter allowing the forearm to air-dry for at least five minutes prior to assessment at time zero.
24. Opposite sides of the demarcated application sites and the corresponding sites on the chromameter base will be marked with reference marks to ensure the exact placement of the chromameter over the site for successive readings.
25. The site closest to the wrist will be labeled as site 1 and the site closest to the elbow

joint will be site 8.

26. Measurements will be done using the mechanically mounted chromameter and visually by the observers, which will start at site 1 to site 8 on the left arm before proceeding to the right arm.
27. Assessment will be done according to the pre-determined assessment times as shown in appendix 2. Assessment will begin for subject 1 at the following times: On day 1 – 08:17, 10:17, 12:17, 14:17, 16:17, 18:17, 20:17, 23:17 and on day 2 – 06:17, 09:17, and 14:17. The subsequent subjects will be assessed at 5 minute intervals.
28. The actual time and/or results of the blanching assessment will be recorded on the forms shown in appendix 3 for each subject.

Note: At least one reading scheduled between 5pm and midnight.

5.6 Posture and Physical Activity

Subjects will not be restricted with respect to posture during the study. However, the application sites on the ventral forearm will be protected by custom made guards to avoid contact with any material or bodily contact, until the subjects are told by the study observers that they are permitted to remove them. Strenuous physical exercise will not be allowed particularly on the forearms.

5.7 Food and fluids

Subjects will not be restricted with respect to food and fluid intake except for those listed in the restricted terms.

5.8 Subject Monitoring

4. The study physician will be contactable by phone for the duration of the study.
5. Subjects will be asked open-ended questions about their health at the time of each assessment and any adverse reactions will be recorded.
6. Subjects will be reminded of study restrictions at the time of each assessment.

6 Data Analysis

The chromameter raw data of each skin blanching response vs. time profile will be adjusted with the baseline value and the untreated control site at that site. Adjustment with the baseline value will be the difference between the measurement after product removal and the baseline measurement for all treated and untreated sites on that arm. Adjustment with the untreated control site will be calculated by taking the treated baseline-adjusted site reading and subtracting it from the average of the untreated control site reading on that arm. The area under the effect curve (AUEC) for each baseline-adjusted, untreated control site-corrected dose duration will be calculated using the trapezoidal rule. AUEC vs. dose duration data will be fitted based on the assumption of a nonlinear mixed effect model using suitable software.

Only the data of the "detectors" i.e. subjects whose AUEC values at D_1 and D_2 are both negative and meet the dose duration-response criterion below, will be included in the data analysis. The dose duration-response criterion is:

$$\text{AUEC at } D_2 / \text{AUEC at } D_1 \geq 1.25$$

Where:

AUEC at D_2 = 0.5 [AUEC at D_2 (left arm) + AUEC at D_2 (right arm)];

AUEC at D_1 = 0.5 [AUEC at D_1 (left arm) + AUEC at D_1 (right arm)];

The bioequivalence comparison should be based on the AUEC values generated from the values obtained at the different assessment times. These values should be computed according to the dose duration corresponding approximately to ED_{50} of the test and reference product.

7 Statistical Analysis

The 90% confidence interval will be calculated to determine the bioequivalence between the test and reference product. The 90% confidence interval will be calculated for the ratio of the average AUEC response of the test product (average of four replicates) to the average AUEC response of the reference product (average of four replicates) using Locke's method. The data used to calculate the confidence interval requires the average AUEC values of the

detectors only. In addition, the data will be plotted to produce a graph of response vs. time to illustrate whether visual assessment is comparable to chromameter assessment.

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APPENDIX 1
APPLICATION TEMPLATES

TEMPLATE 1

ANTECUBITAL FOSSA

Right Arm		Left Arm	
site no.	treatment type	site no.	treatment type
8	D_1	8	D_2
7	T	7	R
6	UNT	6	UNT
5	R	5	T
4	UNT	4	UNT
3	T	3	R
2	D_2	2	D_1
1	R	1	T

WRIST

TEMPLATE 2

ANTECUBITAL FOSSA

Right Arm		Left Arm	
site no.	treatment type	site no.	treatment type
8	UNT	8	UNT
7	R	7	T
6	D_2	6	D_1
5	T	5	R
4	R	4	T
3	D_1	3	D_2
2	T	2	R
1	UNT	1	UNT

WRIST

TEMPLATE 3

ANTECUBITAL FOSSA

Right Arm		Left Arm	
site no.	treatment type	site no.	treatment type
8	T	8	R
7	D_1	7	D_2
6	R	6	T
5	UNT	5	UNT
4	D_2	4	D_1
3	T	3	R
2	UNT	2	UNT
1	R	1	T

WRIST

TEMPLATE 4

ANTECUBITAL FOSSA

Right Arm		**Left Arm**	
site no.	treatment type	site no.	treatment type
8	R	8	T
7	UNT	7	UNT
6	T	6	R
5	D_2	5	D_1
4	UNT	4	UNT
3	R	3	T
2	T	2	R
1	D_1	1	D_2

WRIST

APPENDIX 2

PRODUCT APPLICATION AND REMOVAL SCHEDULE

	Reference Product	Reference Product	Test Product	Reference Product	
Subject no.	**D_2**	**ED_{50}**	**ED_{50}**	**D_1**	**Wash Off**
1	7:00	7:36	7:36	7:54	8:12
2	7:05	7:41	7:41	7:59	8:17
3	7:10	7:46	7:46	8:04	8:22
4	7:15	7:51	7:51	8:09	8:27
5	7:20	7:56	7:56	8:14	8:32
6	7:25	8:01	8:01	8:19	8:37
7	7:30	8:06	8:06	8:24	8:42
8	7:35	8:11	8:11	8:29	8:47
9	7:40	8:16	8:16	8:34	8:52
10	7:45	8:21	8:21	8:39	8:57
11	7:50	8:26	8:26	8:44	9:02
12	7:55	8:31	8:31	8:49	9:07

APPENDIX 3

Subject no.	ASSESSMENT TIMES AND ACTUAL TIMES															
	Day 1															
	Clock Time	Actual Time	Clock Time	Actual Time	Clock Time	Actual Time	Clock Time	Actual Time	Clock Time	Actual Time	Clock Time	Actual Time	Clock Time	Actual Time	Clock Time	Actual Time
1	08:17		10:17		12:17		14:17		16:17		18:17		20:17		23:17	
2	08:22		10:22		12:22		14:22		16:22		18:22		20:22		23:22	
3	08:27		10:27		12:27		14:27		16:27		18:27		20:27		23:27	
4	08:32		10:32		12:32		14:32		16:32		18:32		20:32		23:32	
5	08:37		10:37		12:37		14:37		16:37		18:37		20:37		23:37	
6	08:42		10:42		12:42		14:42		16:42		18:42		20:42		23:42	
7	08:47		10:47		12:47		14:47		16:47		18:47		20:47		23:47	
8	08:52		10:52		12:52		14:52		16:52		18:52		20:52		23:52	
9	08:57		10:57		12:57		14:57		16:57		18:57		20:57		23:57	
10	09:02		11:02		13:02		15:02		17:02		19:02		21:02		00:02	
11	09:07		11:07		13:07		15:07		17:07		19:07		21:07		00:07	
12	09:12		11:12		13:12		15:12		17:12		19:12		21:12		00:12	

	ASSESSMENT TIMES AND ACTUAL TIMES					
Subject no.	**Day 2**					
	Clock Time	**Actual Time**	**Clock Time**	**Actual Time**	**Clock Time**	**Actual Time**
1	06:17		09:17		14:17	
2	06:22		09:22		14:22	
3	06:27		09:27		14:27	
4	06:32		09:32		14:32	
5	06:37		09:37		14:37	
6	06:42		09:42		14:42	
7	06:47		09:47		14:47	
8	06:52		09:52		14:52	
9	06:57		09:57		14:57	
10	07:02		10:02		15:02	
11	07:07		10:07		15:07	
12	07:12		10:12		15:12	

STUDY PARTICIPATION INFORMED CONSENT FORM

A DOUBLE-BLIND SKIN BLANCHING PIVOTAL STUDY OF CLOBETASOL 17-PROPIONATE 0.05% CREAM IN HEALTHY VOLUNTEERS

Study no. WL2 Protocol version 2

INFORMED CONSENT

I ..... born on .....

Current address .....

.....

hereby give permission that corticosteroid formulations, containing 0.05% of the active ingredient clobetasol propionate, be administered to me during the course of this study.

My consent is given freely and I realise that it may be withdrawn at any time, without penalty to me.

I have been fully informed by ..... regarding the possible adverse effects of the drug, procedures to be used in this study and the risks thereof, as detailed in the "Patient Information Brochure".

- I undertake to comply with all the relevant conditions contained in the Patient Information Brochure and confirm that I accept any consequences arising as a result of my withholding or misrepresenting any information required of me. I agree to undergo the necessary pre- and post-study medical screenings as listed in the Patient Information Brochure.
- I undertake to inform the supervising medical personnel immediately of any symptoms - expected or unexpected - which I might experience.
- I agree to my medical records being reviewed in the event of an audit, enquiry, monitoring and/or inspection on the understanding that my anonymity will be maintained.
- I have been informed that protocol violation may result in my exclusion from the study and forfeiture of the agreed upon remuneration. I acknowledge that instructions relating to my participation in this study have been communicated to me both verbally and in writing, and that I understand them.
- I also declare that I have made the necessary arrangements regarding the attendance of lectures and other academic activities.
- I acknowledge that I will receive R200.00 for full participation in this study and that I will receive a pro-rated amount if I withdraw from the study before the end.
- Signing this consent form does not in any way waive my individual legal rights.

Sign Volunteer .....Date: .....

Sign Witness .....

PATIENT INFORMATION BROCHURE

A Double-Blind Pivotal Study of Clobetasol 17-Propionate 0.05% Cream in Healthy Volunteers.

Study No. WL2 protocol version 2

Introduction

You have been invited to take part in a research study. Before you decide whether or not to participate, it is important for you to understand why the study is being done and what it will involve if you agree to participate.

Please read this document carefully. It provides information about this study to help you decide if you wish to participate. Discuss it with your friends and relatives if you wish. Please feel free to ask for explanations on anything that you do not understand. You will be given as much time as you need to decide on whether you would like to participate or not.

Your participation is completely voluntary. You do not have to give any reasons for not wanting to participate or withdrawing during the study. If you decide to take part in the study, you will be asked to confirm your agreement by signing the consent form.

Purpose of the study

The rate and extent of drug absorption from a drug formulation taken orally can be assessed indirectly from blood samples. However, this can not be applied to topical formulations that are meant for local use with no intent of entry into the systemic circulation.

The purpose of this study is to determine the *in vivo* bioequivalence of two formulations containing 0.05% clobetasol 17-propionate by measuring the skin blanching response and comparing the two assessment methods (visually and with a chromameter). The use of topical corticosteroids results in vasoconstriction which manifests as a change in skin colour and can be detected by the eye. This vasoconstriction is also known as skin blanching phenomenon. Cornell and Stoughton have shown that the intensity of the induced blanching is directly proportional to the clinical efficacy of the corticosteroid. Because this method is acceptable (to the FDA) for testing such formulations it forms a basis of comparison against other available methods such as tape stripping, suction blister formation and microdialysis to

mention some.

Study Drug – Clobetasol 17-Propionate cream 0.05%

Clobetasol 17-propionate is a highly potent synthetic corticosteroid that has anti-inflammatory, antipruritic, and vasoconstrictive properties. It is used in the topical treatment of various skin conditions such as eczema, some forms of psoriasis and seborrhoeic dermatitis. Clobetasol 17-propionate is indicated for use in short courses for skin conditions which do not respond satisfactorily to less active steroids.

Duration of the study

The whole study will last for approximately two days. You will need to stay within the BRI clinic for the first day and night. On the second day, you will be allowed to leave the clinic but must return approximately 10 minutes before the next specified assessment time until completion of the study.

Blanching Response Study Procedure

- On the morning of the study you must check into the BRI clinic at 6:00am to undergo a brief medical examination, inclusion and exclusion criteria check and study restriction check. You will then be prepared for product application by having adhesive labels applied to your forearms.
- From 7:00am onwards you will have cream applied to small areas of your forearms. This cream will be left in contact with the skin for a certain period of time.
- After 72-minutes the adhesive labels will be gently removed and your forearms washed with a mild soap and water and then dried.
- Skin blanching will be assessed at the following time intervals: 5 minutes after washing and then again at 2, 4, 6, 8, 10, 12, 15, 21, 26 and 32 hours after removal of the creams. You will be given a timetable of your scheduled assessment times.
- Assessments will be done visually and by a chromameter.

You are not restricted with regard to food and drink or activities during the study period as long as you abide by the study restrictions. Professional medical assistance (registered

medical practitioner and/or a registered nurse) will be available throughout the course of the study. Should any new and significant information about the study medication become available during the course of the study, this will be communicated to you.

Side Effects and Contraindications

Upon application of the cream on the skin, you may experience burning sensation, stinging of the skin and itching.

Potent corticosteroid creams should only be used for short courses. Prolonged and intensive treatment with the corticosteroid cream may cause local atrophic changes such as stretch marks, thinning of the skin, loss of elasticity and blanching effect. However, these are more likely to occur on the face, the use of occlusive dressings e.g. plasters, or where skin folds are present.

Administration should be avoided in persons who exhibit allergic responses to clobetasol 17-propionate or to any ingredient in the preparation or to other corticosteroids. It is contraindicated in the presence of acute dermatological infections e.g. scalp infection. It is also contraindicated in pregnancy.

Subject Inclusion Criteria

16. Mentally competent subjects who are available for the entire study period and willing to adhere to the protocol requirements and able to give informed consent by signing the informed consent form, which s also signed by a witness.
17. Subjects must be able to demonstrate adequate vasoconstriction to the topical corticosteroids and meet the criteria to be a ‘detector’.
18. Male or female between 18 and 50 years of age.
19. Normal in terms of dermatological medical history at the pre-study screening medical, or in the case of an abnormality, the medical practitioner considers the abnormality to be clinically insignificant.
20. Normal in terms of dermatological examination at the pre-study screening medical, or in the case of an abnormality, the medical practitioner considers the abnormality to be clinically insignificant.

Subject Exclusion Criteria

40. Smoking within one week of study.
41. Caffeine intake greater than 500mg per day prior to or during the study. (A cup of coffee contains about 85mg of caffeine)
42. Clinically significant history of alcoholism or drug abuse.
43. Use of topical dermatologic drug therapy on ventral forearms, including prior dosing of topical corticosteroid in pharmacodynamic study to a particular skin site, within one month prior to study.
44. Adverse reactions to topical or systemic corticosteroids.
45. Any current or past medical condition, including active dermatitis or any other dermatologic condition, which might significantly affect pharmacodynamic response to the administered drug.
46. Persons who would require shaving ventral forearms to insure consistent dose on skin surface.
47. Use of any vasoactive (constrictor or dilator) medication, prescription or OTC, that could modulate blood flow. Examples of such drugs include nitroglycerin, antihypertensives, antihistamines, NSAID's, aspirin, and OTC cough/cold products containing antihistamines and/or either phenypropanolamine or phentolamine.
48. Any obvious difference in skin color between arms.
49. Any mental deficiency or handicap.
50. Any contact allergy to the label adhesive on labels used to demarcate application sites.
51. Consumption of alcohol or other enzyme inducing agents within 24 hours of the start of the study (all barbiturates, corticosteroids, phenylhydantoins, etc.)
52. Participation in natural or artificial sunbathing within 6 weeks of the study start date.

Restrictions during the study

22. No exercise with either arm and no strenuous exercise overall, for study duration.
23. No bathing or showering during the periods of drug application and assessment of skin blanching.
24. No use of creams, emollients, or similar products on forearms for 24 hours prior to and throughout the study.

25. No alcohol may be taken by subjects from 24 hours prior to product application until the last visual assessment has been completed.
26. No caffeine intake greater than 500mg per day during the study. (A cup of coffee contains about 85mg of caffeine)
27. No prescription medication and OTC medication (e.g. cold preparations, vitamins, natural products used for therapeutic benefits, antacids) will be allowed for at least one week prior to the study.

    With the exception of study products no concomitant medication will be taken by subjects during the study.
28. Smoking will not be permitted during the course of the study.

Criteria for Removal from the Study

13. Voluntary withdrawal by the subject due to any reason.
14. Illness or injury during the study if regarded as clinically important by the study physician, Principal or Co-investigator.
15. Any adverse reactions or signs of toxicity if regarded as clinically important by the study physician, Principal or Co-investigator.
16. Failure of the subject to comply with or be uncooperative towards any study requirements or restrictions if regarded as clinically important by the study physician, Principal or Co-investigator.

Remuneration

You will receive a gratuity of R200.00 for full participation in this study. Payment following withdrawal from the study will be calculated on a pro-rated basis.

For any questions or queries please contact the principal investigating researcher:
Genevieve Au (BPharm. R.U.)
Email: **g02a1351@campus.ru.ac.za**
Cell: **072 612 0972**

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CLINICAL STUDY VOLUNTEERS

The Biopharmaceutics Research Group is looking for

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If You

1. Are Healthy, and Over 18 Years of Age
2. Respond positively to a simple test to see if your skin temporarily pales on the application of a medicated (corticosteroid) cream to a small area on your arm.

Then You May Be Eligible to Participate

You Will Be Remunerated For Participating

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Or by Phone at: 046 -6038189 (work)

This advert has been approved by the Rhodes University Ethical Standards Committee

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APPENDIX III

MASS BALANCE OF TS STUDY DATA

Please refer to the data included in the attached CD

APPENDIX IV

RESEARCH PROTOCOL

APPLICATION OF TAPE STRIPPING FOR THE ASSESSMENT OF THE BIOAVAILABILITY OF CLOBETASOL PROPIONATE IN HEALTHY VOLUNTEERS

STUDY NUMBER: WL_TS1

PROTOCOL VERSION 1

NOVEMBER 2007

Reference product	**Dermovate® Cream** (Clobetasol propionate 0.05% m/m) Glaxo Wellcome South Africa
Test products	**Dovate® Cream** (Clobetasol propionate 0.05% m/m) Aspen Pharmacare South Africa **Dermovate® Ointment** (Clobetasol propionate 0.05% m/m) Sekpharma South Africa
Principal Investigator	**Wai Ling Au** ***BPharm (Rhodes)*** Biopharmaceutics Research Group (BRG) Faculty of Pharmacy Rhodes University Grahamstown 6140 South Africa
Supervisor	**Professor Isadore Kanfer** ***BSc (Pharm), BSc (Hons), PhD (Rhodes)*** Rhodes University Grahamstown, 6140 South Africa

APPROVAL OF PROTOCOL

Study number	WL_TS1
Study title	Application of tape stripping for the assessment of the bioavailability of clobetasol propionate in healthy volunteers
Reference product	**Dermovate® Cream** (Clobetasol propionate 0.05% m/m) Glaxo Wellcome, South Africa
Test product	Dovate® Cream **(Clobetasol propionate 0.05% m/m)** **Aspen Pharmacare, South Africa** Dermovate® Ointment **(Clobetasol propionate 0.05% m/m)** **Sekpharma, South Africa**

_____ _____

Principal Investigator **Date (yyyy-mm-dd)**

Wai Ling Au

BPharm (Rhodes)

_____ _____

Supervisor **Date (yyyy-mm-dd)**

Professor Isadore Kanfer

BSc (Pharm), BSc (Hons), PhD (Rhodes)

INVESTIGATORS AND FACILITIES

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CONTENTS

1 BACKGROUND INFORMATION

1.1 Clobetasol propionate

1.1.1 Molecular structure

Clobetasol propionate is a synthetic corticosteroid that has a molecular weight of 467.0g/mol. The molecular structure of clobetasol propionate is represented below. The empirical formula for clobetasol propionate is $C_{25}H_{32}ClFO_5$ [1].

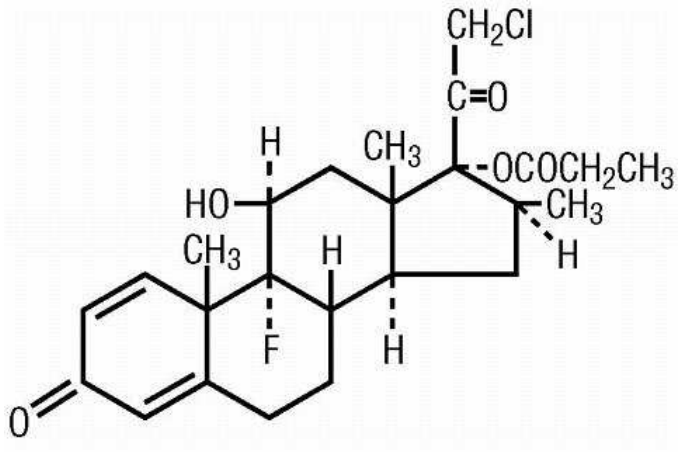

Fig. 1 Chemical structure of clobetasol propionate

1.1.2 Mode of action and uses

The mode of action of corticosteroids is still not clear, however there are a few postulations on their anti-inflammatory activities. One hypothesis that has been widely accepted proposed that the inflammatory process results from the release of hydrolytic enzymes of the lysosomes and that the glucocorticoids stabilize lysosomal membranes, preventing the rupture of the organelles with the accompanying effect of release of hydrolytic enzymes and consequent development of inflammation [2,3]. It has been investigated in view of the fact that prostaglandins have been recovered from several types of inflammatory reactions and are capable of mediating most of the components of inflammation, the possibility arises that corticosteroids may owe their anti-inflammatory activity to part of the inhibition of prostaglandin biosynthesis process [4]. There are suggestions that corticosteroids inhibit the release of prostaglandin by preventing the transport of prostaglandin from inside the fat cell to the extracellular space where it would normally act on the blood vessels [5]. It was also postulated that the corticosteroids block arachidonic acid release from phospholipids from the production of lipocortin which inhibits the activity of phospholipase A_2 [6,7]. The activity of topical corticosteroids is thought to be due to the binding of the drug to steroid receptors located in the cytoplasm or on the outside of the nuclear membrane to form complexes that enter the cell nucleus. These complexes then bind to DNA and modify transcription of mRNA and subsequent synthesis of proteins ultimately responsible for specific effects such as anti-inflammatory activities [8,9]. It is also said that corticosteroids inhibit DNA synthesis in many types of cells thus assists in the treatment of proliferative inflammations such as keloids

[10]. These postulations may perhaps be responsible for one of the anti-inflammatory effects of the corticosteroids, since the arachidonic acid cascade generates several inflammatory mediators. As mentioned earlier, clobetasol 17-propionate is a highly potent synthetic corticosteroid, and it was found that high-potency corticosteroids appear to bind more effectively to the receptors [11].

Clobetasol propionate is a highly potent synthetic topical corticosteroid that has anti-inflammatory, anti-pruritic and vasoconstrictive properties. It is generally used for short term management of various skin disorders. It is widely used in the management of various skin disorders such as eczema, some forms of psoriasis (moderate to severe) and seborrhoeic dermatitis [1,11,].

1.1.3 Pharmacokinetics

Pharmacokinetics after topical application of drugs differs from pharmacokinetics after all other routes of administration in that the site of action (the skin) is accessible to immediate drug analysis. The development of new topical glucocorticoids has two aims [12]:

1) To produce preparations which assure sufficiently high concentrations at the site of action in the skin, and

2) To keep systemic corticosteroid load as low as possible to reduce the risk of systemic effects.

The pharmacokinetic properties of topical corticosteroids remain poorly understood. However, it is certain that the corticosteroids are metabolized in the liver and in other tissues, and are excreted via urine. Most derivatives of this class of drug are absorbed through the skin into dermal blood vessels, with some of the compound being metabolized and excreted as occurs when they are systemically administered. Some compounds are metabolized extensively during skin absorption, for many, metabolism will be small or undetectable [13]. Cutaneous metabolism occurs at the epidermis where certain enzymes found in the liver (including a cytochrome P450 system) may be induced. The corticosteroids may undergo hydrolysis and sulphate conjugation within the epidermis [8].

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1.1.4 Adverse effects

Upon topical application of clobetasol 17-propionate, one may experience burning sensation, pruritus and stinging of the skin. Headache and other irritation symptom(s) may occur [1].

The onset of local side effects from topical corticosteroids is due to application on the same skin site over a long period of time at reasonably high concentrations. The more common local side effects consist of atrophy, striae, purpura, acne, and telangiectases. The less commonly occurring side effects are local hypertrichosis hypopigmentation, and allergic contact dermatitis [14,15]. Systemic side effects usually occur due to an increase in steroid penetration through the skin by applying the corticosteroids to large surface areas, under occlusion, higher concentrations or more potent derivatives especially in children [16-18]. The potential systemic adverse effects upon topical administration include hypothalamic-pituitary-adrenal (HPA)

axis suppression, retarded growth in children, and hyperglycemia [15,19]. Majority of the side effects are reversible upon stopping or reducing the potency of the topical corticosteroid used.

1.1.5 Contraindications

Administration should be avoided in persons who exhibit hypersensitivity to clobetasol 17-propionate or to any ingredient in the preparation or to other corticosteroids. It is contraindicated in the presence of acute dermatological infections e.g. scalp infection [1].

A copy of the product information leaflet of the topical formulation to be used in this study is located in Appendix I.

1.2 Tape stripping technique

1.2.1 Overview of tape stripping

Tape stripping is a non-invasive technique employed to study the penetration, the distribution and the dermatopharmacokinetics of topically applied drugs and cosmetics products [20-23] within the stratum corneum. It is commonly used to disrupt the epidermal barrier to enhance the delivery of drugs *in vivo* [24,25] and to obtain information about stratum corneum function [22]. Tape stripping has been used extensively in dermatological and pharmaceutical fields to measure the stratum corneum mass and thickness, to collect stratum corneum lipids and protein samples, detect proteolytic activity associated with the stratum corneum, quantitatively estimate enzyme levels and activities in the stratum corneum and allow the detection of metal in the stratum corneum [22]. Although in use for over five decades there are no universally accepted protocols for tape stripping [26], however it has been identified to be of sufficient utility to have been proposed by the FDA as part of a standards method to evaluate the bioequivalence of topical dermatological dosage forms [27].

1.2.2 Principles of tape stripping

Tape stripping involves the sequential removal of microscopic layers (typically 0.5 - 1 μm) of the stratum corneum [21,28] by placing a strip of adhesive tape onto the skin surface with uniform pressure, which is then removed [20,21]. Drug uptake into the stratum corneum *in vivo* can be measured by harvesting the stratum corneum previously exposed to a topical product with adhesive tapes, which are subsequently extracted and quantified for drug concentration by a validated analytical method [29]. The number of tape strips needed to remove the stratum corneum varies with age, gender, anatomical site, skin condition and possibly ethnicity [22]. Tape stripping is putatively simple, inexpensive and has been described as a minimally invasive technique [20].

1.2.3 Invasiveness of tape stripping

Although tape-stripping is considered to be essentially non-invasive, stripped sites in certain dark-skinned individuals may remain hyperpigmented for several months after healing. This effect will be communicated to the human volunteers before entering the study [21].

2 STUDY PRODUCTS

2.1 Description

	Reference product	Test products	
Commercial name	Dermovate® cream	Dovate® cream	Dermovate® ointment
Generic name	Clobetasol propionate cream	Clobetasol propionate cream	Clobetasol propionate ointment
Dosage form	cream	cream	ointment
Strength	0.05% m/m	0.05% m/m	0.05% m/m
Manufacturer	Glaxo Wellcome, South Africa	Aspen Pharmacare, South Africa	Sekpharma, South Africa
MCC registration details	F/13.4.1/176	27/13.4.1/0316	F13.4.1/175
Expiry date	01/2009	05/2009	04/2009
Description	A white to off-white cream free from lumps and visible impurities	A soft, smooth, white cream	A soft greyish-white translucent mass, free of lumps and foreign particles
Dosage	± 5 mg per 1cm^2 application site	± 5 mg per 1cm^2 application site	± 5 mg per 1cm^2 application site

2.2 Supply, storage and use

Sufficient products will be timeously supplied to the clinic by the principle investigator prior to the study. Clobetasol 17-propionate is available in cream, ointment or lotion dosage forms. It should be used externally only. The product should be kept in the container it came in, tightly closed and stored at room temperature away from excess heat and moisture [30]. Dispensing and administration of test products will be recorded and administered only to subjects for the purpose of this study.

3 OBJECTIVES

The objective of this study is to measure dermal concentrations of clobetasol propionate using tape stripping to assess *in vivo* bioavailability from topical cream and ointment formulations and therefore obtain valuable information which will lay the foundation in an attempt to determine bioequivalence of clobetasol propionate topical formulations using these two techniques. In addition, the technique will be compared for its effectiveness in the assessment of dermal bioavailability.

4 STUDY POPULATION AND MEDICAL ASSESSMENT

4.1 Number of subjects

Similar studies have made use of 6 - 18 human subjects for this type of study [25,31-35] with equal numbers of male and female subjects. This study will make use of approximately 15 human subjects and will make use of male and female subjects. Three application sites on the right arm of the subjects will be used for the tape stripping studies. Preliminary statistical analysis (Section 8) will be performed on the subjects completing the study. If needed, an add-on study of up to 10 subjects will be conducted to obtain a degree of variability of less than 30% if possible. If no further study is required, preliminary statistical analysis will be finalised and presented in the final report. If an add-on study is conducted, data for all evaluable subjects from both Group 1 and Group 2 will be used in preparation of the final report. Homogeneity testing will be conducted between the two groups.

4.2 Inclusion/Exclusion criteria

Inclusion criteria

Only those subjects meeting the following criteria will be included in the study:

i. Female subjects who are using reliable contraception or abstaining.
ii. Subjects who are aged between 18 and 50.
iii. Subjects who are in general good health.
iv. Subjects who will be available for the entire study period.

Exclusion criteria

Subjects meeting the following criteria will be excluded from the study.

i. Female subjects who are breast feeding.
ii. Female subjects who are contemplating becoming pregnant in the time immediately following the study.
iii. Female subjects who are pregnant.
iv. Subjects who have a known allergy/hypersensitivity to clobetasol propionate or any corticosteroid.
v. Subjects who have any history of drug or alcohol abuse.
vi. Subjects who have any mental deficiency or handicap.

vii. Subjects who have hairy ventral forearm surfaces and/or abrasions on the underside of their forearms.
viii. Subjects who have engaged in any sun-tanning or taken any sunny vacations within the last month.
ix. Subjects who have participated in another topical corticosteroid dermal microdialysis or tape stripping study within 2 months of the study date.
x. Subjects who have used any topical corticosteroid within the last three months.
xi. Subjects who suffer from any allergic conditions (hayfever, allergic rashes, asthma or childhood eczema).
xii. Subjects who suffer from any skin disorder such as psoriasis, eczema or other relevant skin disorder.
xiii. Subjects who take regular medicine or tablets or used any creams within the last week (contraceptive pills excluded).
xiv. Subjects with a history of any neurological, kidney or liver disorders.

4.3 Subject restrictions

i. No prescription medication and OTC medication (e.g. cold preparations, vitamins, natural products used for therapeutic benefits, antacids, herbal or traditional remedies) will be allowed for at least one week prior to the study.
ii. With the exception of study product no concomitant medication may be taken by subjects during the study.
iii. No alcohol may be taken by subjects from 24 hours prior to product application and during the study.
iv. No strenuous physical activity may be undertaken by subjects from 12 hours before product application and during the study.
v. Subjects must not smoke more than 10 cigarettes per day and will not be allowed to smoke during the study.
vi. Subjects must refrain from applying emollients, skin conditioning creams and/or tanning lotions to their forearms for a period of 24 hours prior to the scheduled time of product application.

Subjects will be informed of the above restrictions and each subject will be specifically questioned on these points prior to product application. Any deviations from the above restrictions which are made known to the investigators either voluntarily or on questioning will be recorded on the appropriate CRF (Appendix VI). A decision as to whether the affected subject continues with the study will be taken by the principal investigator and the supervisor in consultation with the study nurse.

4.4 Criteria for removal from the study

Any subject may be withdrawn from the study at any time due to the following:

i. Voluntary withdrawal by the subject due to any reason.

ii. Illness or injury during the study if regarded as clinically significant by the principal investigator and the supervisor in consultation with the study nurse.

iii. Any adverse event or signs of toxicity if regarded as clinically significant by the principal investigator and the supervisor in consultation with the study nurse.

iv. Failure of the subject to comply with or be uncooperative towards any study requirements or restrictions if regarded as clinically significant by the principal investigator and the supervisor in consultation with the study nurse.

Subjects withdrawn or dropped out of the study will be fully documented and accounted for in the CRF.

5 STUDY PROCEDURE

5.1 Pre- and post-study medical screening

Pre-study screening will be conducted not more than 30 days prior to the start of the study. The pre-study evaluations will be conducted as listed in the table below.

Screening test	Pre-study	Post-study
Medical history	√[1]	√[4]
Dermatological assessment	√[2]	√[5]
Adhesive sensitivity	√3	x

1. Medical history: Demographic data (date of birth, age, sex, origin), skin (dermatological), allergies, alcohol consumption, smoking habits, dietary habits and sporting commitments.
2. Dermatological: General assessment of the volar aspect of the forearm and any dermatological condition which may influence the barrier function of the skin and impact on the absorption of topically applied topical corticosteroids.
3. Adhesive sensitivity: Assessment of subject's sensitivity to adhesive on application site demarcation tape.
4. Medical history: Since start of study.
5. Dermatological: Examination of forearms and application sites.

If any study related abnormalities are observed at the post-study medical, appropriate follow-up action will be taken and re-examination and re-testing conducted until the abnormality returns to normal or until the study nurse considers the abnormality to be clinically insignificant.

5.2 Medical tape sensitivity screening

No less than 2 days prior to the start of the study, volunteers will undergo an assessment of their tolerance to the adhesive on medical tape used to demarcate application sites and for tape stripping procedures.

The following screening protocol will be utilised.

i. The forearm of the subject will be washed 1 hour prior to the adhesion of the medical tape.

ii. Adhesion of the medical tape will occur at 0800 hours.

iii. Two different types of adhesive tapes to be employed as templates and for tape stripping will be used on the right arm.

iv. The medical tapes will be removed after four hours.

v. The forearm of the subject will be assessed for allergies to the adhesive medical tapes.

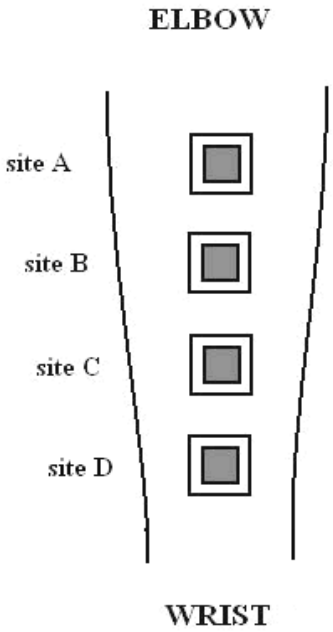
Subjects who exhibit allergies will not be eligible as candidates for the study.

5.3 Check-in and confinement

Subjects will check-in at the clinic (Room T17) in the Faculty of Pharmacy building, Rhodes University, Grahamstown at 0800 hours or 1400 hours of the study day when they will undergo a brief medical examination, inclusion and exclusion criteria check and study restriction check. Subjects accepted into the study will then be prepared for product application. Subjects will remain in the study room for the entire duration of the study.

5.4 Study design

The study will comprise of a single phase sequential design conducted on 15 subjects in the first instance, followed by another 10 subjects if necessary (see section 4.1). A maximum of four subjects will be studied on any study day and the time between the first and the fifteenth subject is expected to be approximately 1 week. A total of four application sites will be employed on the right arm on each subject for the tape stripping study as shown in the diagram below. The application of the formulations will be randomized between sites and individuals. The duration of the study for each subject will be approximately 4 hours.



Templates for use with the tape stripping study will be affixed on the right arm of the subjects using adhesive labels and Scotch® tape. These will be used to demarcate (2 cm x 2 cm) the stratum corneum sampling site. The templates will be designed to have four sampling sites, three sites for the application of the formulations and the other for use as control. One formulation will be applied to one application site and after a 120 minute exposure period the excess drug will be removed by wiping with a cotton swab. Adhesive tapes (3M Scotch® Magic tape no.810) will be applied concurrently to both application sites with uniform pressure and then subsequently removed. 15 successive strips will be made from each site. Transepidermal water loss measurements will be recorded from the control application site. The tape stripping procedure will take approximately 1 hour.

5.5 Pre-study day activities and procedures

This study is dependent on a number of preparations prior to the study. The following is a list of items that will be made available at least 24 hours prior to the study.

i. Calibration standards and mobile phase for clobetasol propionate analysis will be prepared.
ii. The required number of tape strips will be cut for stratum corneum harvesting.
iii. The checklist will be performed (Appendix II).
iv. The general setup of the clinic will be ensured.

5.6 Study day activities and procedures

i. Adhesive tape strips (3M Scotch® Magic Tape no.810) for the tape striping study will be weighed out in the morning prior to start of study.
ii. The adhesive label template (2 x 2 cm) for use with tape stripping will be affixed onto the right forearm with 3M Scotch® Magic Tape (no.810) tapes to delineate the application sites on the stratum corneum.
iii. The reference and test formulations (20 mg) will each be applied to the assigned application sites while one site will be reserved as control.
iv. Excess formulation will be removed from the application site after a 120 minute exposure period using two cotton buds and allowed to air dry for 10 minutes.
v. Tape strips will be applied with uniform pressure to both sites and then subsequently removed.
vi. 15 successive strips will be removed from each site.
vii. The removal of strips will be done in a clockwise manner (N, E, S, W) ensuring that equal amount of stratum corneum is removed in each of the 4 sites.
viii. Transepidermal water loss measurements will be measured from the control site after each tape strip.
ix. Each tape strip will be weighed after tape stripping.
x. The template will be removed from the subject.
xi. The forearm will be rinsed to remove any residual drug.

The actual time of study procedures and/or results/comments obtained during the study will be recorded on the '*Registration of Data during Tape Stripping Form*' (Appendix III).

5.8 Product application

Just prior to the product application, an Eppendorf® (0.5 ml) pipette will be filled with either the reference or test products. The products will be dispensed twice (3 on the dial of the Eppendorf® dispenser) at each application site to allow ± 20 mg of the products (corresponding to 0.01 mg of clobetasol propionate) to be applied at each site for tape stripping. The Eppendorf® dispenser will be used to ensure that an accurate amount of test product is dispensed to each application site for each subject. A pre-weighed glass rod will be used to spread the product and weighed following the spreading to determine the accurate amount of formulation applied to each site. Application of the test product will be done by the principal investigator.

5.9 Posture and physical activity

Strenuous exercises will not be permitted as described in section 4.3.

5.10 Food and fluids

Subjects will not be restricted with respect to food and fluid intake.

5.11 Subject monitoring

The principal investigator will be present at all times during the study. Subjects will be asked open-ended questions about their health at the time of each assessment and any discomfort observed during the experience will be recorded in the CRF (Appendix VI). The study nurse will be contactable by phone for the duration of the study.

6 SAMPLE ANALYSIS

Samples will be analysed for clobetasol propionate using a validated extraction procedure and HPLC analytical method. Analysis will be done within 48 hours after sample collection. Samples will be kept in the fridge at (4 – 8 °C) to prevent any degradation.

7 DATA ANALYSIS

Individual tape strip amount vs. fraction of skin thickness profiles will be generated and the individual total dermal drug amount will be calculated for the tape stripping study. Deletion of data from analysis will be

justified. All individual subject data will be documented and individual tape strip amount vs. fraction of skin thickness curves will be presented in linear/linear and log/linear scale.

7.1 Statistical analysis

Pharmacokinetic and statistical parameters will be determined by the principal investigator using GraphPad Prism version 4. The statistical analysis will estimate the variance associated with subject-to-subject variability . Summary statistics such as median, minimum and maximum will be given.

8 ETHICAL AND REGULATORY REQUIREMENTS

8.1 Ethical and institutional review

Approval by the Rhodes University Ethical Standards Committee (RUESC) (departmental) will be obtained before the study commences. The original signed copy of the ethical approval will be retained by the principal investigator.

The study will be conducted in accordance with the recommendations of the guidelines as set out in the Declaration of Helsinki (1964) and its amendments of Tokyo (1975), Venice (1983), Hong Kong (1989), Somerset West (1996) and Edinburgh (2000), according to ICH Good Clinical Practice (GCP) guidelines and in compliance with the Biopharmaceutics Research Group's (BRG) SOPs, RUESC requirements and guidelines on the conduct of clinical trials in South Africa.

8.2 Written informed consent

Preceding the study, the nature, purpose and risk of participating in the study will be explained to all volunteers. If volunteers desire, they will be given time to consider the information and any questions that they might have will be answered. They will also be informed that they may withdraw from the study at any time without penalty to themselves (other than a reduced remuneration) but that they will be encouraged to be committed to completing the study prior to their enrolment. They will sign a consent form in the presence of a witness. Subjects will receive written, detailed instructions concerning the study performance and restrictions.

8.3 Case report form

The Case Report Form (CRF) for this study will be designed and supplied by the principal investigator (Appendix VI). All case report forms will be quality assured and all major events such as final acceptance of a subject, adverse events and final release from the study will be signed by both the principal investigator and the study nurse.

8.4 Record retention

All source documents, study reports and other study documentation for which the principal investigator is responsible will be archived and retained by the Faculty of Pharmacy, Biopharmaceutics Research Group. Results will be published in the scientific journals and/or presented in a thesis submitted to Rhodes University in fulfilment of the requirement for the degree of Master of Science (Pharmacy) after the completion of the study.

8.5 Insurance

Subjects will be insured against any permanent adverse effect on their health which may arise in connection with the conduct of the study. A copy of the insurance certificate (Appendix VII) will be provided to RUESC (departmental) as part of the application to conduct this study. Adequate insurance cover in the event of negligence on the part of principal investigator, the supervisor and the study nurse will be ensured.

8.6 Termination of the study

The principal investigator reserves the right to terminate the study in the interests of subject welfare following consultation with the supervisor and the study nurse. The supervisor may terminate the study at any time for scientific or safety reasons. If the study is prematurely terminated or suspended for any reason the principal investigator will promptly explain to the subjects, take appropriate steps as deemed necessary under the circumstances to assure the subjects and where applicable follow up with therapy and inform the RUESC (departmental).

8.7 Adherence to protocol

Excluding an emergency situation in which proper treatment is required for the protection, safety and well-being of study subjects, the study will be conducted as described in the approved protocol. Any deviation from the protocol will be recorded and explained.

Should amendments to the protocol be required, the amendments will be documented and signed by the principal investigator and the supervisor. If the protocol amendment(s) has an impact on the safety of subjects, such as a change in dosing regimen or additional formulations, the amendment will be submitted to the RUESC (departmental) for approval.

8.8 Blinding

Subjects will not be blinded and will be informed about the products for use at the application sites.

8.9 Adverse events/Adverse drug reactions

Subjects will be questioned on their health status at check-in, during the course of the study and before leaving the clinic at the end of the study. During the study, open-ended questions will be asked. If any adverse events are reported, the principal investigator together with the study nurse will monitor the adverse event, initiate appropriate treatment if required and decide whether or not to withdraw the subject from the study. Signs and symptoms of any adverse events which occur during the study will be fully documented in the appropriate CRF (Appendix VI). If necessary adverse events will be referred to a suitably qualified medical practitioner for assessment and follow up.

Adverse events (which include illnesses, subjective and objective signs and symptoms that have appeared or worsened during the course of the study) will be assessed by the principal investigator and the supervisor in consultation with the study nurse during and after the study to determine whether or not they are related to the investigational test product (i.e. ADR), to the study procedure or other. The outcome of this assessment will be recorded in the appropriate CRF (Appendix VI).

AEs classified as severe or serious will be reported to the supervisor/study nurse, Rhodes University Ethical Standards Committee (departmental) within 24 hours.

ADRs classified as serious and unexpected will be subject to expedited reporting as detailed in the ICH E2A and E2B guidelines on Clinical Safety Data Management and Data Elements for Transmission of Individual Case Report Forms respectively.

9 REPORTS

A full report on the study will be compiled by the principal investigator in the format requested by the supervisor and submitted to the supervisor. The analytical report will include results for all standard and quality control samples. A representative number of chromatograms or other raw data will be included covering the whole concentration range for all standards and quality control samples. The validation report will also be submitted.

ABBREVIATIONS

ADR	Adverse Dug Reaction
AE	Adverse Effect/Event
ANOVA	Analysis of Variance
AUC	Area Under the Curve
BRG	Biopharmaceutics Research Group
C_{max}	Maximum Concentration
CRF	Case Report Form

CRO	Clinical Research Organisation
GCP	Good Clinical Practice
ICH	International Conference of Harmonisation
IRB	Internal Review Board
QA	Quality Assurance
RUESC	Rhodes University: Ethical Standards Committee
SOP	Standard Operating Procedure
t_{max}	Maximum Time
HPLC	High Pressure Liquid Chromatography

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APPENDIX I

PRODUCT INFORMATION LEAFLET

GlaxoWellcome

Scheduling status:

S4

Proprietary name (and dosage form):

Dermovate® Cream or Ointment

Composition:
Each 100 g contains clobetasol propionate 0,05 g.
The cream contains chlorocresol 0,075% m/m as preservative.

Pharmacological classification:
A13.4.1 Dermatological preparations – corticosteroid without anti-infective agent.

Pharmacological action:
Clobetasol propionate is a highly active topical corticosteroid which is indicated for use in short courses for conditions which do not respond satisfactorily to less active steroids.

Indications:
DERMOVATE Cream or Ointment are of particular value when used for the treatment of more resistant dermatoses such as psoriasis, excluding widespread plaque psoriasis; lichen planus; discoid lupus erythematosus; atopic discoid and stasis eczemas; prurigo; neurodermatoses including lichen simplex; senile pruritus; seborrhoeic dermatitis; intertrigo; contact sensitivity reactions; otitis externa; generalised erythroderma.

Contra-indications:
Rosacea, acne vulgaris, peri-oral dermatitis, peri-anal and genital pruritus.
Skin lesions caused by infection with viruses (e.g. herpes simplex, vaccinia or varicella), fungi (e.g. candidiasis, tinea) or bacteria (e.g. impetigo); dermatoses in children under one year of age, including dermatitis and napkin eruptions.
Hypersensitivity to the preparation.
Corticosteroids have been shown to be teratogenic in animals following dermal application. As these agents are absorbed percutaneously, teratogenicity following topical application cannot be excluded. Therefore, DERMOVATE should not be used during pregnancy.

Dosage and directions for use:
Apply sparingly to the affected area once or twice daily until improvement occurs. As with other highly active topical steroid preparations, therapy should be discontinued when control is achieved. In the more responsive conditions this may be within a few days. If a longer course is necessary, it is recommended that treatment should not be continued for more than four weeks without the patient's condition being reviewed.
Repeated short courses of DERMOVATE may be used to control exacerbations. If continuous steroid treatment is necessary, a less potent preparation should be used.
In very resistant lesions, especially where there is hyperkeratosis, the anti-inflammatory effect of DERMOVATE can be enhanced, if necessary, by occluding the treatment area with polythene film. Overnight occlusion only is usually adequate to bring about a satisfactory response. Thereafter improvement can usually be maintained by application without occlusion.

Side-effects and special precautions:
Potent topical corticosteroids should be used for short courses only as prolonged and intensive treatment may cause local atrophic changes in the skin, such as striae, thinning, loss of elasticity, dilatation of the superficial blood vessels, telangiectasia and ecchymoses. These changes are particularly likely to occur on the face, where occlusive dressings are used, or where skin folds are involved.
Pigmentation changes and hypertrichosis have been reported with the use of topical corticosteroids.
As with other topical corticosteroids, prolonged use of large amounts, treatment of extensive areas, or application to damaged skin, and when the occlusive dressing technique is applied, can result in sufficient systemic absorption to produce the features of hypercorticism such as depression of the hypothalamic-pituitary-adrenal axis with consequent suppression of the adrenal gland. These effects are more likely to occur in infants and children.
In infants, the napkin may act as an occlusive dressing, and hence these preparations should not be used in the nappy areas for flexural eruptions. Ideally, they should not be used in infants and young children at all. Regular review should be made of the necessity for continuing therapy.
In rare instances, treatment of psoriasis with corticosteroids (or its withdrawal) is thought to have provoked the pustular form of the disease.
If signs of hypersensitivity to DERMOVATE appear, application should stop immediately. The least potent corticosteroid which will control the disease should be selected.
Topical corticosteroids should be used with particular caution in facial dermatoses and only for short periods. A steroid rosacea-like facies may be produced. This must be borne in mind when treating such conditions as psoriasis, discoid lupus erythematosus and severe eczema with DERMOVATE.
Topical corticosteroid preparations should be used with caution near the eyes; application to the eyelids may cause glaucoma.
Corticostoeroids should never be used in the presence of infection except in conjunction with effective chemotherapy. Any spread of infection requires withdrawal of topical corticosteroid therapy and systemic administration of antimicrobial agents. Bacterial infection is encouraged by the warm, moist conditions induced by occlusive dressings, and the skin should be cleansed before a fresh dressing is applied.
There is a possibility that DERMOVATE could cause staining and users should take adequate precautions.

Known symptoms of overdosage and particulars of its treatment:
Excessive or prolonged use of DERMOVATE preparations may result in systemic absorption of steroid and complications of steroid therapy, especially growth retardation in children, suppression of pituitary-adrenal function, increased susceptibility to infection, hypoglycaemia, Cushingoid state and benign intracranial hypertension. Cessation of treatment, with appropriate steps in cases of adrenal suppression should correct these symptoms.

Identification:
White to off-white cream or ointment.

Presentation:
Tubes of 25 g

Storage instructions:
Cream and Ointment: Store below 30°C.
Keep out of reach of children.

Registration numbers:
Cream: F/13.4.1/176
Ointment: F/13.4.1/175

Zimbabwe:
Cream: 74/14.2.1/340 PP
Ointment: 74/14.2.1/339 PP

Name and business address of the applicant:
Glaxo Wellcome South Africa (Pty) Ltd
Old Pretoria Road
Midrand, South Africa

Date of publication of this package insert:
11 June 1981

Pro-Print C1800/0899

SEKPHARMA

Scheduling status:

S4

Proprietary name (and dosage form):

Dermovate® Cream or Ointment

Composition:
Each 100 g contains clobetasol propionate 0,05 g.
The cream contains chlorocresol 0,075 % m/m as preservative.

Pharmacological classification:
A13.4.1 Dermatological preparations - corticosteroid without anti-infective agent.

Pharmacological action:
Clobetasol propionate is a highly active topical corticosteroid which is indicated for use in short courses for conditions which do not respond satisfactorily to less active steroids.

Indications:
DERMOVATE Cream or Ointment are of particular value when used for the treatment of more resistant dermatoses such as psoriasis, excluding widespread plaque psoriasis; lichen planus; discoid lupus erythematosus; atopic discoid and stasis eczemas; prurigo; neurodermatoses including lichen simplex; senile pruritus; seborrhoeic dermatitis; intertrigo; contact sensitivity reactions; otitis externa; generalised erythroderma.

Contra-indications:
Rosacea, acne vulgaris, peri-oral dermatitis, peri-anal and genital pruritus.
Skin lesions caused by infection with viruses (eg. herpes simplex, vaccinia or varicella), fungi (eg. candidiasis, tinea) or bacteria (eg. impetigo); dermatoses in children under one year of age, including dermatitis and napkin eruptions.
Hypersensitivity to the preparation.
Corticosteroids have been shown to be teratogenic in animals following dermal application. As these agents are absorbed percutaneously, teratogenicity following topical application cannot be excluded. Therefore, **DERMOVATE** should not be used during pregnancy.

Dosage and directions for use:
Apply sparingly to the affected area once or twice daily until improvement occurs. As with other highly active topical steroid preparations, therapy should be discontinued when control is achieved. In the more responsive conditions this may be within a few days. If a longer course is necessary, it is recommended that treatment should not be continued for more than four weeks without the patient's condition being reviewed.
Repeated short courses of **DERMOVATE** may be used to control exacerbations. If continuous steroid treatment is necessary, a less potent preparation should be used.
In very resistant lesions, especially where there is hyperkeratosis, the anti-inflammatory effect of **DERMOVATE** can be enhanced, if necessary, by occluding the treatment area with polythene film. Overnight occlusion only is usually adequate to bring about a satisfactory response. Thereafter improvement can usually be maintained by application without occlusion.

Side-effects and special precautions:
Potent topical corticosteroids should be used for short courses only as prolonged and intensive treatment may cause local atrophic changes in the skin, such as striae, thinning, loss of elasticity, dilatation of the superficial blood vessels, telangiectasia and ecchymoses. These changes are particularly likely to occur on the face, where occlusive dressings are used, or where skin folds are involved.
Pigmentation changes and hypertrichosis have been reported with the use of topical corticosteroids.
As with other topical corticosteroids, prolonged use of large amounts, treatment of extensive areas, or application to damaged skin, and when the occlusive dressing technique is applied, can result in sufficient systemic absorption to produce the features of hypercorticism such as depression of the hypothalamic-pituitary-adrenal axis with consequent suppression of the adrenal gland. These effects are more likely to occur in infants and children.
In infants, the napkin may act as an occlusive dressing, and hence these preparations should not be used in the nappy areas for flexural eruptions. Ideally, they should not be used in infants and young children at all. Regular review should be made of the necessity for continuing therapy.
In rare instances, treatment of psoriasis with corticosteroids (or its withdrawal) is thought to have provoked the pustular form of the disease.
If signs of hypersensitivity to **DERMOVATE** appear, application should stop immediately. The least potent corticosteroid which will control the disease should be selected.
Topical corticosteroids should be used with particular caution in facial dermatoses and only for short periods. A steroid rosacea-like facies may be produced. This must be borne in mind when treating such conditions as psoriasis, discoid lupus erythematosus and severe eczema with **DERMOVATE**.
Topical corticosteroid preparations should be used with caution near the eyes; application to the eyelids may cause glaucoma.
Corticostoeroids should never be used in the presence of infection except in conjunction with effective chemotherapy. Any spread of infection requires withdrawal of topical corticosteroid therapy and systemic administration of antimicrobial agents. Bacterial infection is encouraged by the warm, moist conditions induced by occlusive dressings, and the skin should be cleansed before a fresh dressing is applied.
There is a possibility that **DERMOVATE** could cause staining and users should take adequate precautions.

Known symptoms of overdosage and particulars of its treatment:
Excessive or prolonged use of **DERMOVATE** preparations may result in systemic absorption of steroid and complications of steroid therapy, especially growth retardation in children, suppression of pituitary-adrenal function, increased susceptibility to infection, hypoglycaemia, Cushingoid state and benign intracranial hypertension. Cessation of treatment, with appropriate steps in cases of adrenal suppression should correct these symptoms.

Identification:
Cream: A smooth white to off-white cream free from lumps and visible impurities.
Ointment: A soft greyish-white translucent mass, free of lumps and foreign particles.

Presentation:
Tubes of 25 g

Storage instructions:
Cream and Ointment: Store below 30 °C.
Keep out of reach of children.

Registration numbers:
Cream: F/13.4.1/176
Ointment: F/13.4.1/175

Name and business address of the applicant:
Sekpharma (Pty) Ltd
Fredman Towers
13 Fredman Drive
Sandton, Gauteng
2196

Date of publication of this package insert:
11 June 1981

P3981/0306

SCHEDULING STATUS: S4

PROPRIETARY NAME: **DOVATE CREAM**

COMPOSITION:
Each 5 g of cream contains 0,0025 g of clobetasol propionate. Preservative:Chlorocresol 0,1% m/m.

PHARMACOLOGICAL CLASSIFICATION:
A 13.4.1 Dermatological preparations - corticosteroids without anti-infective agents.

PHARMACOLOGICAL ACTION:
Clobetasol propionate is a highly potent topical corticosteroid.

INDICATIONS:
Short term use in the treatment of steroid responsive dermatoses resistant to other less potent topical corticosteroids such as: seborrhoeic dermatitis, atopic dermatitis, lichen simplex chronicus, pruritus ani, psoriasis, later phase of allergic contact dermatitis, later phase of irritant dermatitis, discoid lupus erythematosus and lichen planus.

CONTRA-INDICATIONS:
Dovate cream should not be used in the presence of bacterial (including tuberculous), fungal or viral infections of the skin.
Avoid use in ulcerative skin lesions and in rosacea.
Application to ulcers of the leg.
Long-term use is contra-indicated in patients with diabetes mellitus or tuberculosis.
The more potent corticosteroids have been shown to be teratogenic in animals following dermal application. As these agents are absorbed percutaneously, teratogenicity following topical application cannot be excluded. Therefore Dovate should not be used during pregnancy.
Known sensitivity to corticosteroids.

WARNING:
FOR EXTERNAL USE ONLY.

DOSAGE AND DIRECTIONS FOR USE:
Apply once or twice daily sparingly to the affected area until improvement occurs. Therapy should be discontinued when control is achieved. In the more responsive conditions this may be within a few days. If a longer course is necessary, it is recommended that treatment should not be continued for longer than four weeks without the patient's condition being reviewed. Repeated short courses of Dovate may be used to control exacerbations. If continuous steroid treatment is necessary, a less potent preparation should be used.
In very resistant lesions, especially where there is hyperkeratosis, the treatment area may be occluded for part of each 24 hours. Thereafter improvement can usually be maintained by application without occlusion.

SIDE-EFFECTS AND SPECIAL PRECAUTIONS:
Dovate may under certain circumstances, be absorbed through the skin in sufficient amounts to produce systemic effects, since the pharmacokinetic pathway is similar to systemically administered corticosteroids. As Dovate contains a highly potent corticosteroid, the risk associated with systemic absorption is enhanced.
Systemic absorption may cause suppression of the pituitary-adrenal axis. Even small amounts absorbed may cause growth retardation in children. Absorption (and a risk of subsequent toxicity) is also enhanced by application for prolonged periods under occlusive dressings, by application to extensive areas or when the skin is broken.
Application of Dovate to the skin has led to loss of skin collagen and subcutaneous atrophy; local hypopigmentation of deeply pigmented skins has been reported.
In the treatment of severe psoriasis Dovate may induce generalised pustular psoriasis during therapy or on withdrawal.
Other local effects include atrophy of the epidermis and dermal collagen (causing atrophic striae), drying of the skin and telangiectasiae.
Increased fragility of cutaneous vessels may result in bruising and purpura.
Rosacea-like dermatitis, perioral dermatitis and acneiform eruptions may occur.
Occlusive dressings are associated with maceration of the skin and miliaria.
Local infection may be worsened and spread enhanced.
Special care should be exercised in infants and children.
Areas of the body most likely to suffer local damage are the face and eyelids; the intertriginous areas, the neck, axillae, etc. are more permeable. The topical application of Dovate to the eyes has produced corneal ulcers, raised intra-ocular pressure, and reduced visual function.
Long-term topical use is best avoided, especially in children.
Children may absorb proportionally larger amounts of Dovate because of a larger skin surface area to body-mass ratio, and thus be more susceptible to systemic toxicity. Tight fitting nappies or plastic pants should not be used on an infant being treated in the nappy area, as these garments may constitute occlusive dressings.
Use with care in porphyrics.

KNOWN SYMPTOMS OF OVERDOSAGE AND PARTICULARS OF ITS TREATMENT:
See "Side-Effects and Special Precautions".
Treatment is supportive and symptomatic.

IDENTIFICATION:
A soft, smooth, white cream.

PRESENTATION:
25 g Tube.

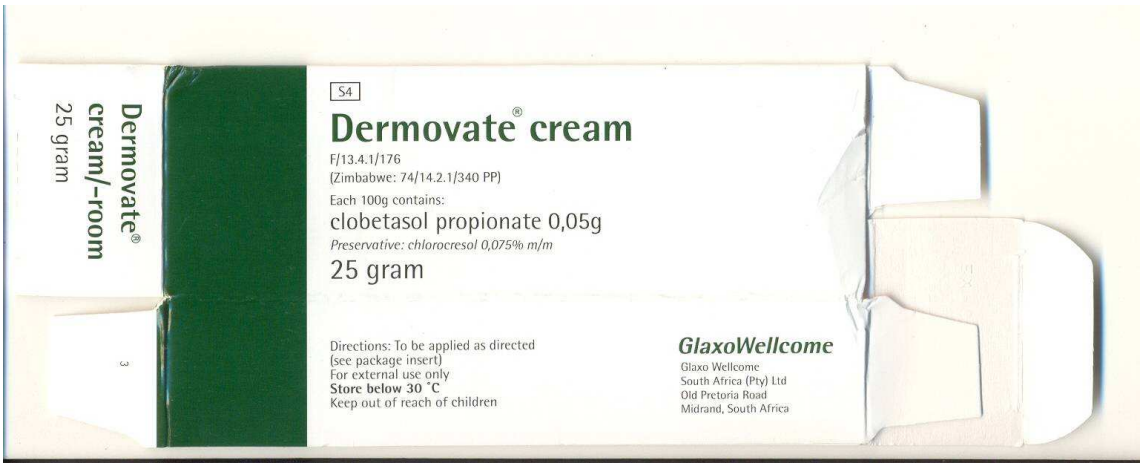
STORAGE INSTRUCTIONS:
Store below 25°C. Keep tube tightly closed. KEEP OUT OF REACH OF CHILDREN.

REGISTRATION NUMBER:
27/13.4.1/0316

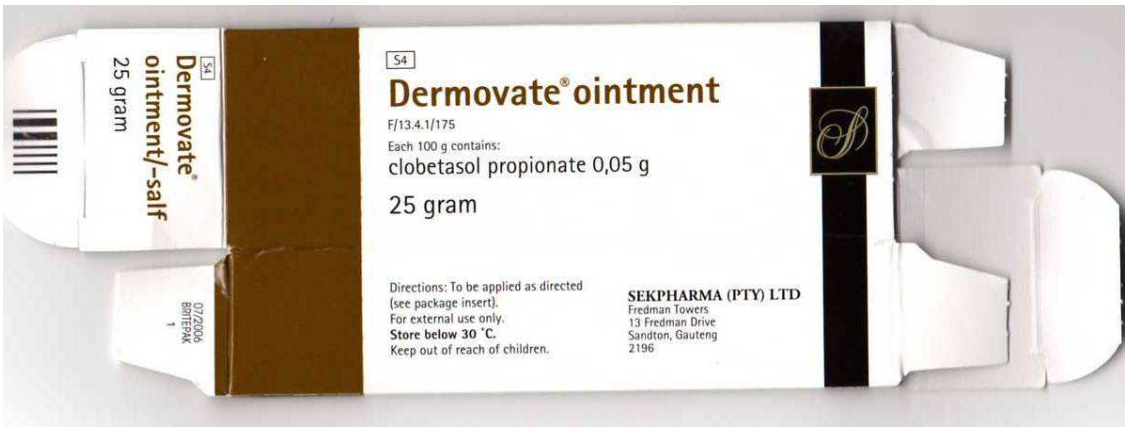
NAME AND BUSINESS ADDRESS OF APPLICANT:
Pharmacare Limited, 7 Fairclough Road, PORT ELIZABETH 6001

DATE OF PUBLICATION OF THIS PACKAGE INSERT:26/8/1993.

G404A
KOHLER C&P P.E.


Dermovate® cream/-room
25 gram
S4
Dermovate® cream
F/13.4.1/176
(Zimbabwe: 74/14.2.1/340 PP)
Each 100g contains:
clobetasol propionate 0,05g
Preservative: chlorocresol 0,075% m/m
25 gram
Directions: To be applied as directed
(see package insert)
For external use only
Store below 30 °C
Keep out of reach of children
GlaxoWellcome
Glaxo Wellcome
South Africa (Pty) Ltd
Old Pretoria Road
Midrand, South Africa


S4 27/13.4.1/0316
25 g
dovate® cream
clobetasol propionate
cream
aspen
cream
COMPOSITION: Each 5 g cream contains 0,0025 g Clobetasol propionate.
Preservative: Chlorocresol 0,1% m/m.
DOSAGE AND DIRECTIONS FOR USE: See enclosed package insert.
E703633
EXP 05 2009
For external use only. Store below 25 °C. Keep container tightly closed.
KEEP OUT OF REACH OF CHILDREN.
Applicant/Applikant: Pharmacare Limited/Beperk.
HOTLINE/BLITSLYN: 0800 122 912


S4
Dermovate® ointment/-salf
25 gram
07/2006
BRITEPAK
1
S4
Dermovate® ointment
F/13.4.1/175
Each 100 g contains:
clobetasol propionate 0,05 g
25 gram
Directions: To be applied as directed
(see package insert).
For external use only.
Store below 30 °C.
Keep out of reach of children.
SEKPHARMA (PTY) LTD
Fredman Towers.
13 Fredman Drive
Sandton, Gauteng
2196

APPENDIX II

CHECKLIST

SUBJECT NAME		**SUBJECT INITIALS**		**DATE**	
STUDY NUMBER	**WL_TS1**	**VOLUNTEER REFERENCE**			
3M Micropore™ tape (1530)					
3M Scotch® tape (810)					
Alcohol swabs					
Biological waste container (yellow)					
Calculator					
Cotton swabs					
Eppendorf® preparations					
Glass rod					
Human subject under study					
Pen/marker					
Pre-cut Scotch® tape (810)					
Ruler					
Scissors					
Protective arm guards (non-occlusive)					
Soap and towel					
Templates for sampling					
Thermometer					
Timer					
Tweezers					
CHECKED BY:			**SIGNATURE:**		

APPENDIX III

REGISTRATION OF DATA DURING TAPE STRIPPING EXPERIMENTS

Date		**Subject initials**	
Subject/Volunteer name		**Volunteer Reference**	
Age		**Study number**	**WL_TS1**
Sex			
Race			
Formulation			
Applied dose			
Site			
Principal Investigator			
Assistant principal investigator			
Room temperature			
Relative Humidity			

TIME SCHEDULE

Task	**Time start**	**Time stop**	**Comment**
Forearm wash			
Demarcation of sites on right arm			
Application and removal of product:			
Control (site _____)	**N/A**	**N/A**	
Reference (site _____)			
Test 1, Dovate® (site _____)			
Test 2, Dermovate® (site _____)			
Stripping of application sites:			
Control (TEWL)			
Reference			
Test 1			
Test 2			
Removal adhesive labels and tapes			
Forearm wash			

WRIST ELBOW

A B C D

Mass of rod + Cream/ointment = _____ _____ _____ _____

Mass of rod = _____

MASS OF STRATUM CORNEUM AND TEWL

CONTROL

Tape strip no.	Mass before	Mass after	Mass of stratum corneum	TEWL
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				

REFERENCE PRODUCT

Tape strip no.	Mass before	Mass after	Mass of stratum corneum
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			

TEST PRODUCT (Dovate®)

Tape strip no.	Mass before	Mass after	Mass of stratum corneum
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			

TEST PRODUCT (Dermovate®)

Tape strip no.	Mass before	Mass after	Mass of stratum corneum
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			

APPENDIX IV

INFORMATION FOR VOLUNTEERS BROCHURE

APPLICATION OF TAPE STRIPPING FOR THE ASSESSMENT OF THE BIOAVAILABILITY OF CLOBETASOL PROPIONATE IN HEALTHY VOLUNTEERS

STUDY NUMBER: WL_TS1
PROTOCOL VERSION 1
BROCHURE VERSION WLTS1
NOVEMBER 2007

1 Study objective

The study involves research to assess the rate and extent to which clobetasol propionate diffuse into the skin from a topical product, using tape stripping as a measure of efficacy. This study will provide valuable insight for use in the assessment of sameness and differences of proprietary clobetasol propionate formulations.

Reference product	**Dermovate® Cream**
Drug	Clobetasol propionate 0.05% m/m
Company	Glaxo Wellcome
	South Africa

Test product	**Dovate® Ointment**
Drug	Clobetasol propionate 0.05% m/m
Company	Aspen Pharmacare
	South Africa

Test product	**Dermovate® Ointment**
Drug	Clobetasol propionate 0.05% m/m
Company	Sekpharma Ltd.
	South Africa

The study will involve approximately 15 subjects.

2 Ethical considerations and standards of practice

Innovator drug companies who develop a new drug have patent protection for the drug for a number of years, typically 15 - 20. Once the patent has expired, generic drug companies are permitted to make and market their own products as competitors to the innovator's product, usually at a substantially reduced cost.

However, a prerequisite for registration with national drug registration bodies (and subsequent marketing) is that the rate and extent to which the drug is absorbed from the generic product is shown to be equivalent to that from the innovator product. This is an indirect way of demonstrating that the generic product will be as effective clinically as the innovator and is demonstrated by what are known as 'comparative bioavailability or bioequivalence studies'.

2.1 Ethical and institutional review

Approval by the Rhodes University Ethical Standards Committee (RUESC) (departmental) is required before the study can commence. The study will be conducted in accordance with guidelines set out in the Declaration of Helsinki (1964) and its amendments of Tokyo (1975), Venice (1983), Hong Kong (1989), Somerset West (1996) and Edinburgh (2000). This Declaration sets out ethical principles, which protect your rights participating in such studies. Guidelines on the conduct of clinical trials in South Africa will be adhered to.

The study will also be conducted according to the ICH (International Conference on Harmonisation) Guidelines for GCP (Good Clinical Practice), the Biopharmaceutics Research Group's Standard Operating Procedures and the requirements of RUESC.

2.2 Written informed consent

Preceding the study, the nature, purpose and risk of participating in the study will be explained to you. Should you wish, you will be given time overnight to consider the information and any questions that you might have will be answered. The nature of the insurance cover will also be explained. If you decide to participate in the study you will sign a consent form in the presence of a witness. You are encouraged to consult your parents or personal medical doctor for approval in this study.

3 Voluntary nature of participation

Your participation in this study is entirely voluntary and you may withdraw from the study at any time, without prejudice. Should you decide to participate, we ask that you try to be committed to completing the study if at all possible. Should you encounter any problems along the way, please speak to me so that every effort can be made to assist you.

4 Dates and duration of the study phases

The study consists of one phase only which will last for approximately four hours.

5 Place of study

The study will be conducted in the clinic (Room T17) in the Faculty of Pharmacy building at Rhodes University. Room T17 is on the top floor of the Pharmaceutical and Chemical Sciences building in Artillery Road on the Rhodes University campus. The principal investigator, supervisor and the study nurse will be suitably qualified, trained and experienced to perform the study procedures.

6 Background information

Clobetasol propionate is used in the management and treatment of patients with skin disorders such as psoriasis, eczema and dermatitis. It is for adult use only. The cream must be applied sparingly once to twice daily to affected areas only.

Tape stripping is a technique also employed to study the penetration of drugs to the skin. Tape stripping involves the sequential removing of microscopic layers (typically 0.5 - 1 μm) of outer dead layers skin (stratum corneum) by placing an adhesive tape strip onto the skin surface with uniform pressure which is then subsequently removed. Tape stripping is simple and has been described as a minimal invasive technique. Although tape stripping is considered to be essentially non-invasive, stripped sites may remain darker than usual for several months after healing.

7 Study design

Templates for use with the tape stripping study will be affixed on your right arm with Scotch® tape (810). These will be used to demarcate (2 cm x 2 cm) the stratum corneum sampling site. The templates will be designed to have three sampling sites, two sites for the application of the formulations and the other for use as control. The test formulation will be applied to one application site and after a 120 minute exposure period the excess drug will be removed by wiping with a cotton swab. Adhesive tapes (3M Scotch® Magic Tape no.810) will be applied concurrently to both application sites with uniform pressure and then subsequently removed. 15 successive strips will be made from each site. The amount of moisture loss measurements will be recorded from the control application site using a small instrument which is placed on the skin. This is also entirely pain free.

8 Adverse effects

For topical preparations, delayed hypersensitivity may occur at the site of the application, but this is uncommon. It is advised that clobetasol propionate cream preparations should not be used on open wounds or lesions on the skin or near the eye. The skin reactions are however reversible on discontinuation of therapy. Clobetasol propionate must not be administered to healthy or ill individuals who have an allergy to this compound or to any of the ingredients in the formulation. In this study, a single application to a limited area is unlikely to invoke any adverse effects but procedures will be in place to address any discomfort noted during and after the study.

Tape stripping technique is safe and no adverse reactions have been reported so far. Although tape stripping is considered to be essentially non-invasive, stripped sites may remain hyperpigmented for several months after healing.

9 Conditions of participation in the study

To participate in this study you must:-

i. Undergo and pass a medical assessment which includes giving a general medical history, recording of vital signs, body height and weight measurements and an assessment of your skin.
ii. Undergo a skin test to assess any possible allergy or sensitivity to the adhesive in the medical tape that will be used. After 3 hours the tape is removed, the forearm washed and the skin inspected for any signs of reaction to the tape adhesive.
iii. Fulfil certain inclusion and exclusion criteria which have been set out in the protocol.
iv. Agree to be fully committed to the study and conscientiously abide by the restrictions required of you as listed in section 11.

NB: It is extremely important that you divulge any past medical history and abide by the rules for participation in this study. This is to protect you from unnecessary risks and to help ensure the reliability of the data gathered.

10 Volunteer inclusion/exclusion criteria

You will be considered for this study if you are aged between 18 and 50, in general good health, available for the entire study period and if female you are on reliable contraception or abstaining from sex.

Please do not consider participating in this study if you

i. are breast feeding.
ii. are contemplating becoming pregnant in the time immediately following the study.
iii. are pregnant.
iv. have a known allergy/hypersensitivity to clobetasol propionate or any corticosteroid.
v. have any history of drug or alcohol abuse.
vi. have any mental deficiency or handicap.
vii. have hairy ventral forearm surfaces and/or abrasions on the underside of their forearms.
viii. have engaged in any sun-tanning or taken any sunny vacations within the last month.
ix. have participated in another corticosteroid dermal microdialysis or tape stripping study within 2 months of the study date.
x. have used any corticosteroids within the last three months.
xi. suffer from any allergic conditions (hayfever, allergic rashes, asthma or childhood eczema).
xii. suffer from any skin disorder such as psoriasis, eczema or other relevant skin disorder.
xiii. take regular medicine or tablets or used any creams within the last week (contraceptive pills excluded).
xiv. have a history of any neurological, kidney or liver disorders.

11 Study restrictions

Restricted item	Duration of restriction	Examples of restriction	Comments
Prescription	From 1 week before the start of the study, until the end of the	- all medication obtained on prescription	This includes all long

Restricted item	Duration of restriction	Examples of restriction	Comments
drugs	study.	- antibiotics, vaccinations - anti-inflammatories - anti-asthmatic drugs - anti-acne drugs etc.	term medication.
Over-the-counter (OTC) drugs	From 1 week before the start of the study, until the end of the study.	- anti-flu drugs, sports supplements, antacids, paracetamol - vitamins, minerals -homeopathics	This includes herbs, natural products & all medications that can be bought without a prescription.
Alcohol	From 24 hours before the start of the study and during the study. **No alcohol for a total of just over 2 days**	All alcoholic drinks and alcohol containing foods.	**It is important that this requirement is taken seriously and observed, as alcohol can significantly affect the liver.**
Strenuous Physical Exercise	From 12 hours before the start of the study.	Rugby Squash Rowing Gym Tennis etc.	Light exercise such as walking is permitted.
Smoking	No smoking will be permitted during the study.	Cigarettes	No cigars or pipe smoking.
Moisturising creams	You must refrain from applying any type of skin conditioning creams to your forearms from 24 hours before the study until the end of the study	All skin creams, e.g. moisturizers, Vaseline, medicated creams, aqueous cream and tanning lotions.	This could interfere with the absorption of the study formulation.

NB: Random checks will be done to ascertain whether you have managed to adhere to the above restrictions. If you have not been able to adhere to the study restrictions please inform us immediately or else you may not be able to participate with a reduced remuneration as detailed in section 15.

12 Procedures and duration of the study

The study consists of a single phase. The procedures and duration of the study are detailed below.

i. On the morning or afternoon of the study you must check into the clinic (Room T17) in the Faculty of Pharmacy building at 0800 or 1400 hours respectively. At check-in you will be:-

a. questioned and undergo a brief medical examination to establish whether you still fulfil all the inclusion and exclusion criteria since your pre-study medical examination and that you have complied with the study restrictions.
b. prepared for product application by having medical tape applied to your forearms.

ii. The study will commence at 0800 or 1400 hours and you will be expected to remain in the clinic throughout the course of the study.

iii. You are not restricted with regard to food and drink during the study period.

iv. Professional medical assistance (registered study nurse and/or a registered nurse) will be available throughout the course of the study.

v. Should any new and significant information about the study medication become available during the course of the study, this will be communicated to you.

vi. A tube of 0.1% m/m hydrocortisone cream will be given to you to self medicated your arm twice daily for 2 weeks.

13 Benefits

Since you have been screened as healthy before the start of the study, there is no medical benefit to you from participating in this study.

14 Financial compensation

You will receive a gratuity of **R 100.00** for full participation in the study. Payment following withdrawal from the study will be calculated on a pro-rated basis.

15 Adverse medical events

You will be monitored prior to, during and after each study day for any adverse events whether or not they are thought to be related to the investigational products or procedures. If any adverse events are reported, the study nurse will decide whether or not to withdraw you from the study and what treatment is appropriate. In addition, the Rhodes University Ethical Standards Committee (departmental) will be notified of any serious adverse events (SAEs). All adverse events will be monitored and treated until recovery to your pre-study status. You will be referred to a medical doctor if necessary and all medical costs will be covered by the BRG. All adverse events will be monitored and treated appropriately until a satisfactory outcome is attained.

16 Insurance

You are covered by an insurance policy taken out by the BRG, in case of claims arising from the medication or procedures as outlined in the protocol, and in case of negligence on the part of the BRG. If you have personal insurance e.g. life insurance /assurance your participation in this study may affect your policy. You are advised to determine this prior to participating in the study. If you need any assistance in this regard please feel free to contact me. During the clinical study you are not entitled to participate in other studies and you should not participate in any other study/s for thirty (30) days after completion of this study to ensure that there are no interactions. Insurance policies will be available from the principal investigator for scrutiny.

17 Confidentiality

Your medical history and physical examination records and any other information or data generated during this study will be kept confidential. However, you must agree that all the above mentioned documentation and data can be released for any lawful purpose and released for publication in scientific journals and/or presentation in a thesis submitted to Rhodes University in fulfilment of the requirement for the degree of Master of Science (Pharmacy) after the completion of the study. In such cases your name will be removed from all documentation to ensure your anonymity. In signing the consent form for the study, you agree to the granting of access to your medical data. Your medical data will be provided to you upon request and you will be informed of significant abnormalities identified before or after the study.

Your consent is also required to permit the study staff to consult with any medical practitioner who is normally responsible for your care if the need arises. The onus, however, is on you to inform such a practitioner of your intention to participate in such a study, should you so wish.

18 Amendments/Changes

Should there be any changes made to the trial protocol these will be communicated to you verbally and in writing in time to enable you to reconsider your decision to participate in the study.

19 Withdrawal

You may withdraw from the study at any time due to the following:

i. Voluntary withdrawal by yourself due to any reason.
ii. Illness or injury during the study if regarded as clinically significant by the principal investigator and the supervisor in consultation with the study nurse.
iii. Any adverse event or signs of toxicity if regarded as clinically significant by the principal investigator and the supervisor in consultation with the study nurse.
iv. Failure to comply with or be uncooperative towards any study requirements or restrictions if regarded as clinically significant by the principal investigator and the supervisor in consultation with the study nurse.

It is your right to withdraw from the study at any time. However, by signing the Informed Consent form and participating in the study, you agree to be committed to completing the study if at all possible.

20 Termination of the study

The BRG reserves the right to terminate the study prematurely in the interests of your welfare.

21 Emergency contacts

You are obliged to notify the principal investigator as soon as possible if you are unable to follow the procedures or if you suffer any adverse event we have not told you about. In particular, you should make every effort to contact me if you suffer a Serious Adverse Events (SAEs) or need to take additional medication of any kind. This applies to out of hours, as to normal work time.

In such cases of medical emergencies during the study, or if you have any urgent questions relating to adverse effects or unrelated illness, please feel free to telephone the study nurse, Sr. Kay Wentworth at any time at the following phone numbers:

Work 046 603 8189 Home 046 622 6384 Cell 072 299 8230

22 Contacts for additional information

The following individuals are responsible for conducting this study and as such may be approached for more information:

Portfolio	**Name**	**Contact details**	
Principal Investigator	**Wai Ling Au** *BPharm (Rhodes)*	Work Cell Email	046 603 8412 072 612 0972 g02a1351@campus.ru.ac.za
Supervisor	**Professor Isadore Kanfer** *BSc (Pharm), BSc (Hons), PhD (Rhodes)*	Work Email	046 603 8381/8382 i.kanfer@ru.ac.za

If you have questions about this study which have not been answered adequately by the principal investigator, supervisor or the study nurse, you should first discuss them with your doctor. After you have consulted your doctor and are still dissatisfied you may contact the Rhodes University Ethics Standards Committee (departmental) at:

Professor Beverley Wilson
Rhodes University Ethics Standards Committee (departmental)
RHODES UNIVERSITY
Tel:. 046 603 8381
Fax:. 046 636 1205
Email:. b.wilson@ru.ac.za

APPENDIX V

STUDY PARTICIPATION INFORMED CONSENT FORM

APPLICATION OF TAPE STRIPPING FOR THE ASSESSMENT OF THE BIOAVAILABILITY OF CLOBETASOL PROPIONATE IN HEALTHY VOLUNTEERS

STUDY NUMBER: WL_TS1

PROTOCOL VERSION 1

CONSENT VERSION 1

NOVEMBER 2007

I ..... Born on.....

Present address .....

.....

hereby give permission that the tape stripping procedure to be conducted as described to me by the principal investigator and that the formulation stated below be applied to my skin during the course of this study.

Reference product: Dermovate® Cream (clobetasol propionate 0.05% m/m) – Glaxo Wellcome, South Africa
Test products: Dovate® Cream (clobetasol propionate 0.05% m/m) – Aspen Pharmacare, South Africa
Dermovate® Ointment (clobetasol propionate 0.05% m/m) – Sekpharma South Africa.

My consent is given freely and I realise that it may be withdrawn at any time, without penalty to me. Furthermore, I understand that I do not give up any of my legal rights by signing this consent form.

I have been fully informed by ..... regarding the possible adverse effects of the medication, procedures to be used in this study and the risks thereof, as detailed in the "Information for Volunteers Brochure". I will receive a copy of the information brochure and signed consent form for my records.
I undertake to comply with all the relevant conditions contained in the Information to Volunteers Brochure and confirm that I understand that it is important not to withhold or misrepresent any information asked of me. I agree to undergo the necessary pre- and post-study medical investigations as listed in the protocol.

I undertake to inform the study nurse and the principal investigator immediately of any symptoms - expected or unexpected - which I might experience.

I agree to my medical records being reviewed in the event of an audit, enquiry, monitoring and/or inspection on the understanding that my anonymity will be maintained.

I agree and consent that the BRG may consult with any study nurse who is normally responsible for care of study subjects. _____ Initial

I have been informed that if I do not adhere to the protocol, it may result in my exclusion from the study and forfeiture of the agreed upon remuneration. I acknowledge that instructions relating to my participation in this study have been communicated to me both verbally and in writing, and that I understand them.

I also declare that I have made the necessary arrangements regarding the attendance of lectures and other academic activities.

I understand that a policy to cover volunteers in clinical studies against death or disablement arising as a direct result of participation in such clinical studies has been taken out by the Biopharmaceutics Research Group. I accept the conditions of the policy as set out in the insurance policies.

I acknowledge that I will receive **R 100.00** for full participation in this study and that I will receive a pro-rated amount if I withdraw from the study before it has been completed.

_____ _____

Signature of volunteer **Date (yyyy-mm-dd)**

_____ _____

Signature of a witness **Date (yyyy-mm-dd)**

_____ _____

Principal Investigator **Date (yyyy-mm-dd)**

Wai Ling Au

BPharm (Rhodes)

Contact details of subject	
Telephone number	
Cell number	

APPENDIX VI

CASE REPORT FORM

APPLICATION OF TAPE STRIPPING FOR THE ASSESSMENT OF THE BIOAVAILABILITY OF CLOBETASOL PROPIONATE IN HEALTHY VOLUNTEERS

STUDY NUMBER: WL_TS1

PROTOCOL VERSION 1

CASE REPORT FORM VERSION 1

NOVEMBER 2007

Reference product	**Dermovate® Cream (clobetasol propionate 0.05% m/m) – Glaxo Wellcome, South Africa**
Test product	**Dovate® Cream (clobetasol propionate 0.05% m/m) – Aspen Pharmacare, South Africa** **Dermovate® Ointment (clobetasol propionate 0.05% m/m) – Sekpharma, South Africa**

Principal Investigator	**Wai Ling Au** ***BPharm (Rhodes)***
Supervisor	**Professor Isadore Kanfer** ***BSc (Pharm), BSc (Hons), PhD (Rhodes)***

Subject initials	
Volunteer reference number	
Study subject number	
Date of screening medical	

Pre-study examiner:	
Name and initials	
Qualifications	

MEDICAL HISTORY

DEMOGRAPHIC DATA

Birth Date	Age in Years	Sex (M/F)	Origin				Other
			White	Black	Oriental	Indian	

Height (m)	Weight (kg)	BMI (Body Mass Index) To be calculated by BRG Investigators	Not required

REVIEW OF PAST ILLNESS

System/Site	Illness? Y/N	Describe Abnormalities
Skin -Connective Tissue Any other illness?		

GENERAL SYSTEMS EXAMINATION

System/Site	Illness? Y/N	Describe Abnormalities
Skin - General Skin - Forearms		
Medical tape allergy pass		

INCLUSION/EXCLUSION CRITERIA

(NB: Unshaded areas - acceptable: Shaded areas - unacceptable)

	YES	NO
Will you be available for the entire study period?		
Are you in general good health?		
Are you aged between 18 and 50?		
Do you have eczema or scratch marks on the underside of your forearms?		
Do you suffer from any skin disorder such as psoriasis or other relevant skin disorder?		
Do you suffer from any neurological, kidney or liver disorders?		
Do you suffer from any allergic conditions (hayfever, allergic rashes, asthma or childhood eczema)?		
Have you engaged in any sun-tanning or taken any sunny vacations within the last month?		
Do you take any regular medicine (prescription or OTC)?		
Have you taken any medicine or tablets or used any creams within the last week (contraceptive pills excluded)?		
Have you participated in another corticosteroid dermal study within 2 months of the study date?		
Have you used any corticosteroids within the last three months?		
Do you have any mental deficiency or handicap?		
Are you pregnant?		
Have any history of drug or alcohol abuse?		
Are you contemplating becoming pregnant in the time immediately following the study?		
Are you breastfeeding?		
Are you using reliable contraception (The contraceptive pill, minipill, IUD or abstinence)?		
Do you have a known allergy/hypersensitivity to clobetasol propionate or any other corticosteroids?		

VOLUNTEER ACCEPTABILITY /COMMENTS WITH REGARDEXAMINATION/INCLUSION/ EXCLUSION CRITERIA

Acceptable		Not Acceptable	
Notes/Comments			
Sign _____ Date _____ Principal Investigator (dd/mm/yy)			

POST-STUDY REPORT/MEDICAL

SUBJECT/STUDY STATUS

Study Completed		Study Not Completed	
Comments (If "no" give reasons and procedure/follow-up undertaken)			
Sign _____ Date _____ Study nurse (dd/mm/yy)			

CLINICAL EXAMINATION/SUBJECT RELEASE

Test		**Status/Comments**	
Medical Update			
Application Site Assessment			
Subject Released		Subject Retained for Further Investigation	
Sign _____ Date _____ Study nurse (dd/mm/yy)			
Sign _____ Date _____ Principal Investigator (dd/mm/yy)			

APPENDIX VII

CERTIFICATE OF INSURANCE

Alexander Forbes
RISK SERVICES

Attention: Mike Skinner 3rd November 2007

Dear Mr Skinner

SUBJECT: Confirmation of Insurance

TO WHOM IT MAY CONCERN

This is to confirm that clinical trial volunteers are insured for accidental bodily injury and / or illness as a direct result of participation in the clinical study no. WL_TS1 subject to the terms and conditions of certificate 10/725/113 underwritten by Llyods of London. This policy is renewable annually on 1 January. The next renewal date is therefore 1 January 2008.

The compensation limits are as follows (per volunteer but not exceeding R5,000,000 in respect of all claims arising from one common tests):

Death	: R500,000
Permanent Disability	: Such percentage of R500,000 as is specified for the particular disability
Medical Expenses	: R100,000 (first amount payable R250)

If you have any queries, please call.

Kind Regards

Debra Tuohy
Direct Line: 041 392 8514

Certified Management System ISO 9001:2000

Alexander Forbes Risk Services (Pty) Ltd
Co. Reg. No : 2007/018288/07
An Authorised Financial Services Provider - FSB/FSP Licence No : 9299

Directors: AJ Ossip JJ Erwee BL McClatchie

P O Box 27155, Greenacres, Port Elizabeth, 6057
270 Cape Road, Newton Park, Port Elizabeth, 6045
Tel: +27 (41) 392 8300 (s/b)
For your convenience, please refer to our direct lines
Website: www.alexanderforbes.co.za

APPENDIX VIII

SCREENING MEDICAL CONSENT FORM

APPLICATION OF TAPE STRIPPING FOR THE ASSESSMENT OF THE BIOAVAILABILITY OF CLOBETASOL PROPIONATE IN HEALTHY VOLUNTEERS

STUDY NUMBER: WL_TS1

PROTOCOL VERSION 1

CONSENT FORM VERSION 1

NOVEMBER 2007

I ..... Born on.....

Present address .....

.....

hereby:

i. Confirm that my consent is given freely and I realise that it may be withdrawn at any time, without penalty to me.
ii. Confirm that I have read the information contained in Appendix A and have been informed of the tests to be undertaken.
iii. Confirm that I have been informed of the general procedure for clinical studies undertaken by the BRG.
iv. Confirm that I have attended an informed consent session specific for the clinical study for which I am volunteering at which the contents of the 'Information for Volunteers' Brochure, Version WLTS1 were explained to me and a copy given to me to take home and read before making my decision.
v. Recognise that undergoing a screening medical does not ensure automatic inclusion into the clinical study.
vi. Agree to my medical records being reviewed in the event of an audit or enquiry on the understanding that my anonymity will be maintained.

_____ _____

Signature of volunteer **Date (yyyy-mm-dd)**

_____ _____

Principal Investigator **Date (yyyy-mm-dd)**

Wai Ling Au

BPharm (Rhodes)

APPENDIX IX

VERBAL DELIVERY OF INFORMATION TO STUDY VOLUNTEERS

The announcement will be posted around campus.

Students then contact the principal investigator directly and relay their interest in participating in the study. A short and factual explanation is given of the study and female participants are specifically asked whether they are using reliable contraception (the contraceptive pill, minipill or an IUD) and whether any participant suffers from eczema or psoriasis.

The principal investigator will provide the participants an information brochure regarding the study. The information brochure will be given to the particpants no less than 2 days prior to the study; this allows ample time for the reading through of this information.

At the first meeting in the clinic room in the Faculty of Pharmacy building, 30 minutes are allocated to a verbal explanation of the study and any questions. This provides an undisturbed physical environment and the time allocation of 30 minutes is realistic.

Study volunteers will be given approximately 2 days in which to consider the written information and formulate questions. Should there be any additional questions regarding the preceding verbal information, study volunteers have an opportunity to consider these answers before a final decision is made with regard to participation.

Should the study volunteer continue to be interested in participating, the study volunteer signs the study participation informed consent form as well as the screening medical consent form which are also signed by the principal investigator and in the presence of a witness if required. Study volunteers are hereby included in the study and are given a copy of the Information for Volunteers Brochure and the signed consent forms as well as written instructions.

BRG

ADVERTISEMENT
VALID 1 DECEMBER – 13 DECEMBER 2007

CLINICAL STUDY VOLUNTEERS

The **B**iopharmaceutics **R**esearch **G**roup is looking for

HEALTHY FEMALE and MALE VOLUNTEERS

to participate in clinical studies on a medicated cream applied to the skin using tape stripping

If You Are:

1. Over 18 years
2. Generally healthy and currently medicine free
3. Willing to spend half a day in the clinic

Then You May Be Eligible to Participate

You Will Be Remunerated For Participating

If You Are Interested Please Contact **Genevieve** as soon as possible for More Information:

Preferably by E-mail at: g02a1351@campus.ru.ac.za

Or by Phone at: 046 603 8412

This advert has been approved by the Rhodes University Ethical Standards Committee

Please take a tear-off slip below

BRG Microdialysis Study
g02a1351@campus.ru.ac.za
046 603 8412

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g02a1351@campus.ru.ac.za
046 603 8412

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046 603 8412

BRG Microdialysis Study
g02a1351@campus.ru.ac.za
046 603 8412

AMENDMENTS TO THE TS RESEARCH PROTOCOL

APPLICATION OF TAPE STRIPPING FOR THE ASSESSMENT OF THE BIOAVAILABILITY OF CLOBETASOL PROPIONATE IN HEALTHY VOLUNTEERS

STUDY NUMBER: WL_TS1

PROTOCOL VERSION 1

NOVEMBER 2007

Amendment no. 1 (30 June 2008)

1. **Section 4.1 Number of Subjects**
   From: If needed, an add-on study of up to 10 subjects will be conducted to obtain a degree of variability of less than 30% if possible.
   To: If needed, an add-on study of up to 15 subjects will be conducted to obtain a degree of variability of less than 30% if possible.
2. **Section 5.4 Study Design**
   From: The study will comprise of a single phase sequential design conducted on 15 subjects in the first instance, followed by another 10 subjects if necessary (see section 4.1)
   To: The study will comprise of a single phase sequential design conducted on 15 subjects in the first instance, followed by another 15 subjects if necessary (see section 4.1)

_____ _____

Principal Investigator **Date (yyyy-mm-dd)**

Wai Ling Au

BPharm (Rhodes)

_____ _____

Supervisor **Date (yyyy-mm-dd)**

Professor Isadore Kanfer

BSc (Pharm), BSc (Hons), PhD (Rhodes)

APPENDIX V

RESEARCH PROTOCOL

APPLICATION OF DERMAL MICRODIALYSIS FOR THE ASSESSMENT OF THE BIOAVAILABILITY OF CLOBETASOL PROPIONATE IN HEALTHY VOLUNTEERS

STUDY NUMBER: WL_002

PROTOCOL VERSION 2

November 2008

Reference product	Clobetasol Propionate Ethanolic Solution **(Clobetasol propionate 4% m/v)** **Rhodes University** **South Africa**

Principal Investigator	Wai Ling Au **BPharm (Rhodes)** **Biopharmaceutics Research Group (BRG)** **Faculty of Pharmacy** **Rhodes University** **Grahamstown 6140** **South Africa**
Supervisor	Professor Isadore Kanfer **BSc (Pharm), BSc (Hons), PhD (Rhodes)** **Rhodes University** **Grahamstown, 6140** **South Africa**

FINAL APPROVAL OF PROTOCOL

Study number	WL_002
Study title	**Application of dermal microdialysis for the assessment of the bioavailability of clobetasol propionate in healthy volunteers**
Reference product	Clobetasol Propionate Ethanolic Solution **(Clobetasol propionate 4% m/v)** **Rhodes University, South Africa**

_____ _____

Principal Investigator — Date (yyyy-mm-dd)

Wai Ling Au

BPharm (Rhodes)

_____ _____

Supervisor — Date (yyyy-mm-dd)

Professor Isadore Kanfer

BSc (Pharm), BSc (Hons), PhD (Rhodes)

INVESTIGATORS AND FACILITIES

Position	Name and qualifications	Contact address
Principal Investigator/ Study site/Statistics/ Final report	Wai Ling Au **BPharm (Rhodes)**	**Rhodes University** **Faculty of Pharmacy** **Biopharmaceutics Research Group** **PO Box 94** **Grahamstown 6140** **South Africa** **Tel:. +27 (0) 72 612 0972** **Fax:. +27 (0) 46 636 1205** **Email:. g02a1351@campus.ru.ac.za**
Supervisor	Professor Isadore Kanfer **BSc (Pharm), BSc (Hons), PhD (Rhodes)**	**Rhodes University** **Faculty of Pharmacy** **PO Box 94** **Grahamstown 6140** **South Africa** **Tel:. +27 (0) 46 603 8399** **Fax:. +27 (0) 46 636 1205** **Email:. i.kanfer@ru.ac.za**
Dermatological Consultant	Dr. Eva Benfeldt **M.D., PhD (University of Copenhagen)**	**Department of Dermatology** **University of Copenhagen** **Gentofte Hospital** **DK-2900 Hellerup** **Denmark** **Tel:. +45 35 31 31 59** **Fax:. +45 35 31 31 13** **Email:. benfeldt@post5.tele.dk**
Consultant	Professor Roger Verbeeck **BSc (Pharm), PhD (KUL)**	School of Pharmacy Université Catholique de Louvain PMNT 7360 Avenue Mounier 73 B-1200 Brussels Belgium Tel:. + 32 (0) 2 764 7353 Fax:. +32 (0) 2 764 7398 Email:. roger.verbeeck@uclouvain.be
Consultant	Dr. Mike Skinner **BPharm (Hons), PhD (Rhodes)**	**Rhodes University** **Biopharmaceutics Research Institute** **PO Box 94** **Grahamstown 6140** **South Africa** **Tel:. +27 (0) 46 603 8189** **Fax:. +27 (0) 46 636 1205** Email:. m.skinner@ru.ac.za

CONTENTS

1 BACKGROUND INFORMATION

1.1 Clobetasol propionate

1.1.1 Molecular structure

Clobetasol propionate is a synthetic corticosteroid that has a molecular weight of 467.0g/mol. The molecular structure of clobetasol propionate is represented below. The empirical formula for clobetasol propionate is $C_{25}H_{32}ClFO_5$ [1].

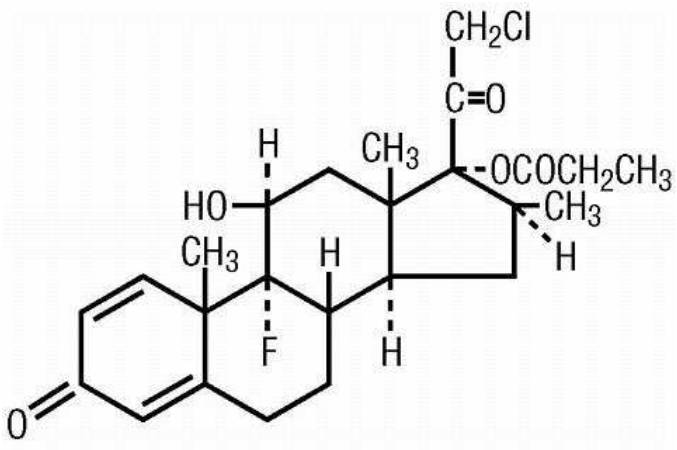


Fig. 1 Chemical structure of clobetasol propionate

1.1.3 Mode of action and uses

The mode of action of corticosteroids is still not clear, however there are a few postulations on their anti-inflammatory activities. One hypothesis that has been widely accepted proposed that the inflammatory process results from the release of hydrolytic enzymes of the lysosomes and that the glucocorticoids stabilize lysosomal membranes, preventing the rupture of the organelles with the accompanying effect of release of hydrolytic enzymes and consequent development of inflammation [2,3]. It has been investigated in view of the fact that prostaglandins have been recovered from several types of inflammatory reactions and are capable of mediating most of the components of inflammation, the possibility arises that corticosteroids may owe their anti-inflammatory activity to part of the inhibition of prostaglandin biosynthesis process [4]. There are suggestions that corticosteroids inhibit the release of prostaglandin by preventing the transport of prostaglandin from inside the fat cell to the extracellular space where it would normally act on the blood vessels [5]. It was also postulated that the corticosteroids block arachidonic acid release from phospholipids from the production of lipocortin which inhibits the activity of phospholipase A_2 [6,7]. The activity of topical corticosteroids is thought to be due to the binding of the drug to steroid receptors located in the cytoplasm or on the outside of the nuclear membrane to form complexes that enter the cell nucleus. These complexes then bind to DNA and modify transcription of mRNA and subsequent synthesis of proteins ultimately responsible for specific effects such as anti-inflammatory activities [8,9]. It is also said that corticosteroids inhibit DNA

synthesis in many types of cells thus assists in the treatment of proliferative inflammations such as keloids [10]. These postulations may perhaps be responsible for one of the anti-inflammatory effects of the corticosteroids, since the arachidonic acid cascade generates several inflammatory mediators. As mentioned earlier, clobetasol 17-propionate is a highly potent synthetic corticosteroid, and it was found that high-potency corticosteroids appear to bind more effectively to the receptors [11].

Clobetasol propionate is a highly potent synthetic topical corticosteroid that has anti-inflammatory, anti-pruritic and vasoconstrictive properties. It is generally used for short term management of various skin disorders. It is widely used in the management of various skin disorders such as eczema, some forms of psoriasis (moderate to severe) and seborrhoeic dermatitis [1,11,].

1.1.3 Pharmacokinetics

Pharmacokinetics after topical application of drugs differs from pharmacokinetics after all other routes of administration in that the site of action (the skin) is accessible to immediate drug analysis. The development of new topical glucocorticoids has two aims [12]:

1) To produce preparations which assure sufficiently high concentrations at the site of action in the skin, and

2) To keep systemic corticosteroid load as low as possible to reduce the risk of systemic effects.

The pharmacokinetic properties of topical corticosteroids remain poorly understood. However, it is certain that the corticosteroids are metabolized in the liver and in other tissues, and are excreted via urine. Most derivatives of this class of drug are absorbed through the skin into dermal blood vessels, with some of the compound being metabolized and excreted as occurs when they are systemically administered. Some compounds are metabolized extensively during skin absorption, for many, metabolism will be small or undetectable [13]. Cutaneous metabolism occurs at the epidermis where certain enzymes found in the liver (including a cytochrome P450 system) may be induced. The corticosteroids may undergo hydrolysis and sulphate conjugation within the epidermis [8].

.

1.1.4 Adverse effects

Upon topical application of clobetasol 17-propionate, one may experience burning sensation, pruritus and stinging of the skin. Headache and other irritation symptom(s) may occur [1].

The onset of local side effects from topical corticosteroids is due to application on the same skin site over a long period of time at reasonably high concentrations. The more common local side effects consist of atrophy, striae, purpura, acne, and telangiectases. The less commonly occurring side effects are local hypertrichosis hypopigmentation, and allergic contact dermatitis [14,15]. Systemic side effects usually occur due to an increase in steroid penetration through the skin by applying the corticosteroids to large surface areas, under occlusion, higher concentrations or more potent derivatives especially in children [16-18]. The potential systemic adverse effects upon topical administration include hypothalamic-pituitary-adrenal (HPA)

axis suppression, retarded growth in children, and hyperglycemia [15,19]. Majority of the side effects are reversible upon stopping or reducing the potency of the topical corticosteroid used.

1.1.5 Contraindications

Administration should be avoided in persons who exhibit hypersensitivity to clobetasol 17-propionate or to any ingredient in the preparation or to other corticosteroids. It is contraindicated in the presence of acute dermatological infections e.g. scalp infection [1].

1.2 Dermal microdialysis sampling

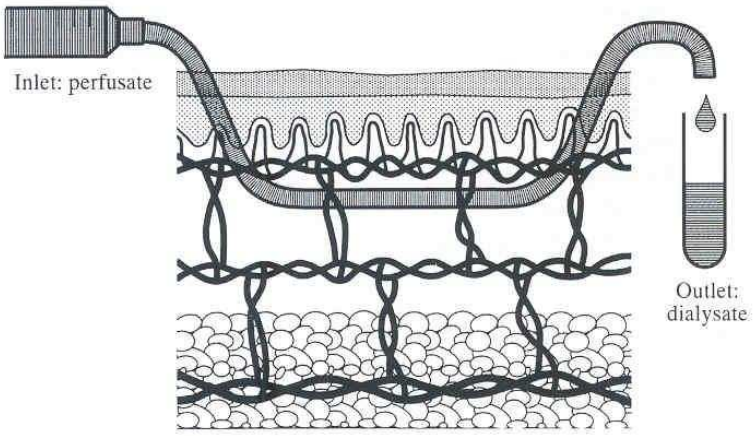
1.2.1 Rationale for dermal microdialysis sampling

During recent decades the advantages of cutaneous and percutaneous drug delivery has gained increasing attraction for novel drug formulations. The percutaneous route may be an attractive solution for systemic delivery of very potent drugs with low oral bioavailability, low systemic clearance and narrow therapeutic window, due to the avoidance of hepatic first-pass metabolism and potential of long-term controlled release. However, the greatest potential for the topical administration route is targeted drug delivery to the skin itself, where dramatically higher skin-to-plasma ratios are attained compared to systemic drug delivery. This route of administration can therefore maintain therapeutically effective drug concentrations in the target organ without the risk of inducing side-effects due to high systemic exposures [20].

In the clinical setting, there are currently no sampling techniques which can be used to demonstrate dermal bioavailability or bioequivalence of topically applied agents which are both minimally invasive and which also give an indication of tissue concentration at a target site in the skin with time. Dermal microdialysis has the potential to address the gap in available sampling techniques due to its minimally invasive nature and its ability to generate concentration-time profiles at the target site with good resolution provided a sufficiently sensitive analytical method is available [21].

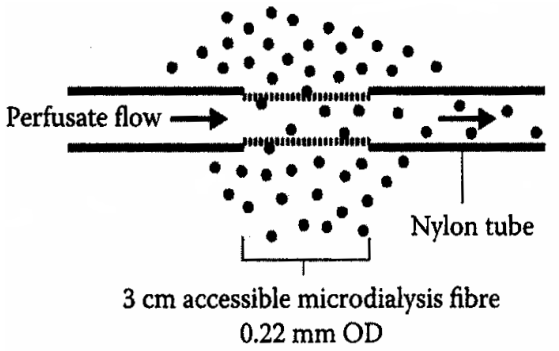
1.2.2 Overview of microdialysis

Microdialysis is a technique for sampling of endogenous and exogenous substances in the extracellular space in living tissue [22,23]. The technique was originally developed in neuropharmacological sciences but has now been used extensively in other tissues in animal models and human studies [21,23]. In human studies, this technique has been employed in adipose tissue, brain, heart, lung, solid tumours, skin etc [20,24,25]. Initial clinical studies using microdialysis have demonstrated the suitability of this method for the measurement of drug concentrations in the interstitium of target tissues and allow for the description of relative changes of concentration-time courses of solutes against a baseline [25]. The figure below shows a linear microdialysis probe inserted into the dermis prior to topical application [22].



1.2.3 Principles of microdialysis

A microdialysis fibre consists of a semipermeable membrane forming a thin hollow 'tube' (typically 0.22 mm ID), which functionally resembles a blood vessel. The fibre allows the passage of molecules with a volume smaller than the cut-off value of the membrane. The fibre has one end connected to an impermeable tube which leads to a micropump and the other end to an efferent sampling tube. For dermal microdialysis, the probe is implanted in the dermis of the skin via a guide cannula. The microdialysis fibre is slowly perfused with physiological solution, which equilibrates with extracellular fluid of the surrounding tissue exchanging substances smaller than the cut-off value of the membrane during the passage through the fibre. Entering the microdialysis fibre, the solution is termed perfusate and following dialysis of substances, the solution exiting the fibre is termed dialysate [20]. The diffusion of substances across the dialysis membrane will occur by passive diffusion, driven across the membrane by the concentration gradient and the speed of equilibrium is consequently proportional to the size of the gradient and diffusion rate of the medicine in the medium, in addition to the surface area of the fibre membrane. Using continuous flow through the probe, compounds will be recovered by the membrane and sampled for further analysis. As substances are able to diffuse in both directions through the membrane, microdialysis can principally be used to both extract and deliver substances in tissues [20].



The figure above depicts the principle of microdialysis sampling by a linear probe [23]. The perfusate is pumped through the probe at a preset, low flow rate. During the passage through the membrane, the diffusion of small molecules will take place.

1.2.4 Insertion trauma and invasiveness of dermal microdialysis sampling

Dermal microdialysis sampling has been described by authors as a minimally invasive technique [21,23]. The insertion of microdialysis probes creates slight but reversible trauma which initially causes an acute inflammatory reaction characterised by increased blood flow, erythema and skin thickness. This is caused by the use of a 23G guide cannula/hypodermic needle for insertion of the linear microdialysis probe. An equilibration time of 60 minutes has been reported to be sufficient to allow the skin to normalise [23]. Histological examination of skin biopsies showed no signs of an inflammatory reaction in the tissue around the probe 8 to 10 hours after probe insertion. Ice is used to induce anaesthesia during probe insertion to provide minimum discomfort to the human volunteer. No significant discomfort is observed when the cannulae have been removed. Hydrocortisone 0.1% m/m cream applied twice daily for 2 weeks is used to arrest any residual inflammation after the completion of the study. Sterile techniques will be employed during the study to ensure the minimum risk of skin infections.

2 STUDY PRODUCTS

2.1 Description

	Reference product
Commercial name	**-**
Generic name	**Clobetasol propionate ethanolic solution**
Dosage form	**Ethanolic Solution**
Strength	**4% m/v**
Manufacturer	**Rhodes University, South Africa**
MCC registration details	**-**
Expiry date	**-**
Description	**A clear, slightly yellow solution**
Dosage	**infinite dose**

2.2 Supply, storage and use

Extemporaneous clobetasol propionate ethanolic solution will be freshly prepared on the day before the study commences and stored in the refrigerator. Sufficient products will be timeously supplied to the clinic by the

principle investigator prior to the study. It should be used externally only. The product should be kept in the container, tightly closed and stored at room temperature away from excess heat and moisture [26]. Dispensing and administration of reference products will be recorded and administered only to subjects for the purpose of this study.

3 OBJECTIVES

The objective of this study is to measure dermal concentrations of clobetasol propionate using microdialysis to assess *in vivo* bioavailability from an extemporaneous topical ethanolic solution and therefore obtain valuable information which will lay the foundation in an attempt to determine bioavailability and bioequivalence of other clobetasol propionate topical formulations using this technique. In addition, the technique will be assessed for its effectiveness in the assessment of dermal bioavailability.

4 STUDY POPULATION AND MEDICAL ASSESSMENT

4.1 Number of subjects

Similar studies have made use of 6 - 18 human subjects for this type of study [27-31] with equal numbers of male and female subjects. This study will make use of approximately 10 human subjects and will make use of an equal number of male and female subjects. Four application sites on the left arm of the subjects will be used for the microdialysis study. Preliminary statistical analysis (Section 8) will be performed on the subjects completing the study. If needed, an add-on study of up to 14 subjects will be conducted to obtain a degree of variability of less than 30% if possible. If no further study is required, preliminary statistical analysis will be finalised and presented in the final report. If an add-on study is conducted, data for all evaluable subjects from both Group 1 and Group 2 will be used in preparation of the final report. Homogeneity testing will be conducted between the two groups.

4.2 Inclusion/Exclusion criteria

Inclusion criteria

Only those subjects meeting the following criteria will be included in the study:

- Female subjects who are using reliable contraception or abstaining.
- Subjects who are aged between 18 and 50.
- Subjects who are in general good health.
- Subjects who will be available for the entire study period.

Exclusion criteria

Subjects meeting the following criteria will be excluded from the study.

- Female subjects who are breast feeding.
- Female subjects who are contemplating becoming pregnant in the time immediately following the study.

- Female subjects who are pregnant.
- Subjects who have a known allergy/hypersensitivity to clobetasol propionate or any corticosteroid.
- Subjects who have a known allergy/hypersensitivity to eggs/soy/nuts products (Intralipid®).
- Subjects who have any history of drug or alcohol abuse.
- Subjects who have any mental deficiency or handicap.
- Subjects who have hairy ventral forearm surfaces and/or abrasions on the underside of their forearms.
- Subjects who have engaged in any sun-tanning or taken any sunny vacations within the last month.
- Subjects who have participated in another topical corticosteroid dermal microdialysis study within 2 months of the study date.
- Subjects who have used any topical corticosteroid within the last three months.
- Subjects who suffer from any allergic conditions (hayfever, allergic rashes, asthma or childhood eczema).
- Subjects who suffer from any skin disorder such as psoriasis, eczema or other relevant skin disorder.
- Subjects who take regular medicine or tablets or used any creams within the last week (contraceptive pills excluded).
- Subjects who test positive for HIV and Hepatitis B*.
- Subjects with a history of any neurological, kidney or liver disorders.

4.3 Subject restrictions

i. No prescription medication and OTC medication (e.g. cold preparations, vitamins, natural products used for therapeutic benefits, antacids, herbal or traditional remedies) will be allowed for at least one week prior to the study.

ii. With the exception of study product no concomitant medication may be taken by subjects during the study.

iii. No alcohol may be taken by subjects from 24 hours prior to product application and during the study.

iv. No strenuous physical activity may be undertaken by subjects from 12 hours before product application and during the study.

v. Subjects must not smoke more than 10 cigarettes per day and will not be allowed to smoke during the study.

vi. Subjects must refrain from applying emollients, skin conditioning creams and/or tanning lotions to their forearms for a period of 24 hours prior to the scheduled time of product application.

Subjects will be informed of the above restrictions and each subject will be specifically questioned on these points prior to product application. Any deviations from the above restrictions which are made known to the investigators either voluntarily or on questioning will be recorded on the appropriate CRF (Appendix V). A decision as to whether the affected subject continues with the study will be taken by the principal investigator and the supervisor.

* The process outlining HIV and Hepatitis B testing is located in the Screening Medical and HIV Testing Consent Form (Appendix VII).

4.4 Criteria for removal from the study

Any subject may be withdrawn from the study at any time due to the following:

i. Voluntary withdrawal by the subject due to any reason.

ii. Illness or injury during the study if regarded as clinically significant by the principal investigator and the supervisor.

iii. Any adverse event or signs of toxicity if regarded as clinically significant by the principal investigator and the supervisor.

iv. Failure of the subject to comply with or be uncooperative towards any study requirements or restrictions if regarded as clinically significant by the principal investigator and the supervisor.

v. Subjects withdrawn or dropped out of the study will be fully documented and accounted for in the CRF.

5 STUDY PROCEDURE

5.1 Pre- and post-study medical screening

Pre-study screening will be conducted not more than 30 days prior to the start of the study. Post-study follow-up will be conducted within 48 hours after removal of the microdialysis probes from the forearm of the subject either at the end of the study or termination of the study or withdrawal from the study. Pre-study and post-study evaluations will be conducted as listed in the table below.

Screening test	Pre-study	Post-study
Medical tests	√[1]	x
Medical history	√[2]	√[5]
Dermatological assessment	√[3]	√[6]
Adhesive sensitivity	√[4]	x

[1]Medical tests: HIV, Hepatitis B and pregnancy test

[2]Medical history: Demographic data (date of birth, age, sex, origin), skin (dermatological), allergies, alcohol consumption, smoking habits, dietary habits and sporting commitments.

[3]Dermatological: General assessment of the volar aspect of the forearm and any dermatological condition which may influence the barrier function of the skin and impact on the absorption of topically applied topical corticosteroids. Ultrasound scanning of the texture of the subject's skin of the forearm.

[4]Adhesive sensitivity: Assessment of subject's sensitivity to adhesive on application site.

[5]Medical history: Since start of study.

[6]Dermatological: Examination of forearms and application sites.

If any study related abnormalities are observed at the post-study medical, appropriate follow-up action will be taken and re-examination and re-testing conducted until the abnormality returns to normal or until the principal investigator considers the abnormality to be clinically insignificant.

5.2 Medical tape and clear adhesive glue sensitivity screening

Not more than 30 days prior to the start of the study, volunteers will undergo an assessment of their tolerance to the adhesive on medical tapes and glue used to demarcate application sites.

The following screening protocol will be utilised.

i. The forearm of the subject will be washed 1 hour prior to the applications.
ii. Application of the tape and glue will occur at 0700 hours.
iii. Six hours after application, the medical tapes and glue will be removed.
iv. The forearm of the subject will be assessed for allergies to the adhesives.

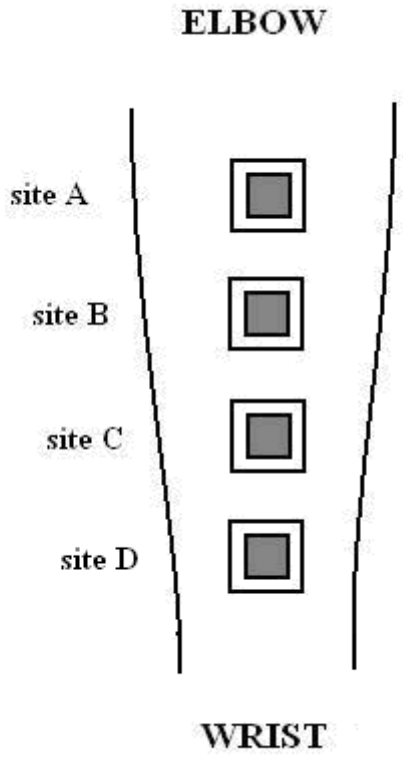
Subjects who exhibit allergies will not be eligible as candidates for the study.

5.3 Check-in and confinement

Subjects will check-in at the Biopharmaceutics Research Group (BRG) clinic, Pharmaceutical Sciences and Chemistry Building, Rhodes University, Grahamstown at 0700 hours on the morning of the study day when they will undergo a brief medical examination, inclusion and exclusion criteria check and study restriction check. Subjects accepted into the study will then be prepared for product application. Subjects will remain in the study room for the entire duration of the study.

5.4 Study design

The study will comprise of a single phase sequential design conducted on 10 subjects in the first instance, followed by another 14 subjects if necessary (see section 4.1). A maximum of two subjects will be studied at any one time on any study day and the time between the first and the tenth subject is expected to be approximately 6 weeks. A total of 4 probes and application sites will be employed on the left arm for the microdialysis study as shown in the diagram below. The duration of the study for each subject will be approximately 7 hours.



Microdialysis probes prepared as described in section 5.6 will be inserted approximately 0.6 to 0.8 mm beneath the skin surface for a distance of 3 cm as described in section 5.7. Probes will be inserted parallel to each other across the volar aspect of the left forearm approximately 3 cm apart using 23G x 1½" hypodermic needles as guide cannulae. Anaesthesia will be obtained by the application of an ice pack to the area of cannulation. No other anaesthetic will be necessary and the procedure during and after probe insertion is essentially pain-free [23]. Probes will remain in place for no longer than 7 hours.

5.5 Pre-study day activities and procedures

This study is dependent on a number of preparations prior to the study. The following is a list of items that will be made available at least 24 hours prior to the study.

- The linear microdialysis probes will be manufactured in-house no more than 24 hours before the study (Section 5.6).
- Extemporaneous 4% m/v clobetasol propionate ethanolic solution (25 ml) will be prepared no more than 24 hours before the study.
- Microlitre glass (300 µl) inserts, 1.5 ml amber HPLC vials and caps will be prepared for use with the analytical procedure.
- Calibration standards and mobile phase for clobetasol propionate analysis will be prepared.
- Pre-weighed sampling vials or centrifuge tubes will be labelled and ready for sample collection.
- Sterilisation solution (70% v/v ethanol) will be prepared.

- CMA microdialysis syringes, a pair of stainless steel scissors, a pair of tweezers, spatula, and a glass rod will be left sterilising overnight in sterilisation solution.
- The checklist will be performed (Appendix I).
- The general setup of the clinic will be ensured.

5.6 Linear microdialysis probe manufacture

The linear probes used for the study will be manufactured in-house by the principal investigator. The probes will be prepared no longer than 24 hours prior to each study day. The following manufacturing protocol will be used to prepare the linear probes for the study.

i. The desired lengths of the Portex® tube (30 cm), Hemophane, (8 cm) and stainless steel wire (9 cm) will be cut.
ii. Stainless steel wire will be degreased with acetone.
iii. Hemophane fibres which will be carefully threaded with the stainless steel wire, which will provide mechanical strength to the probe assembly.
iv. A length of Portex® tube will be glued with cyanoacrylate glue (Loctite®) to one end of the Hemophane fibre - stainless steel wire setup. This assembly will now be referred to as the probe.
v. Probes will be left to dry overnight.
vi. Preliminary leak tests will be performed with double-distilled water.
vii. Blue tubing connectors will be attached to the Portex® end of the probes.
viii. Probes will be stored in a clean glass petri dish with cover and conveyed to the clinic.
ix. Six probes will be manufactured in the unlikely event that a probe fails the preliminary leak test.
x. The probes will be sterilised by soaking in 70% v/v ethanol for 20 minutes before use in subjects.

5.7 Study day activities and procedures

5.7.1 Microdialysis study

1. Study sessions will begin at approximately 0700 hours each study day.
2. Verification by history of adequate washout of excluded drugs.
3. Checklist will be confirmed (Appendix I).
4. CMA microdialysis syringes pre-sterilised over night (Section 5.5) will be flushed and filled with sterile intralipid 20% (perfusate).
5. Probes will be connected to the syringes and a final 20 drop leak test will be manually performed using perfusate prior to installing the syringes into the CMA microdialysis pumps.
6. The syringes connected to the probes will be housed in the CMA microdialysis pump and aligned.
7. Subjects will be advised to take a bathroom visit.
8. The left forearm of the subject will be rinsed with mild soap and water and then blotted dry with a non-abrasive towel.
9. Subjects will be requested to lie on a bed.

10. The wrist will be loosely restricted using a bandage to prevent any sudden jerk during and after the insertion of the probes.
11. Four parallel application sites aligned in a straight line from the elbow joint to the wrist will be demarcated on the ventral surface of the forearm of the subject using a permanent marker.
12. Entry and exit points through which the guide cannulae (23G x 1½") will be inserted will be marked on the forearm at the ends of each application site.
13. Ice packs will be placed on each application site and by employing sterile technique, guide cannulae will be inserted between 0.6 - 0.8 mm below the surface of the skin for a distance of 3 cm transversally across the volar forearm in parallel at the four sites. The application of ice pack at each site on the forearm may have to be repeated during the insertion process.
14. Care will be taken to maintain placement in the superficial dermis in order to minimise intra-probe depth variability.
15. Probes which have been sterilised by soaking into 70% v/v solution will be taken from the ethanol bath and the Hemophane portion introduced through the guide cannulae.
16. The guide cannulae will then be removed leaving the Hemophane portion of the probe in the dermis with the Hemophane/Portex® junction abutting the dermal insertion point.
17. Portex® tubing at the end of each probe will be secured on the arm with Micropore™ tape.
18. Entry and exit points will be sealed with a drop of cyanoacrylate glue.
19. A reservoir (3.2 x 2.0 cm) will be adhered over each demarcated site using Bostik® clear adhesive glue on the forearm.
20. Perfusion of the probes will commence at 1µl/min for 60 minutes.
21. Blank samples will be collected for 60 minutes at a perfusion rate of 1 µl/min .
22. Approximately 4ml of the product will be applied to each site from an Eppendorf® pipette (Section 5.8).
23. Samples of perfusate will be collected continuously in 30 minute aliquots in a period of 4 hours i.e. 8 samples at a perfusion rate of 1 µl/min.
24. At the end of the last sampling period, the Portex® tubing will be severed from the pump setup and if necessary will be further secured on the forearm of the subject.
25. The reservoirs and its contents will be removed from the forearm of the subjects.
26. Ultrasound scanning will be conducted to determine the exact depth of each probe underneath the surface of the skin.
27. Probes will be carefully removed from the forearm of the subject ensuring that both the Hemophane fibre and the stainless steel wire are removed.
28. The application site area will be dressed with alcohol swabs and small adhesive plasters.
29. Subjects will receive a 0.1% m/m hydrocortisone cream to be used twice daily for 2 weeks on the forearm to reduce any swelling caused by the insertion of the probes.
30. The CMA microdialysis syringes, stainless steel scissors, tweezers, and spatula will be left sterilising overnight for use with the next subject.

NB: There will be no re-use of apparatus inserted into subjects. Such apparatus will be discarded as biological waste.

The actual time of study procedures and/or results/comments obtained during the study will be recorded on the '*Registration of Data during Microdialysis Form*' (Appendix II).

5.8 Product application

Just prior to the product application, the setting will be adjusted to 4 ml on the dial of the Eppendorf® pipette to ensure that the correct volume of the product will be dispensed once into each reservoir for microdialysis, respectively. The Eppendorf® pipette will be used to ensure that an accurate volume of the solution is dispensed to each application site for each subject. Application of the product will be done by the principal investigator.

5.9 Posture and physical activity

Subjects will be expected to lie on a bed for the duration of the microdialysis procedure with the forearm restricted loosely with a bandage. Subjects will not be able to walk around for the whole duration of the microdialysis study except for the initial bathroom visit. The duration of the entire study will last for approximately 7 hours. Strenuous exercises will not be permitted as described in section 4.3.

5.10 Food and fluids

Subjects will not be restricted with respect to food and fluid intake. However subjects will be advised to keep fluid intake to a minimum, so as to not develop the urge to visit the bathroom frequently during the study.

5.11 Subject monitoring

The principal investigator will be present at all times during the study. Subjects will be asked open-ended questions about their health at the time of each assessment and any discomfort observed during the experience will be recorded in the CRF (Appendix V).

6 SAMPLE ANALYSIS

Samples will be analysed for clobetasol propionate using a validated extraction procedure and a suitable analytical method. Analysis will be done within 48 hours after sample collection. Extracted samples will be kept in the fridge at (4 – 8 °C) to prevent any degradation.

7 DATA ANALYSIS

For the microdialysis study, concentration-time profiles will be generated and the AUC will be calculated using the trapezoidal rule. The data used to estimate AUC will be reported. Deletion of data from analysis will be justified. All individual subject data will be documented and the individual dermal concentration-time curves will be presented in linear/linear and log/linear scale.

7.1 Statistical analysis

Pharmacokinetic and statistical parameters will be determined by the principal investigator using GraphPad® Prism version 4. The statistical analysis will estimate the variance associated with subject-to-subject variability and the probe-to-probe variability within the subjects. Summary statistics such as median, minimum and maximum will be given.

8 ETHICAL AND REGULATORY REQUIREMENTS

8.1 Ethical and institutional review

Approval by the Rhodes University Departmental Ethical Standards Committee (RUDESC) will be obtained before the study commences. The original signed copy of the ethical approval will be retained by the principal investigator.

The study will be conducted in accordance with the recommendations of the guidelines as set out in the Declaration of Helsinki (1964) and its amendments of Tokyo (1975), Venice (1983), Hong Kong (1989), Somerset West (1996) and Edinburgh (2000), according to ICH Good Clinical Practice (GCP) guidelines and in compliance with the Biopharmaceutics Research Group's (BRG) SOPs, RUDESC requirements and guidelines on the conduct of clinical trials in South Africa.

8.2 Written informed consent

Preceding the study, the nature, purpose and risk of participating in the study will be explained to all volunteers. If volunteers desire, they will be given time to consider the information and any questions that they might have will be answered. The nature of the insurance cover will also be explained. They will also be informed that they may withdraw from the study at any time without penalty to themselves (other than a reduced remuneration) but that they will be encouraged to be committed to completing the study prior to their enrolment. They will sign a consent form in the presence of a witness. Subjects will receive written, detailed instructions concerning the study performance and restrictions.

8.3 Case report form

The Case Report Form (CRF) for this study will be designed and supplied by the principal investigator (Appendix V). All case report forms will be quality assured and all major events such as final acceptance of a subject, adverse events and final release from the study will be signed by the principal investigator.

8.4 Record retention

All source documents, study reports and other study documentation for which the principal investigator is responsible will be archived and retained by the Faculty of Pharmacy, Biopharmaceutics Research Group. Results will be published in the scientific journals and/or presented in a thesis submitted to Rhodes University in fulfilment of the requirement for the degree of Doctor of Philosophy (Pharmacy) after the completion of the study.

8.5 Insurance

Subjects will be insured against any permanent adverse effect on their health which may arise in connection with the conduct of the study. A copy of the insurance certificate (Appendix VI) will be provided to RUDESC as part of the application to conduct this study. Adequate insurance cover in the event of negligence on the part of principal investigator and the supervisor will be ensured.

8.6 Termination of the study

The principal investigator reserves the right to terminate the study in the interests of subject welfare following consultation with the supervisor. The supervisor may terminate the study at any time for scientific or safety reasons. If the study is prematurely terminated or suspended for any reason the principal investigator will promptly explain to the subjects, take appropriate steps as deemed necessary under the circumstances to assure the subjects and where applicable follow up with therapy and inform the RUDESC.

8.7 Adherence to protocol

Excluding an emergency situation in which proper treatment is required for the protection, safety and well-being of study subjects, the study will be conducted as described in the approved protocol. Any deviation from the protocol will be recorded and explained.

Should amendments to the protocol be required, the amendments will be documented and signed by the principal investigator and the supervisor. If the protocol amendment(s) has an impact on the safety of subjects, such as a change in dosing regimen or additional formulations, the amendment will be submitted to the RUDESC for approval.

8.8 Blinding

Subjects will not be blinded and will be informed about the products for use at the application sites.

8.9 Adverse events/Adverse drug reactions

Subjects will be questioned on their health status at check-in, during the course of the study and before leaving the clinic at the end of the study. During the study, open-ended questions will be asked. If any adverse events are reported, the principal investigator will monitor the adverse event, initiate appropriate treatment if required and decide whether or not to withdraw the subject from the study. Signs and symptoms of any adverse events which occur during the study will be fully documented in the appropriate CRF (Appendix V). If necessary adverse events will be referred to a suitably qualified medical practitioner for assessment and follow up.

Adverse events (which include illnesses, subjective and objective signs and symptoms that have appeared or worsened during the course of the study) will be assessed by the principal investigator and the supervisor during and after the study to determine whether or not they are related to the investigational test product (i.e. ADR), to the study procedure or other. The outcome of this assessment will be recorded in the appropriate CRF (Appendix V).

AEs classified as severe or serious will be reported to the supervisor, Rhodes University Ethical Standards Committee within 24 hours.

ADRs classified as serious and unexpected will be subject to expedited reporting as detailed in the ICH E2A and E2B guidelines on Clinical Safety Data Management and Data Elements for Transmission of Individual Case Report Forms respectively.

9 REPORTS

A full report on the study will be compiled by the principal investigator in the format requested by the supervisor and submitted to the supervisor. The analytical report will include results for all standard and quality control samples. A representative number of chromatograms or other raw data will be included covering the whole concentration range for all standards and quality control samples. The validation report will also be submitted.

ABBREVIATIONS

ADR	Adverse Dug Reaction
AE	Adverse Effect/Event
ANOVA	Analysis of Variance
AUC	Area Under the Curve

BRG	Biopharmaceutics Research Group
C_{max}	Maximum Concentration
CRF	Case Report Form
CRO	Clinical Research Organisation
HPLC	High Pressure Liquid Chromatography
GCP	Good Clinical Practice
ICH	International Conference of Harmonisation
IRB	Internal Review Board
QA	Quality Assurance
RUDESC	Rhodes University: Departmental Ethical Standards Committee
SOP	Standard Operating Procedure
t_{max}	Maximum Time

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APPENDIX I

CHECKLIST

SUBJECT NAME		SUBJECT INITIALS		DATE	
STUDY NUMBER	WL_002	VOLUNTEER REFERENCE			
2.5 ml microdialysis syringes (labelled were necessary)					
23G 1½" intravenous needles					
3M Micropore™ tape (1530)					
Alcohol swabs					
Bandages					
Sharps and biological waste container					
Calculator					
CMA 400 microdialysis pumps setup					
Cotton swabs					
Cyanoacrylate glue (Loctite® glue) and Clear adhesive glue (Bostik®)					
Electrical cables and power supply					
25ml of a 4%m/v clobetasol propionate ethanolic solution					
Green cloth underlay					
Human subject under study					
Ice packs					
Pen/marker					
Perfusate solution – *Intralipid 20%*					
Prepared probes attached to blue connectors (leaks pre-checked with water) in glass dish					
Pre-weighed and labelled sampling vials/centrifuge tubes					
Ruler					
Scissors					
Snack/Lunch/Supper					
Soap and towel					
Sterile gloves					
Sterilisation solution (70% v/v ethanol)					
Sterilisation trays					
PVC Reservoirs					
Thermometer					
Timer					
Tweezers					
Ultrasound scanner setup					
CHECKED BY:			SIGNATURE:		

APPENDIX II

REGISTRATION OF DATA DURING MICRODIALYSIS EXPERIMENTS

Date		Subject initials	
Subject/Volunteer name		Volunteer Reference	
Age		Study number	WL_002
Sex			
Race			
Formulation			
Applied dose			
Site			
Principal Investigator			
Assistant principal investigator			
Room temperature			
Relative Humidity			

TIME SCHEDULE

Task	Time start	Comments
Confirm checklist		
Fill syringes with perfusate		
Connect probes to syringes		
Preliminary leak check (20 drops)		
Confirm Inclusion and Exclusion criteria		
Confirm consent form		
Flush and align syringes in CMA 400 pump		
Bathroom visit (volunteer!!)		
Forearm wash		
Wrist bandage to be placed		
Mark entry and exit points on arm		
Probes put in ethanol soak (20 mins)		
Ice pack retrieval		
Insertion 1		
Insertion 2		

Insertion 3		
Insertion 4		
Probe insertions (4 probes)		
23G intravenous needle removal		
Seal entry and exit points (15 mins)		
Place reservoirs over probes		
Secure Portex® on the arm with Micropore™		
Stabilise flow at 1.0 µl/min and collect blank solutions (60 minutes)		
Application of reference formulation and commence sampling every 30 minutes at a flow rate of 1.0 µl/min for 4 hours		
End of microdialysis sampling		
Cut the Portex® tubing to the pump		
Remove the templates from the arm		
Ultrasound scanning		
Probes out of arm		
Dress area with alcohol swabs and bandages		
Ethanol soak of equipment		
End of study		

ULTRASOUND SCANNING

	Probe 1 (mm)		Probe 2 (mm)		Probe 3 (mm)		Probe 4 (mm)	
	Skin	Probe	Skin	Probe	Skin	Probe	Skin	Probe
Scan 1(a)								
Scan 2(b)								
Scan 3(c)								
Mean								
Std Dev								

APPENDIX III

INFORMATION FOR VOLUNTEERS BROCHURE

APPLICATION OF DERMAL MICRODIALYSIS FOR THE ASSESSMENT OF THE BIOAVAILABILITY OF CLOBETASOL PROPIONATE IN HEALTHY VOLUNTEERS

STUDY NUMBER: WL_002
PROTOCOL VERSION 2
BROCHURE VERSION WL02
November 2008

1 Study objective

The study involves research to assess the rate and extent to which clobetasol propionate diffuse into the skin from an extemporaneous topical ethanolic solution, using dermal microdialysis as a measure of efficacy. This study will provide valuable insight for future use in the assessment of amount of drug penetrated into the skin and the sameness of generic and proprietary clobetasol propionate formulations.

Reference product	**Clobetasol Propionate Ethanolic Solution**
Drug	**Clobetasol propionate 4% m/v**
Company	**Rhodes University**
	South Africa

The study will involve approximately 10 subjects.

2 Ethical considerations and standards of practice

Innovator drug companies who develop a new drug have patent protection for the drug for a number of years, typically 15 - 20. Once the patent has expired, generic drug companies are permitted to make and market their own products as competitors to the innovator's product, usually at a substantially reduced cost. However, a prerequisite for registration with national drug registration bodies (and subsequent marketing) is that the rate and extent to which the drug is absorbed from the generic product is shown to be equivalent to that from the innovator product. This is an indirect way of demonstrating that the generic product will be as effective clinically as the innovator and is demonstrated by what are known as ‘comparative bioavailability or bioequivalence studies’.

2.1 Ethical and institutional review

Approval by the Rhodes University Departmental Ethical Standards Committee (RUDESC) is required before the study can commence. The study will be conducted in accordance with guidelines set out in the

Declaration of Helsinki (1964) and its amendments of Tokyo (1975), Venice (1983), Hong Kong (1989), Somerset West (1996) and Edinburgh (2000). This Declaration sets out ethical principles, which protect your rights participating in such studies. Guidelines on the conduct of clinical trials in South Africa will be adhered to.

The study will also be conducted according to the ICH (International Conference on Harmonisation) Guidelines for GCP (Good Clinical Practice), the Biopharmaceutics Research Group's Standard Operating Procedures and the requirements of RUDESC.

2.2 Written informed consent

Preceding the study, the nature, purpose and risk of participating in the study will be explained to you. Should you wish, you will be given time overnight to consider the information and any questions that you might have will be answered. The nature of the insurance cover will also be explained. If you decide to participate in the study you will sign a consent form in the presence of a witness. You are encouraged to consult your parents or personal medical doctor for approval in this study.

3 Voluntary nature of participation

Your participation in this study is entirely voluntary and you may withdraw from the study at any time, without prejudice. Should you decide to participate, we ask that you try to be committed to completing the study if at all possible. Should you encounter any problems along the way, please speak to me so that every effort can be made to assist you.

4 Dates and duration of the study phases

The study consists of one phase only which will run over a day. The study will be conducted from 0700 hours until 1400 hours.

5 Place of study

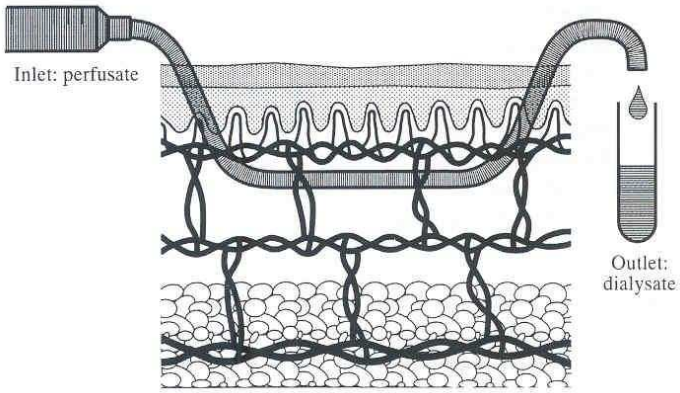
The study will be conducted in the Biopharmaceutics Research Group (BRG) clinic in the Pharmaceutical Sciences and Chemistry Building at Rhodes University. The principal investigator and the supervisor will be suitably qualified, trained and experienced to perform the study procedures.

6 Background information

Clobetasol propionate is used in the management and treatment of patients with skin disorders such as psoriasis, eczema and dermatitis. It is for adult use only.

Microdialysis is a technique for sampling of natural and foreign substances in the space surrounding cells in living tissue. The technique was originally developed for brain research but has now been used extensively in other tissues in animal models and human studies. In human studies, this technique has been employed in adipose tissue, brain, heart, lung, solid tumours, skin etc. A microdialysis fibre consists of a semipermeable membrane forming a thin hollow 'tube' (typically 0.22 mm ID), which acts like a capillary. The fibre has

one end connected to a tube which leads to a micropump and the other end to sampling tube. The probe is implanted in the dermis or the middle layer of the skin via a needle. The microdialysis fibre is slowly perfused with Intralipid and substance in the skin will diffuse across the membrane into the Intralipid which is collected and analysed. Substances move across the dialysis membrane will occur by passive diffusion.



Dermal microdialysis sampling is described as a minimally invasive technique. The insertion of the microdialysis probes creates acute inflammatory reaction characterised by increased blood flow, erythema and swelling demonstrated with the use of a linear microdialysis probe by a 23G needle. This procedure has been shown to be safe and no irritations with the probes have been noted to date. Ice is used as anaesthesia providing minimum discomfort. No more discomfort is observed when the needle has been removed. You will be supplied with a tube of hydrocortisone 0.1% m/m cream to apply twice daily for 2 weeks to arrest any residual inflammation after the completion of the study. Sterile techniques will be employed during the study to ensure the minimum risk of skin infections.

Study design

On the left arm, four very thin microtubules which are 0.2 mm in diameter (*microdialysis probes*) will be inserted approximately between 0.6 - 0.8 mm beneath the skin and approximately 3 cm apart. Ice will be used as a means of anaesthesia to allow for minimum discomfort when the probes are inserted into the skin by means of a needle. Pain should be minimal – if at all. Once the microdialysis probes are in place, you will not be able to move freely, but will have to remain lying on a bed or sitting in an arm chair with the opportunity to watch TV or read. During the study, the washout liquid which is pumped through the probes will be collected every 30 minutes. The microdialysis study will conclude at 1400 hours, after the position of the probes under the skin has been ascertained by means of an ultrasound scanner. This is a quick pain free non-invasive technique which uses high frequency sound to measure the depth of the probe in the skin. Thereafter the probes will be removed from the skin and the area where the probes were inserted will be covered by small adhesive plaster strips.

8 Adverse effects

For topical preparations, delayed hypersensitivity may occur at the site of the application, but this is uncommon. It is advised that clobetasol propionate cream preparations should not be used on open wounds

or lesions on the skin or near the eye. The skin reactions are however reversible on discontinuation of therapy. Clobetasol propionate must not be administered to healthy or ill individuals who have an allergy to this compound or to any of the ingredients in the formulation. In this study, a single application to a limited area is unlikely to invoke any adverse effects but procedures will be in place to address any discomfort noted during and after the study.

Microdialysis technique is safe and no adverse reactions such as irritation or allergic reactions to the probes have been reported so far. The insertion of microdialysis probes creates slight but reversible swelling. This is caused by the use of the needle for insertion of the linear microdialysis probe. No reactions have been reported to date in tissue around the probe 8 to 10 hours after probe insertion. Ice is used to induce anaesthesia during probe insertion to provide minimum discomfort. No significant discomfort is observed when the needles have been removed. Sterile techniques will be employed during the study to ensure the minimum risk of skin infections.

9 Conditions of participation in the study

To participate in this study you must:-

- Undergo and pass a medical assessment which includes giving a general medical history, recording of vital signs, body height and weight measurements and an assessment of your skin.
- Undergo a skin test to assess any possible allergy or sensitivity to the adhesive in the medical tape and clear glue that will be used. After 6 hours the tape and glue is removed, the forearm washed and the skin inspected for any signs of reaction.
- Fulfil certain inclusion and exclusion criteria which have been set out in the protocol.
- Agree to be fully committed to the study and conscientiously abide by the restrictions required of you as listed in section 11.

NB: It is extremely important that you divulge any past medical history and abide by the rules for participation in this study. This is to protect you from unnecessary risks and to help ensure the reliability of the data gathered.

10 Volunteer inclusion/exclusion criteria

You will be considered for this study if you are aged between 18 and 50, in general good health, available for the entire study period and if female you are on reliable contraception or abstaining from sex.

Please do not consider participating in this study if you

xv. are breast feeding.

xvi. are contemplating becoming pregnant in the time immediately following the study.

xvii. are pregnant.

xviii. have a known allergy/hypersensitivity to clobetasol propionate or any corticosteroid.

xix. have any history of drug or alcohol abuse.

xx. have any mental deficiency or handicap.

xxi. have hairy ventral forearm surfaces and/or abrasions on the underside of their forearms.

xxii. have engaged in any sun-tanning or taken any sunny vacations within the last month.

xxiii. have participated in another corticosteroid dermal microdialysis study within 2 months of the study date.

xxiv. have used any corticosteroids within the last three months.

xxv. suffer from any allergic conditions (hayfever, allergic rashes, asthma or childhood eczema).

xxvi. suffer from any skin disorder such as psoriasis, eczema or other relevant skin disorder.

xxvii. take regular medicine or tablets or used any creams within the last week (contraceptive pills excluded).

xxviii. test positive for HIV and Hepatitis B[•].

xxix. have a history of any neurological, kidney or liver disorders.

xxx. have a known allergy/hypersensitivity to egg, soy and/or nut products.

11 Study restrictions

Restricted item	Duration of restriction	Examples of restriction	Comments
Prescription drugs	From 1 week before the start of the study, until the end of the study.	- all medication obtained on prescription - antibiotics, vaccinations - anti-inflammatories - anti-asthmatic drugs - anti-acne drugs etc.	This includes all long term medication.
Over-the-counter (OTC) drugs	From 1 week before the start of the study, until the end of the study.	- anti-flu drugs, sports supplements, antacids, paracetamol - vitamins, minerals -homeopathics	This includes herbs, natural products & all medications that can be bought without a prescription.
Alcohol	From 48 hours before the start of the study and during the study. **No alcohol for a total of just over 2 days**	All alcoholic drinks and alcohol containing foods.	**It is important that this requirement is taken seriously and observed, as alcohol can significantly affect the liver.**
Strenuous Physical Exercise	From 48 hours before the start of the study. **No strenuous exercise for a total of almost 2 days.**	Rugby Squash Rowing Gym	Light exercise such as walking is permitted.

[•] The process outlining HIV and Hepatitis B testing is located in the Screening Medical and HIV Testing Consent Form

Restricted item	Duration of restriction	Examples of restriction	Comments
		Tennis etc.	
Smoking	No smoking will be permitted during the study.	Cigarettes	No cigars or pipe smoking.
Moisturising creams	You must refrain from applying any type of skin conditioning creams to your forearms from 24 hours before the study until the end of the study	All skin creams, e.g. moisturizers, Vaseline, medicated creams, aqueous cream and tanning lotions.	This could interfere with the absorption of the study formulation.

NB: Random checks will be done to ascertain whether you have managed to adhere to the above restrictions. If you have not been able to adhere to the study restrictions please inform us immediately or else you may not be able to participate with a reduced remuneration as detailed in section 15.

12 Procedures and duration of the study

The study consists of a single phase. The procedures and duration of the study are detailed below.

On the morning of the study you must check into the clinic at 0700 hours. At check-in you will be:-

- questioned and undergo a brief medical examination to establish whether you still fulfil all the inclusion and exclusion criteria since your pre-study medical examination and that you have complied with the study restrictions.
- You will be instructed to use the bathroom because when the study starts your wrist will be restricted and you will not be able to leave the bed or the chair for approximately 6 hours.
- The study will commence at 0730 hours and you will be expected to remain in the clinic throughout the course of the study.
- Entertainment and lunch will be provided.
- You are not restricted with regard to food and drink during the study period although minimum fluid intake will be advised so as not to develop an urge to visit the bathroom during the study.
- Should any new and significant information about the study medication become available during the course of the study, this will be communicated to you.
- A tube of 0.1% m/m hydrocortisone cream will be given to you to self medicated your arms twice daily for 2 weeks.

13 Benefits

Since you have been screened as healthy before the start of the study, there is no medical benefit to you from participating in this study.

14 Financial compensation

You will receive a gratuity of R 500.00 for full participation in the study. Payment following withdrawal from the study will be calculated on a pro-rated basis from the start of the study at 0700 hours to the end of the study at approximately 1400 hours.

15 Adverse medical events

You will be monitored prior to, during and after each study day for any adverse events whether or not they are thought to be related to the investigational products or procedures. If any adverse events are reported, the principal investigator/supervisor will decide whether or not to withdraw you from the study and what treatment is appropriate. In addition, the Rhodes University Departmental Ethical Standards Committee will be notified of any serious adverse events (SAEs). All adverse events will be monitored and treated until recovery to your pre-study status. You will be referred to a medical doctor if necessary and all medical costs will be covered by the BRG. All adverse events will be monitored and treated appropriately until a satisfactory outcome is attained.

16 Insurance

You are covered by an insurance policy taken out by the BRG, in case of claims arising from the medication or procedures as outlined in the protocol, and in case of negligence on the part of the BRG. If you have personal insurance e.g. life insurance /assurance your participation in this study may affect your policy. You are advised to determine this prior to participating in the study. If you need any assistance in this regard please feel free to contact me. During the clinical study you are not entitled to participate in other studies and you should not participate in any other study/s for thirty (30) days after completion of this study to ensure that there are no interactions. Insurance policies will be available from the principal investigator for scrutiny.

17 Confidentiality

Your medical history and physical examination records and any other information or data generated during this study will be kept confidential. However, you must agree that all the above mentioned documentation and data can be released for any lawful purpose and released for publication in scientific journals and/or presentation in a thesis submitted to Rhodes University in fulfilment of the requirement for the degree of Doctor of Philosophy (Pharmacy) after the completion of the study. In such cases your name will be removed from all documentation to ensure your anonymity. In signing the consent form for the study, you agree to the granting of access to your medical data. Your medical data will be provided to you upon request and you will be informed of significant abnormalities identified before or after the study.

Your consent is also required to permit the study staff to consult with any medical practitioner who is normally responsible for your care if the need arises. The onus, however, is on you to inform such a practitioner of your intention to participate in such a study, should you so wish.

18 Amendments/Changes

Should there be any changes made to the trial protocol these will be communicated to you verbally and in writing in time to enable you to reconsider your decision to participate in the study.

19 Withdrawal

You may withdraw from the study at any time due to the following:

- Voluntary withdrawal by yourself due to any reason.
- Illness or injury during the study if regarded as clinically significant by the principal investigator and the supervisor.
- Any adverse event or signs of toxicity if regarded as clinically significant by the principal investigator and the supervisor.
- Failure to comply with or be uncooperative towards any study requirements or restrictions if regarded as clinically significant by the principal investigator and the supervisor.
- It is your right to withdraw from the study at any time. However, by signing the Informed Consent form and participating in the study, you agree to be committed to completing the study if at all possible.

20 Termination of the study

The BRG reserves the right to terminate the study prematurely in the interests of your welfare.

21 Emergency contacts

You are obliged to notify the principal investigator as soon as possible if you are unable to follow the procedures or if you suffer any adverse event we have not told you about. In particular, you should make every effort to contact me if you suffer a Serious Adverse Events (SAEs) or need to take additional medication of any kind. This applies to out of hours, as to normal work time.

In such cases of medical emergencies during the study, or if you have any urgent questions relating to adverse effects or unrelated illness, please feel free to telephone the supervisor at any time at the following phone numbers or contact via email:

Work 046 603 8399 Cell 082 802 1845 Email i.kanfer@ru.ac.za

22 Contacts for additional information

The following individuals are responsible for conducting this study and as such may be approached for more information:

Portfolio	Name	Contact details
Principal Investigator	Wai Ling Au **BPharm (Rhodes)**	**Work 046 603 8412** **Cell 072 612 0972** **Email g02a1351@campus.ru.ac.za**
Supervisor	Professor Isadore Kanfer **BSc (Pharm), BSc (Hons), PhD (Rhodes)**	**Work 046 603 8399** **Email i.kanfer@ru.ac.za**

If you have questions about this study which have not been answered adequately by the principal investigator, supervisor, you should first discuss them with your doctor. After you have consulted your doctor and still dissatisfied you may contact the Rhodes University Ethics Standards Committee (departmental) at:

Ms Carmen Oltmann

Chairperson: Rhodes University Departmental Ethics Standards Committee

RHODES UNIVERSITY

Tel:. 046 603 8494

Fax:. 046 636 1205

Email:. c.oltmann@ru.ac.za

APPENDIX IV

STUDY PARTICIPATION INFORMED CONSENT FORM

APPLICATION OF DERMAL MICRODIALYSIS FOR THE ASSESSMENT OF THE BIOAVAILABILITY OF CLOBETASOL PROPIONATE IN HEALTHY VOLUNTEERS

STUDY NUMBER: WL_002

PROTOCOL VERSION 2

CONSENT VERSION 2

November 2008

I ..... Born on.....

Present address .....
.....
hereby give permission that the necessary probes may be inserted into the surface of my skin and the microdialysis procedure conducted as described to me by the principal investigator and that the formulation stated below be applied to my skin during the course of this study.

Reference product: Clobetasol Propionate Ethanolic Solution (Clobetasol propionate 4% m/v) – Rhodes University, South Africa

My consent is given freely and I realise that it may be withdrawn at any time, without penalty to me. Furthermore, I understand that I do not give up any of my legal rights by signing this consent form.

I have been fully informed by ..... regarding the possible adverse effects of the medication, procedures to be used in this study and the risks thereof, as detailed in the "Information for Volunteers Brochure". I will receive a copy of the information brochure and signed consent form for my records.

I undertake to comply with all the relevant conditions contained in the Information to Volunteers Brochure and confirm that I understand that it is important not to withhold or misrepresent any information asked of me. I agree to undergo the necessary pre- and post-study medical investigations as listed in the protocol.

I undertake to inform the principal investigator immediately of any symptoms - expected or unexpected - which I might experience.

I agree to my medical records being reviewed in the event of an audit, enquiry, monitoring and/or inspection on the understanding that my anonymity will be maintained.

I agree and consent that the BRG may consult with consultants/supervisor who are normally responsible for care of study subjects. _____ Initial

I have been informed that if I do not adhere to the protocol, it may result in my exclusion from the study and forfeiture of the agreed upon remuneration. I acknowledge that instructions relating to my participation in this study have been communicated to me both verbally and in writing, and that I understand them.

I also declare that I have made the necessary arrangements regarding the attendance of lectures and other academic activities.

I understand that a policy to cover volunteers in clinical studies against death or disablement arising as a direct result of participation in such clinical studies has been taken out by the Biopharmaceutics Research Group. I accept the conditions of the policy as set out in the insurance policies.

I acknowledge that I will receive R 500.00 for full participation in this study and that I will receive a pro-rated amount if I withdraw from the study before it has been completed.

_____ _____

Signature of volunteer Date (yyyy-mm-dd)

_____ _____

Signature of a witness Date (yyyy-mm-dd)

_____ _____

Principal Investigator Date (yyyy-mm-dd)

Wai Ling Au

BPharm (Rhodes)

Contact details of subject	
Telephone number	
Cell number	

APPENDIX V

CASE REPORT FORM

APPLICATION OF DERMAL MICRODIALYSIS FOR THE ASSESSMENT OF THE BIOAVAILABILITY OF CLOBETASOL PROPIONATE IN HEALTHY VOLUNTEERS

STUDY NUMBER: WL_002

PROTOCOL VERSION 2

CASE REPORT FORM VERSION 2

November 2008

Reference product	**Clobetasol Propionate Ethanolic Solution** (clobetasol propionate 4% m/v) – Rhodes University, South Africa

Principal Investigator	**Wai Ling Au**
	BPharm (Rhodes)
Supervisor	**Professor Isadore Kanfer**
	BSc (Pharm), BSc (Hons), PhD (Rhodes)

Subject initials	
Volunteer reference number	
Study subject number	
Date of screening medical	

Pre-study examiner:	
Name and initials	
Qualifications	

MEDICAL HISTORY (to be completed by the principal investigator)

DEMOGRAPHIC DATA

Birth Date	Age in Years	Sex (M/F)	Origin				Other
			White	Black	Oriental	Indian	
Height (m)	Weight (kg)	BMI (Body Mass Index) To be calculated by BRG Investigators	Not required				

REVIEW OF PAST ILLNESS

System/Site	Illness? Y/N	Describe Abnormalities
Skin -Connective Tissue Any other illness?		

PHYSICAL EXAMINATION (to be completed by the examining principal investigator)

VITAL SIGNS

Blood Pressure - Supine (mmHg)	Pulse (beats/min)	Oral Temperature °C

GENERAL SYSTEMS EXAMINATION

System/Site	Illness? Y/N	Describe Abnormalities
Skin - General Skin - Forearms		
Medical tape allergy pass		

INCLUSION/EXCLUSION CRITERIA

(NB: Unshaded areas - acceptable: Shaded areas - unacceptable)

	YES	NO
Will you be available for the entire study period?		
Are you in general good health?		
Are you aged between 18 and 50?		
Do you have eczema or scratch marks on the underside of your forearms?		
Do you suffer from any skin disorder such as psoriasis or other relevant skin disorder?		
Do you suffer from any neurological, kidney or liver disorders?		
Do you suffer from any allergic conditions (hayfever, allergic rashes, asthma or childhood eczema)?		
Have you engaged in any sun-tanning or taken any sunny vacations within the last month?		
Do you take any regular medicine (prescription or OTC)?		
Have you taken any medicine or tablets or used any creams within the last week (contraceptive pills excluded)?		
Have you participated in another corticosteroid dermal study within 2 months of the study date?		
Have you used any corticosteroids within the last three months?		
Do you have any mental deficiency or handicap?		
Are you pregnant?		
Have any history of drug or alcohol abuse?		
Are you contemplating becoming pregnant in the time immediately following the study?		
Are you breastfeeding?		
Are you using reliable contraception (The contraceptive pill, minipill, IUD or abstinence)?		
Do you have a known allergy/hypersensitivity to clobetasol propionate or any other corticosteroids?		
Have you ever had a reaction to a local anaesthetic injection at the dentist or in casualty? Are you allergic to any soy/egg/nut products?		

VOLUNTEER ACCEPTABILITY /COMMENTS WITH REGARDEXAMINATION/INCLUSION/ EXCLUSION CRITERIA

Acceptable		Not Acceptable	
Notes/Comments			
Sign _____ Date _____ Principal Investigator (dd/mm/yy)			

POST-STUDY REPORT/MEDICAL

SUBJECT/STUDY STATUS

Study Completed		Study Not Completed	
Comments (If "no" give reasons and procedure/follow-up undertaken)			
Sign _____ Date _____ principal investigator (dd/mm/yy)			

CLINICAL EXAMINATION/SUBJECT RELEASE

Test		**Status/Comments**	
Medical Update			
Application Site Assessment			
Subject Released		Subject Retained for Further Investigation	
Sign _____ Date _____ Principal Investigator (dd/mm/yy)			

APPENDIX VI

CERTIFICATE OF INSURANCE

ENDORSEMENT ATTACHING TO AND FORMING PART OF
STATED BENEFITS POLICY NUMBER 765474*001
IN THE NAME OF BIOPHARMACEUTICALS RESEARCH INSTITUTE

In view of the Insured having declared that they would be conducting a Skin Study with 10 Subjects.

The Study Reference number is :Study WL 002

There is a premium of R3 275.00 due to the Insurer.

SUBJECT OTHERWISE TO THE TERMS, CONDITIONS AND EXCEPTIONS OF THE POLICY.

Signed for and on behalf of the Insurers this 9th day of March 2009.

W. Thom

GUARDRISK INSURANCE COMPANY LIMITED
(Insurers)
Reg No. 1992/01639/06
VAT No. 4250138072

APPENDIX VII

SCREENING MEDICAL AND HIV TESTING CONSENT FORM

APPLICATION OF DERMAL MICRODIALYSIS FOR THE ASSESSMENT OF THE BIOAVAILABILITY OF CLOBETASOL PROPIONATE IN HEALTHY VOLUNTEERS

STUDY NUMBER: WL_002

PROTOCOL VERSION 2

HIV CONSENT FORM VERSION 1

November 2008

I ..... Born on.....

Present address .....

.....

hereby:

vii. Confirm that my consent is given freely and I realise that it may be withdrawn at any time, without penalty to me.

viii. Confirm that I have read the information contained in Appendix III and have been informed of the tests to be undertaken.

ix. Confirm that I have been informed of the general procedure for clinical studies undertaken by the BRG.

x. Confirm that I have attended an informed consent session specific for the clinical study for which I am volunteering at which the contents of the 'Information for Volunteers' Brochure, Version WL02 were explained to me and a copy given to me to take home and read before making my decision.

xi. Recognise that undergoing a screening medical does not ensure automatic inclusion into the clinical study.

xii. Agree to my medical records being reviewed in the event of an audit or enquiry on the understanding that my anonymity will be maintained.

xiii. Agree to be screened for Hepatitis B.

xiv. Agree to be screened for the Human Immunodeficiency Virus (HIV), which causes Aquired Immunodeficiency Syndrome (AIDS), and with respect to this test:

a. I understand the information contained in the attached three-page HIV Informed Consent Document (Appendix A)
b. I freely consent to the withdrawal of blood from me.
c. I freely consent to the testing of that blood.
d. I understand that the results will be kept confidential, except for the disclosure by the BRG of any reactive result to the doctor whom performed the screening medical or a doctor of my choice.
e. I have read the information in this document about what a test result means.
f. I understand that the BRG will pay for one session of pre- and post-test counselling which will be conducted by the St Raphael Centre at 11 Donkin Street, Grahamstown 6139.
g. I understand that I should contact the principal investigator or a doctor of my choice for further information and counselling if required.
h. I understand that I have the right to request and receive a copy of this form.

_____ _____

Signature of volunteer Date (yyyy-mm-dd)

_____ _____

Principal Investigator Date (yyyy-mm-dd)
Wai Ling Au
BPharm (Rhodes)

Appendix A HIV SCREENING TEST INFORMATION SHEET

ALL CLINICAL STUDY VOLUNTEERS MUST READ THIS INFORMATION SHEET BEFORE THE TEST FOR HIV IS DONE

INTRODUCTION

This document contains the information that you have a right to be given before agreeing to be tested for HIV antibodies. The HIV antibody test (sometimes called the "AIDS test") is a test that will tell you whether or not you have been infected with Human Immunodeficiency Virus (HIV). Below we set out your rights with respect to this test, information about HIV and AIDS and the AIDS test, any why the BRG wants to test you for HIV antibodies before it decides whether or not to include you in a clinical study.

WHAT ARE MY RIGHTS?

Your rights are:

- Not to be tested for the HIV without your free and informed consent.
- To be given all material information on the harms, risks and benefits of taking, or not taking, the AIDS test.
- To refuse to take the test. If you do this, you will not be able to participate in a clinical study.
- You will receive pre-test counselling which is private and confidential, and which will inform you more about the test and it's implications before you consent to the test. Pre-test counselling will be at the expense of the BRG.
- To have your test result treated confidentially. The result will be made available to your doctor only with your prior consent. A test result will also be stored by the BRG. You have the right to access this information to check that it is correct.
- To one session of post-test counselling at the expense of the BRG, whether the test is positive or negative. After this you will be referred to a healthcare provider who will provide you with further counselling and treatment if a positive result is obtained.

WHAT IS HIV?

HIV is the virus that causes AIDS. While infected with HIV, and before a person develops AIDS, he or she will feel well or healthy. During this time, the person will be able to infect other people with the virus.

WHAT IS AIDS?

AIDS is the name for a number of illnesses that develop as a result of being infected with HIV. The HIV attacks the immune system and leaves it unable to fight various illnesses. More than half of the people infected with the HIV will get AIDS within 10 years of infection.

When you are sick with AIDS, you can usually no longer work. AIDS is a serious disease that eventually leads to death.

WHAT IS THE HIV TEST?

The HIV test checks your blood for antibodies to the HIV. The test cannot tell you the date you were infected, or by whom you were infected. A sample of blood will be drawn from you. It will be sent to a pathologist's laboratory, where it will be tested.

HOW DO I BECOME INFECTED WITH THE VIRUS THAT CAUSES AIDS?

Almost all cases of infection result from sexual intercourse. The HIV is transmitted in this way from one person to another through semen and vaginal fluids. The HIV can also be passed on to babies through the mother's blood or through breast-feeding. Although rare, the HIV can be transmitted by contact with infected blood - for example, through blood transfusions, through sharing needles during drug-of-abuse use or by inadvertent needle-stick injuries or spillage, where health care workers are especially at risk. Most cases of infection are transmitted from women to men, or from men to women. Men and women of all ages, races and religious beliefs can be infected with the HIV. Homosexual transmission also occurs.

IS THERE A CURE FOR HIV AND AIDS?

There is no known cure for HIV or AIDS. Modern medical science, as well as traditional healers, have searched for cures for the HIV. So far these efforts have been unsuccessful.

However, should you be HIV positive, by adopting a healthy life-style and having your HIV managed properly by health care workers, you can greatly enhance your quality of life before AIDS sets in. It is therefore of the utmost importance that you keep yourself both mentally and physically healthy in spite of being HIV positive. It is also possible that a cure may be found over this time.

WHY DOES THE BRG NEED TO TEST FOR THE HIV?

The BRG conducts clinical tests on pharmaceutical formulations. These tests involve administering medications to study subjects and taking blood samples from each subject at various intervals during the study. Blood samples are then centrifuged and prepared for analysis, when the concentration of medication in each sample is measured. During the clinical and analytical process numerous people handle each blood sample e.g. the nurse who takes the sample, the technician who prepares the sample immediately after it is taken and the analyst who prepares the sample for analysis. The BRG is duty bound to ensure that the individuals who handle these blood samples are not at risk from contracting the HIV. The BRG must also ensure that subjects who participate in the study are not at risk from contracting the HIV virus through any possible accidental contact with any blood while participating in the study. In addition, the BRG must satisfy import/export authorities in South Africa and the receiving country that samples in any shipment across international borders are non-infectious.

IS THE TEST ALWAYS CORRECT? CAN THERE BE MISTAKES?

The tests are usually very accurate, and are performed by registered pathology laboratories. If your test result shows that you are infected with the HIV, you can have this confirmed by having further tests done at your own expense, or by going to the nearest clinic or public hospital for a free HIV test.

WHAT DOES IT MEAN IF THE TEST IS NEGATIVE?

If your test result is negative, this does not mean that you may not become infected in the future. If you engage in unprotected sex, you may be infected at some time in the future. You should think very seriously about the ways in which you can ensure that you are not infected in the future. In particular, you should consider using safer sexual practices, for example, a condom.

There is a time of approximately six weeks after infection when an HIV test will not detect the HIV. This happens because the test for antibodies cannot detect them for a short while after infection. This time is called the "window period". If you are in the "window period" your test results will be negative, although you are actually infected with the HIV.

The chance of being in the "window period" is very small. If you suspect that you may have become infected recently and are in the "window period", you can arrange to be tested again in about three or more months' time at your own expense, or go to the nearest clinic or public hospital for a free test and counselling.

WHAT DOES IT MEAN IF THE TEST IS POSITIVE?

If your test result is positive, this means that you have been infected with the HIV. A positive test result will mean that you will not be able to participate in any clinical study conducted by the BRG. The implications of a positive test result should be discussed with your doctor or the doctor who conducted your screening medical. The BRG will pay for one session for you to discuss these implications with a doctor.

WHAT ARE THE HARMS AND RISK OF THE AIDS TEST?

Many people do not understand the facts about infection with the HIV. This has led to people infected with the HIV being stigmatised and isolated by their families and communities. Some people have committed suicides. A positive test can lead to difficulties in seeking housing bonds, employment, as well as medical and dental treatment. Psychological difficulties might also arise. For these reasons, the BRG will keep your test results confidential.

WHAT ARE THE BENEFITS OF THE AIDS TEST?

If the test is negative, this can reassure you and help you make sure you do not become infected with the HIV. A positive test result can offer an opportunity to get early treatment, to change life plans and to prevent infection of your sexual partners.

NOTIFICATION OF TEST RESULTS

If your test result is negative: You will be considered for inclusion into the clinical study.

If your test is positive: Because a trained person should deliver that information so that you can understand clearly what the test result means, you are asked in Appendix B of this document to designate the doctor who conducted the screening medical or a doctor or clinic of your choice to deliver the test result to you.

Consequently it is of the utmost importance that you think carefully about the person who should receive the results. Should you not know who to name, please ask someone for assistance or suggestions.

You will be advised to contact the designated doctor or clinic, so that they can discuss the meaning of the test result with you. Please note that if you are asked or receive a letter to contact the designated doctor, that this does not automatically mean that the AIDS test result is positive, as many other medical impairments may lead to you not being able to participate in the clinical study and which should require further medical follow-up for your benefit.

Appendix B

This page must be detached from the document and taken with you when you go for your HIV test.

Section 1 must be completed by you, the clinical study volunteer.

Sections 2 and 3 must be completed by the person drawing the blood sample.

Section 1: To be completed by the clinical study volunteer.

I designate: i. The doctor who conducted the screening medical as the person to deliver a positive test result to me.

Signature of person being tested _____ Date _____

OR

I designate: ii The following doctor or clinic to deliver a positive test result to me.

Name _____

Address _____

_____

Signature of person being tested _____ Date _____

To be completed by the person drawing the blood sample.

(Please retain this document and forward to the BRG together with the HIV test results)

Section 2: Identification of applicant for all pathological tests (must always be completed)

Passport/Identity Number of person being tested: | | | | | | | | | | | | | |

Name of person being tested _____

Address _____

_____

Signature of person being tested _____ Date _____

Section 3: Identification of and declaration by person drawing the sample.

Name of person drawing the sample _____

Practice number _____

Address _____

_____

Signature of person being tested _____ Date _____

I have satisfied myself that the person being tested has received the Informed Consent Document, and I have verified the identity of the applicant and that he/she has freely consented to have the sample drawn and tested for HIV antibodies.

I have inspected the following document to verify the identity of the volunteer:

☐ valid South African identity document

☐ valid South African passport

☐ valid temporary South African identity document

☐ foreign passport

Signature of person drawing the sample _____ **Date** _____

APPENDIX VIII

VERBAL DELIVERY OF INFORMATION TO STUDY VOLUNTEERS

The announcement will be posted around campus.

Students then contact the principal investigator directly and relay their interest in participating in the study. A short and factual explanation is given of the study and female participants are specifically asked whether they are using reliable contraception (the contraceptive pill, minipill or an IUD) and whether any participant suffers from eczema or psoriasis.

The principal investigator then mails the participants information brochure to the participants to the place of residence of the study volunteer. As the announcement is made 1 - 2 weeks before the commencement of the study, ample time is allowed for the reading through of this information.

At the first meeting in the Seminar Room in the Faculty of Pharmacy building 30 minutes are allocated to a verbal explanation of the study and any questions. This provides an undisturbed physical environment and the time allocation of 30 minutes is realistic.

Study volunteers are then shown the clinic unit where the study will take place as well as the needles for the insertion of the probes etc. Thereafter questions from the study volunteers are answered.

Study volunteers will be given approximately 7 days in which to consider the written information and formulate questions. Should there be any additional questions regarding the preceding verbal information, study volunteers have an opportunity to consider these answers before a final decision is made with regard to participation.

Should the study volunteer continue to be interested in participating, the two days on which the study will be conducted are arranged. Thereafter the study volunteer signs the study participation informed consent form as well as the screening medical and HIV testing consent form which are also signed by the principal investigator and in the presence of a witness if required. Study volunteers are hereby included in the study and are given a copy of the Information for Volunteers Brochure and the signed consent forms as well as written instructions regarding the 2 pre-arranged study days.

The study volunteers will then be contacted to see the principal investigator for medical screening as outlined in the protocol.

BRG

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