# EVALUATION OF THE SAFETY AND EFFICACY OF TOPICAL MOMETASONE FUROATE FORMULATIONS

**by**

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# ABSTRACT

The human skin blanching assay (HSBA) is a well-researched and validated method for the bioequivalence assessment of topical corticosteroids. Traditionally, visual assessment of skin blanching has been used. Such testing methods are not conducive for inter-laboratory comparisons. Regulatory bodies prefer less subjective methods of analysis. The FDA released guidelines on the assessment of bioequivalence for topical corticosteroids that recommends the use of a chromameter as a reliable method to measure skin blanching although the use of visual assessment with acceptable validation is also provided for. However, the FDA does not elucidate on the manipulation and handling of the chromameter during skin blanching measurements. The purpose of this project was several fold, which included investigations to standardize the manipulation and handling of a chromameter. In particular, measures to avoid skin whitening resulting from the effects of pressure on the skin during chromameter use were investigated.

Other methods of analysis should surpass or at least be comparable to the HSBA if such methods are to be used for the assessment of topical corticosteroids. Microdialysis is a relatively new technique for assessing the rate at which drug penetrates the skin. The advantage of using this method is that there are fewer restrictions for selection of an appropriate study population unlike those required for the HSBA where one has to be both a 'responder' and a 'detector' for their results to be used in data analysis.

Microdialysis was investigated by initially conducting experiments in which microdialysis probes were embedded into topical formulations containing mometasone furoate (MF) and the initial results revealed that relatively low drug was released from the formulations. These results indicated that should microdialysis be applied to measure the *in vivo* release of MF from such topical formulations following application to the skin, even lower concentrations of MF would likely result in the dialysate, necessitating the need for ultra-high sensitive methods of analysis. Typically, the availability of an appropriate analytical technique such as liquid chromatography coupled with mass spectrometry (LCMS) would be a pre-requisite for such *in vivo* studies. However, only high-pressure liquid chromatography (HPLC) and other less sensitive equipment was available in the laboratories. The study objectives were therefore focussed on *in vitro*

assessment of the release of MF from topical formulations using microdialysis and Franz cells. In addition, the *in vivo* release of MF was also studied using the HSBA. Data obtained from the microdialysis experiments were compared with the data obtained from the Franz cell diffusion studies in order to provide information on the pharmaceutical availability of MF from the various topical MF dosage forms. Subsequently, pharmaceutical equivalence was investigated from the comparative pharmaceutical availability data using statistical analysis.

An additional objective was to attempt to correlate *in vitro* with *in vivo* data (IVIVC) to establish a model that could be used to assess safety and efficacy of generic topical drug products. The *in vivo* data obtained from the HSBA were processed according to the FDA requirements and these pharmacodynamic data were subsequently compared with the microdialysis and Franz cell results.

In summary the objectives of this project were:

1. To develop a system to improve the reproducibility of the use of a Minolta® chromameter and compare this with the standard/normal manipulation and handling of such instruments.
2. To develop and validate an HPLC method for the analysis of MF for use with *in vitro* diffusion studies using microdialysis and Franz cells.
3. To conduct a comparative HSBA on proprietary MF topical creams from two different countries in accordance with the FDA guidance.
4. To assess the pharmaceutical equivalence of topical formulations containing MF using Franz diffusion cells and *in vitro* microdialysis.
5. To compare the *in vivo* data obtained from the HSBA with those obtained *in vitro* using microdialysis and Franz cells.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| % TPS | percent total possible score |
| AMP | adenosine monophosphate |
| ANOVA | analysis of variance |
| API | active pharmaceutical ingredient |
| ARC | apparent release constant |
| AUC | area under curve |
| CI | confidence interval |
| CIE | *Commission Internationale de l'Eclairage* |
| COMT | catechol *o*-methyl transferase |
| CP | clobetasol 17-propionate |
| DEAE | diethylaminoethyl |
| ECF | extracellular fluid |
| ED | Euclidean distance |
| FDA | Food and Drug Administration |
| GMP | guanosine monophosphate |
| HPLC | high pressure liquid chromatography |
| HSBA | human skin blanching assay |
| MAO | monoamine oxidase |
| MF | mometasone furoate |
| NE | norepinephrine |
| NP | normal phase |
| PEEK | polyetheretherketone |
| PG | propylene glycol |
| RP | reverse phase |

# CHAPTER 1

# INTRODUCTION

## 1.1 Background

Topical corticosteroids have been in use for a considerable time and more recently, following patent expiry of the brand products, many generic topical corticosteroids have become available.[1] Topical corticosteroid products are the most frequently prescribed products used in dermatology[2,3] but unfortunately their increasing availability has resulted in misuse and abuse. For example, topical corticosteroid products are often used as skin lighteners, particularly in black populations. The prevalence of the use of skin lighteners in Nigeria has been reported as being in the region of about 80% and as recent as 2002 black African women in Lagos top the list.[4] Consequently, health professionals have had to reinforce and disseminate information on the disadvantages of topical corticosteroid abuse. This has partially achieved its purpose but has also led to topical corticosteroid phobia often resulting in the under usage of the relevant products in appropriately indicated conditions.[3,5] The perception by patients is that limited (under) use even whilst prescribed will prevent the listed associated side effects and/or the adverse effects, especially skin thinning and other long term effects that may occur.

The numerous modalities available for the treatment of dermatological diseases complicate the choice of the appropriate product. Since topical corticosteroid therapy is used over the full spectrum of patients, from paediatric patients through to postmenopausal and geriatric patients, issues of the efficacy and safety profiles of these corticosteroids are of primary importance.[6] In addition to the different treatment modalities which may also involve different topical corticosteroids, generics products are generally available. The advent of generic products has necessitated the need for comparative bioequivalence studies to be performed whereby the generic (test) product is compared to the innovator (reference) product to establish their safety and efficacy. Furthermore, since different topical corticosteroid compounds have different degrees of

potency, it is convenient to group these various corticosteroids into classes of potency which provide better knowledge for the prescriber for use for a particular indication. Such information provides confidence to the prescriber which is then reflected in the counselling of the patient.[7] An informed patient who has confidence in the prescriber together with knowledge of the efficacy and safety of the preparation will more likely use the correct dose for the prescribed time. Concordance results in cutting down of costs given that more than seven million Americans are affected by psoriasis alone. As early as 1974 in the USA, approximately one hundred million dollars was spent on the purchase of topical corticosteroids.[8]

Mometasone furoate was selected as the topical corticosteroid for study in this research project and topical products containing this particular corticosteroid were evaluated for safety and efficacy using both *in vitro* and *in vivo* methods.

It has previously been established by Mackenzie and Stoughton[9] that topical corticosteroids cause skin blanching. The degree of skin blanching can be related to the amount of topical corticosteroid entering the skin; hence the use of this physiological/pharmacological response can be used as a basis for the comparison of bioequivalence of the various topical corticosteroids, also sometimes known as dermatocorticoids.

The human skin blanching assay (HSBA), which is purported to be based on the degree of vasoconstriction following the application of a topical corticosteroid to the skin, has thus been used as a measure to compare the efficacy of a generic formulation when compared with the innovator equivalent. Generic products contain the same percentage of active pharmaceutical ingredient (API) but may differ in the vehicles/excipients used. Very often, the vehicle may become the rate-limiting step of drug release from the formulation. Bioequivalence studies are therefore a check of these different vehicles/excipients to ascertain whether they have the same influence on the release of the corticosteroid from both the test and the reference dosage form and subsequent efficacy and safety of the product.

## 1.2 Mometasone Furoate

### 1.2.1 Description

Mometasone furoate (MF) (CAS 83919–23–7, Sch 32088)[10] is 9α, 21-dichloro–11β, 17α–dihydroxy–16α–methylpregna–1, 4–diene–3, 20–dione–17–(2–furoate), a synthetic corticosteroid with empirical formula $C_{27}H_{30}Cl_2O_6$ and molecular weight of 521.43 (Figure 1.1).

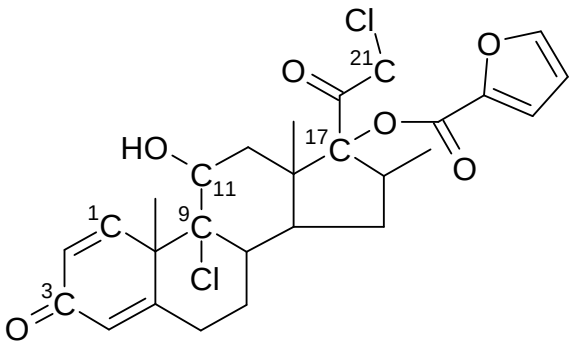



**Figure 1.1 Chemical structure of mometasone furoate**

MF is a white to off-white powder that contains not less than 97.0% and not more than 102.0% of $C_{27}H_{30}C_{l2}O_6$, calculated on the dried basis.[11]

### 1.2.2 Physicochemical Properties

#### 1.2.2.1 Melting Point

MF melts at about 220 ˚C with decomposition.[11] The melting point of anhydrous MF using a hot-stage microscope was found to be between 233 and 242 ˚C. Concomitant degradation was observed with melting.[12]

#### 1.2.2.2 Solubility

MF is practically insoluble in water, freely soluble in acetone and methylene chloride and slightly soluble in alcohol.[11] MF solubility at pH 1, 4, 7, 8 and 10 is described as sparingly soluble.[10]

Solubility studies of MF in Mcllvaine buffer solutions, comprising 0.05 M citric acid and 0.1 M disodium hydrogen phosphate (pH 2.5, 3, 4, 5, 6, 7 and 8), and in water were conducted by adding excess MF to the appropriate solvent and shaken at 37 ˚C in a water bath for 24 hours. The samples were analysed after 24 hours using a validated HPLC method (*vide infra* Chapter 4) and the results of these solubility samples have been summarized (*vide infra* Figure 6.2 and Table 6.2). Solubility in binary compositions of propylene glycol and water was assessed using a Junior Orbit Shaker (Lab-Line Instrument Inc., Melrose Park, Illinois, USA) at room temperature (22 ˚C) and in a water bath at 32 ˚C.

#### 1.2.2.3 Dissociation Constant (pKa)

The acid dissociation constant, pKa, of MF is 13.08 ± 0.20.[10] The dissociation constant measures the tendency of a drug molecule to keep the proton at its ionization centre(s) and defines the extent of ionization of a drug molecule and its predicted chemical and biological behaviour. If a molecule is not readily ionized it tends to stay in non-polar solvents such as the nature of the lipid membrane cells in biological systems as opposed to being in the aqueous tissue compartment.

#### 1.2.2.4 Partition Coefficient (Log P)

Log P is the logarithm of the partition ratio of a drug between octanol and water also known as the partition coefficient. Log P of MF is 4.725 ± 0.523.[10] Using the chromatographic hydrophobicity index method that is correlated with measured log P, a log P value of 3.49 was obtained for MF.[13] Log P gives an insight on drug partitioning between water and lipids *in vivo*. Hence the partition coefficient of a molecule is one of the core properties that may be used to estimate the absorption, distribution and transportation potential of a drug in biological systems.[14] Drugs with a partition coefficient between 2 and 3 have been shown to have the optimum percutaneous absorption.[15]

Higher log P values, (higher lipophilicity) are associated with correspondingly higher permeability coefficients.[15] MF intake into the *stratum corneum* is thus high and there is greater retention within the tissue. Since the skin is the site of action, retention thus should give favourable results. The furoate in the 17α position increases the lipophilicity of MF thereby enhancing the ratio of topical to systemic potency and increasing the interaction of the molecule with the glucocorticoid receptor.

### 1.2.2.5 Ultraviolet Absorption Spectrum

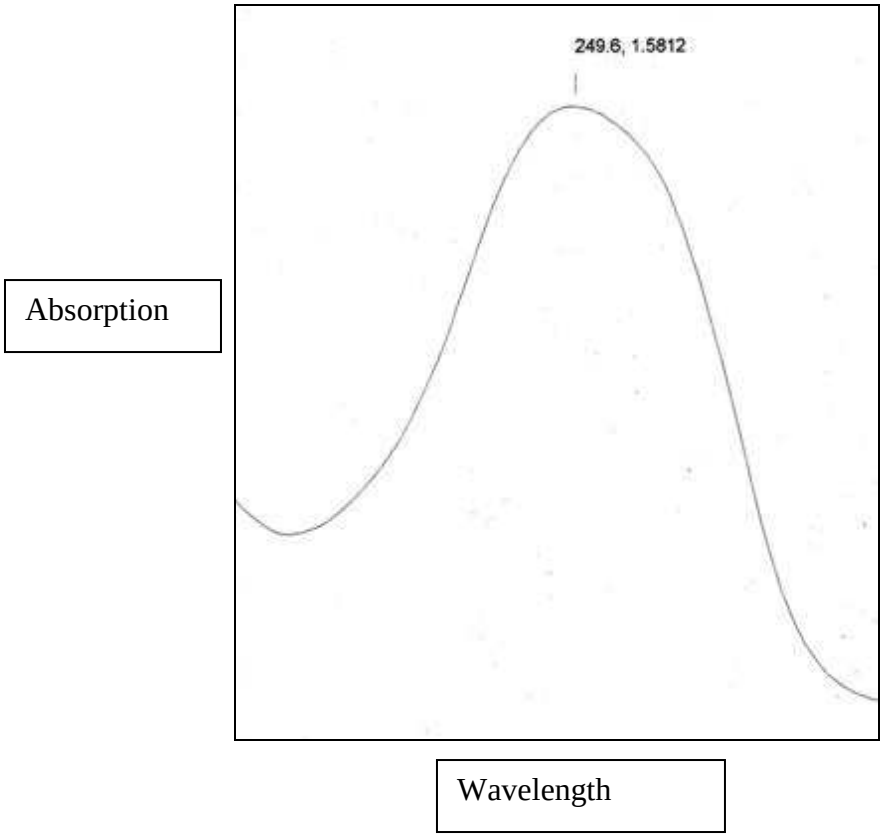



**Figure 1.2 Ultraviolet absorption spectrum of mometasone furoate**

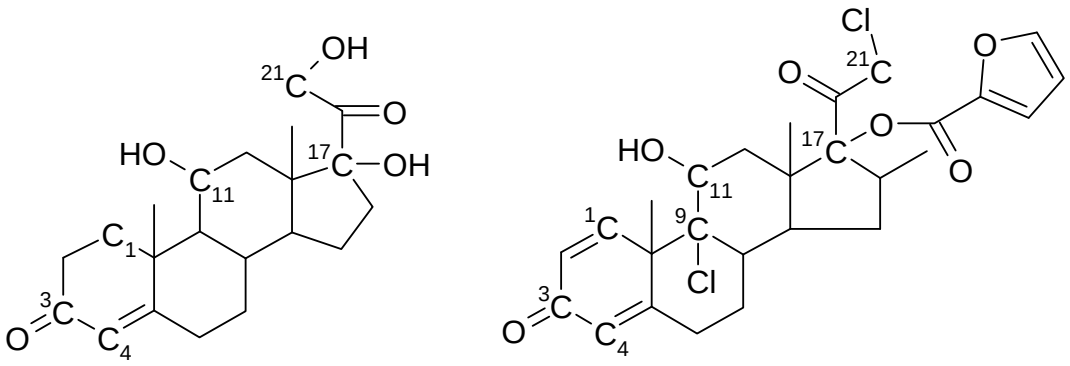
A methanolic solution of MF was scanned using a double beam GBC UV/VIS, Model 916 Spectrometer (GBC Scientific Equipment Pty Ltd, Victoria, Australia) and the maximum absorption of MF was found to be 249.6 nm. (Figure 1.2)

### 1.2.2.6 Stability

Storage conditions for MF products should be between 2 and 30 ˚C for the different topical preparations cream, ointment and lotion. If stored at this temperature then the expiration date is two years following date of manufacture.[16]

### 1.2.2.7 Structure Activity Relationship

Topical corticosteroids such as MF are synthesized from the natural steroid, cortisol. The structures of MF and cortisol are depicted in Figure 1.3.




Cortisol Mometasone furoate

**Figure 1.3 Chemical structures of cortisol and mometasone furoate**

The 4, 5 double bond and the 3-keto groups are essential for mineralocorticoid and glucocorticoid activity of cortisol. These were selectively left intact in ring A. The 1, 2 double bond increased the glucocorticoid activity of MF but slowed its metabolism. The 11β-hydroxyl through its electron withdrawing effect increases the glucocorticoid and anti-inflammatory effects imparted by the chlorine atom at the 9α position. The 16α-methyl group eliminates the mineralocorticoid activity.

Where MF has the 16α-methyl group, beclomethasone has the β configuration and the chlorine at position 21 of MF replaces the hydroxyl group in beclomethasone. The structural differences between beclomethasone and MF increase MF topical anti-inflammatory activity. The esterification of the 17-hydroxyl group also increases the anti-inflammatory activity of MF as compared to mometasone.

## 1.2.3 Pharmacological Properties

### 1.2.3.1 Classification

MF is a synthetic 16α-methyl analogue of beclomethasone. It is classified as a medium potent glucocorticoid for dermatological use.[17] It is available as 0.1% cream, ointment and lotion formulations and is sold under the trade name Elocon® or Elocom, depending on the country where it is marketed. MF shows higher affinity for the glucocorticoid receptor *in vitro* than betamethasone dipropionate. It is better at suppressing erythema and has greater activity and a longer duration of action than both betamethasone dipropionate and betamethasone valerate.[18]

### 1.2.3.2 Indications

MF is used for the treatment of glucocorticoid-responsive dermatoses, in the management of patients with atopic dermatitis, seborrheic dermatitis, eczema, allergic contact dermatitis, scalp psoriasis and psoriasis vulgaris where it is generally applied as a 0.1% cream twice a day. The ointment is for use on dry, scaly fissured lesions whereas the cream is suitable for moist lesions and is cosmetically acceptable on the face. The ointments are associated with lower incidence of hypersensitivity because of the absence of preservatives.[3] Elocon® lotion is applied to scalp sites and other affected skin areas by massaging a few drops of the lotion thoroughly into application site until the medication has disappeared.[17]

### 1.2.3.3 Dosage

This chlorinated corticosteroid is lipid soluble and long acting; as such MF offers the convenience of a once-daily topical dose. Application to moist areas such as the perianal region, results in greater absorption hence drug formulations should be applied sparingly over such affected areas.[17,19] Care should be taken in the case of application in the nappy area of babies. The nappy acts as an occlusive dressing hence will result in increased absorption.

### 1.2.3.4 Mode of Action

Steroids act on target cells by regulating their gene expression and protein biosynthesis.[17] The steroid enters the cytoplasm of a target cell by passive diffusion, binds to the steroid receptor proteins and then proceeds to the nucleus. Activation of the steroid-receptor takes place resulting in dimerisation. This dimer interacts with particular regions of the DNA known as hormone responsive elements (HRE) located in the 5'-upstream promoter region, inducing or suppressing gene transcription.[6] Translation of the mRNA gives a variety of proteins responsible for the plethora of effects within the inflammatory cascade. Corticosteroids inhibit the cytokine production including inteleukin-1 (IL-1), IL-2, IL-2 receptor, interferon-a (INF-a), tumour necrosis factor (TNF) and various colony stimulating factors (CSFs) such as IL-3.[6] Lipocortin inhibits the action of $pLA_2$, an enzyme that catalyzes the conversion of arachidonic acid into the eicosanoids, prostaglandins and leukotrienes. These eicosaniods give rise to noxious effects. In addition, corticosteroids inhibit the attraction of inflammatory cells to the site of an allergic reaction.

Endogenous cortisol inhibits vasodilation, leakage of capillaries and may block chemotaxis.[19] Cortisol also acts as an immunosuppressant inhibiting lymphocyte action which if permitted to act leads to type IV allergic reactions when lymphokines access target cells. Synthetic topical corticosteroids act in the same way as endogenous cortisol but their effectiveness is due to a combination of their higher intrinsic activity and better bioavailability.[20] The therapeutic effectiveness of synthetic topical corticosteroids in general is based on their anti-inflammatory and anti-pruritic activities. In addition, they show anti-mitotic effects on the human epidermis accounting for the additional action in psoriasis and other dermatologic diseases that have increased turnover of basal cells of the epidermis.[20,21] However, inhibition of DNA synthesis is not without consequence, atrophy of the epidermis and dermis occurs after prolonged treatment.

The above traditional genomic theory does not explain the rapid effects of corticosteroids. Currently a nongenomic theory has been put forward. Nongenomic actions are mediated by a distinct membrane receptor. This membranous receptor

compares well with the cytoplasmic receptor, implicated in the genomic theory, in terms of hormone-binding properties. The membrane receptor is thought to be linked to several intracellular signalling pathways that act via G-protein-coupled receptors and a number of kinase pathways. Kinases and phosphatases associated with the glucocorticoid receptor and released on hormone binding may be accountable for the rapid induction of tyrosine kinases in some cells.[22]

#### 1.2.3.5 Adverse Effects

MF is a well tolerated drug with mild to moderate transient side effects such as stinging, burning, folliculitis and dryness. Serious adverse effects can occur after prolonged and extensive use. Long term continuous treatment with MF should be avoided as far as possible as this may cause atrophic changes in the skin leading to thinning, loss of elasticity, and dilatation of superficial blood vessels, telangiectasiae and ecchymoses. These changes are likely to occur on the face or when occlusive dressings are used. Even though these adverse effects can occur it has been shown that for an equivalent dose of betamethasone 17-valerate MF produces approximately half of the suppressive effect on the hypothalamic-pituitary-adrenal axis.[17]

In addition, the atrophogenic potential is low and no greater than that of other glucocorticoids in the class. No clinical or histological signs of atrophy were observed after a once a day dose application for one year.[18] Signs of skin atrophy have been described with steroids of lower or similar efficacy when applied under occlusion.[23] Thus it can be concluded that MF has a favourable adverse drug reaction profile.

#### 1.2.3.6 Contraindications

MF is not for use in patients hypersensitive to the drug or other corticosteroids. It is contraindicated in most viral infections of the skin, tuberculosis, acne rosacea, peri-oral dermatitis, fungal skin infections and ulcerative conditions.[17]

Ideally MF should not be used in children under the age of two or in pregnant women. In children treatment should not be for more than three weeks as adrenal suppression is more likely to occur.[17] Corticosteroids exert a suppressive effect on growth by decreasing the release of the growth hormone. Corticosteroids have the potential to reduce bone formation, increase bone resorption, decrease calcium absorption and renal calcium reabsorption leading to osteoporosis. However, the use of topical corticosteroids with minimal systemic bioavailability limits the effects on growth, thus at recommended doses no growth retardation is observed.[6] Prolonged use on the face, flexures and intertriginous areas is undesirable.

## 1.2.4 Pharmacokinetics of Topical Mometasone Furoate

### 1.2.4.1 Absorption

The systemic absorption of MF is extremely low. Following topical applications in animals only 2–6% of the drug was absorbed systemically whereas from intact human skin, only 0.4% was detected consequent to systemic absorption when Elocon® cream (0.1%) was applied as a single dose for 8 hours. When Elocon® ointment 0.1% was used, about 0.7% of the dose was absorbed systemically.[17]

### 1.2.4.2 Distribution

Being more lipophilic, MF remains in the skin with lower systemic absorption in comparison to similar corticosteroids.[6] MF exhibits high topical potency and high affinity for the glucocorticoid receptor with low systemic absorption. When inhaled, MF has a total systemic bioavailability of less than 11%.[13] MF demonstrates a strong anti-inflammatory activity, rapid onset of action, low systemic bioavailability and a favourable ratio between local and systemic side effects,[24] consequently, it shows a comparably longer duration of action and at the same time has low potential to cause adverse effects such as suppression of the hypothalamic-pituitary-adrenal (HPA) axis.

### 1.2.4.3 Metabolism

Absorbed corticosteroids are handled in a similar way to systemically administered corticosteroids. Mometasone, the free alcohol, is one metabolite of MF which results via hydrolysis of the furoate ester. Mometasone has a high binding affinity for the glucocorticoid receptor.[25] Metabolism of MF includes hydroxylation resulting in the formation of 6β–OH-MF, 6β–OH-mometasone[25] and substitution of the C21–chlorine group by a hydroxyl group to form 21-OH–MF. These metabolites have high binding affinities for the glucocorticoid receptor hence are potentially responsible for the systemic activity.[25]

### 1.2.4.4 Excretion

MF undergoes virtually complete hepatic metabolism.[13] Excretion is via kidneys and some metabolites are found in bile. The products resulting from the different metabolic pathways are shown in Figure 1.4.

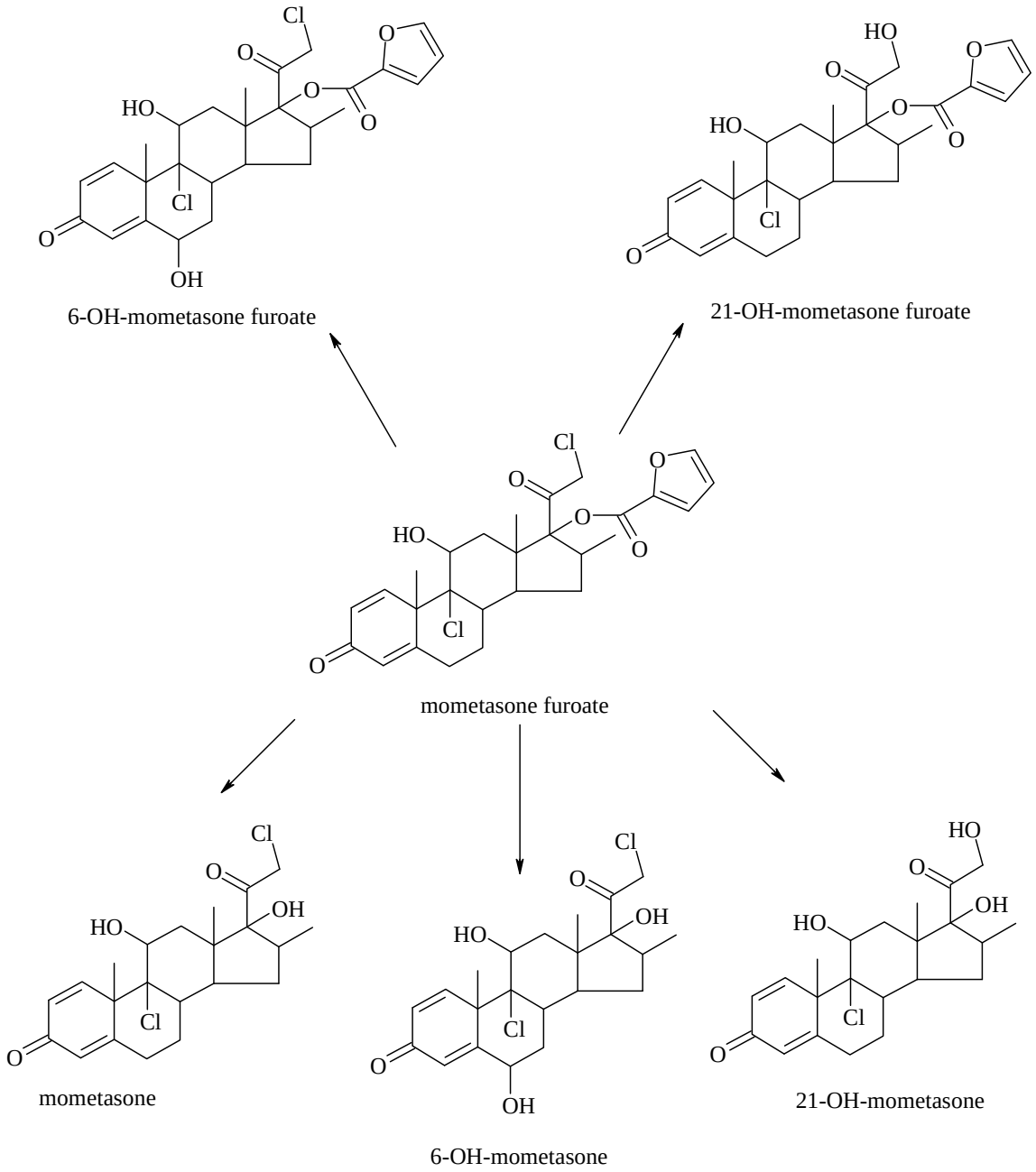



**Figure 1.4 Metabolic pathway of MF[23]**

## 1.2.5 Preparations

MF preparations are classified as endodermal preparations.[26] For a formulation to be described as an endodermal preparation, the drug contained in it must pass the *stratum corneum*, penetrate deeper into the dermis but not reach the general circulation. Such preparations are intended for local treatment of tissues below the site of application.[27]

Corticosteroid receptors are located between the *stratum corneum* and the upper dermis layer.[28]

Mometasone furoate is available as different formulations; cream, ointment and lotion. The creams are cosmetically acceptable on the face whereas the lotion is useful for application to the scalp and ointments are used for dry lesions. The innovator company manufacturing MF products is the Schering Corporation, USA. Several other companies have subsequently developed generics and these include Fougera, Perrgio Co, Warrick Pharm and Clay-Park Labs.[16]

# CHAPTER 2

# HUMAN SKIN BLANCHING ASSAY

## 2.1 Literature Review Including Background

Blanching is the whitening (pallor) of skin as a result of topical corticosteroid application. This pharmacodynamic response was first observed by Hollander in 1950.[29] Later, in 1962, the observation that the degree of blanching could be employed as an index of percutaneous absorption of the active drug was noted by McKenzie and Stoughton.[30] This observation has formed the basis of one of the best established human bioassays for assessing topical corticosteroid products and is commonly referred to as the human skin blanching assay (HSBA).[31] Generally, the intensity of the blanching response is related to the degree of percutaneous absorption of the topically applied corticosteroid. Earlier blanching tests involved the application of ethanolic topical corticosteroid solutions or suspensions or other preparations for 6-16 hours, followed by visual assessment at a single time point usually two hours post removal of application.[32,33] An all-or-none evaluation of the vasoconstriction response was done visually. The assay methodology has undergone many modifications since it was first suggested. Barry[34] in 1976 modified the McKenzie–Stoughton blanching test by taking readings of pallor over a longer period of time (up to 96 hours). The all-or-none system of evaluation was replaced by the use of a graded scoring schedule.[29] Subsequently, the HSBA was further modified to include multiple dose duration applications, a method which is currently used to assess bioequivalence of topical corticosteroid preparations.[30,32] The assay has been further improved by optimizing the study design facilitating the acquisition of dose-response data and the application of appropriate statistical procedures.[20,32,35] The advancement in technology and statistical approach has resulted in a refinement of the bioequivalence procedure for topical corticosteroid assessment.[28] The assessment of the bioequivalence of topical formulations to-date can generally only be accomplished by clinical trials except in the case of topical generic corticosteroids where the HBSA can be readily applied for this purpose. Clinical trials are time consuming, costly, inconvenient, and

laborious, require the incorporation of large numbers of patients[29] and often lack sensitivity.[32] In addition, the application of a placebo is also required. Furthermore, patients suffering from dermatological diseases are not ideal subjects as standardized lesions are difficult to obtain yet these are required for the bioequivalence assessment of topical formulations. Clinical trials usually use parallel group design where patients are allocated to different groups where either a test (generic) or reference (innovator) product or a placebo is applied. The disadvantage is that inter-subject variability cannot be controlled hence such study designs are not ideal for bioavailability or bioequivalence studies.[36] Consequently other methods for topical corticosteroid product assessment need to be developed.

HSBA has been the preferred method compared to tape stripping, ultraviolet irradiation, antigranuloma assays and cytological studies for topical corticosteroid assessment as it is less tedious, does not require the use of animals and is pain free.[30,37] Also, the HSBA is non-invasive, inexpensive, and reproducible.[28] An important advantage of the HBSA is that healthy volunteers are used and the simultaneous comparison of more formulations may be made in the same subject.[30] In addition, the FDA preferentially recommends the assessment of bioequivalence using pharmacodynamic effect studies as compared to clinical trials, *in vivo* animal studies or *in vitro* studies.[32]

### 2.1.1 Mechanism of Blanching

Several theories to explain blanching have been put forward[29,38], amongst which is the inference that blanching could be due to vasoconstriction. Studies conducted following topical corticosteroid application suggest that topical corticosteroids cause vasoconstriction indirectly by sensitizing the vascular musculature to norepinephrine (NE).[31] NE is a hormone closely related to adrenaline and with similar actions. It is secreted by the medulla of the adrenal gland and is the neurotransmitter of most postganglionic sympathetic nerve fibres. When NE is released it diffuses across the synaptic cleft, it binds briefly to the adrenergic receptors, thereby activating them and causing a physiologic response. This process is terminated by the uptake of NE into the prejunctional neurons where it is degraded by monoamine oxidase (MAO) and also

extraneuronally by the action of catechol O-methyltransferase (COMT).[39] NE release results in constriction of small blood vessels.

It has been suggested that corticosteroids augment vascular tone by potentiating the actions of vasoconstrictor hormones such as NE and angiotensin II. Potentiation has been linked to the indirect influence of topically applied corticosteroids on NE metabolism or re-uptake[29] and up-regulation of vasoconstrictor receptors in vascular smooth muscle cells.[38] Direct action of topical corticosteroids on vascular smooth muscle cells that are independent of vasoconstrictor hormones[38] has also been implicated. It is proposed that the corticosteroid attaches itself to a receptor site, subsequently causing the release of adenosine or guanosine monophosphate (AMP or GMP).[29] Some researchers have suggested that topically applied corticosteroids stimulate the release of NE held in cutaneous stores in nerve endings, hence tachyphylaxis may result when these sources are depleted.[29] Where some researchers have found evidence for, others have found evidence against – Fritz and Levine[29,38] observed the potentiation of vasoconstrictors by corticosteroids, however, Sessa[40] and Bockman[41] did not whereas Mihayara[42] actually observed 50% reduction in contractions due to NE.

Since the mechanism of blanching has not been fully elucidated this bioassay should be referred to as the HSBA and not the vasoconstriction assay.[29]

### 2.1.2 Bioequivalence Assessment of Topical Corticosteroid Formulations

HSBA has been shown to be a useful tool for the comparison of potency of different topical corticosteroid formulations. The assay is discriminatory hence it is used for regulatory purposes in bioequivalence testing.[32] A direct correlation has been demonstrated between the intensity of corticosteroid-induced skin blanching and therapeutic potency.[29,32] Since the 1960s the quantitative assessment of induced skin blanching has been carried out subjectively by visual observation and grading. HSBA has been criticized since visual assessment is subjective.[28,43,44]

The FDA suggested that with increasingly sophisticated methods of detecting physical and chemical changes, the ability of the human observer to assess the magnitude of the pharmacodynamic effect was perceived to be inadequate.[32] In attempting to standardise the technique of *in vivo* topical corticosteroid formulation bioequivalence assessment, the FDA, released a guidance document entitled Topical Dermatologic Corticosteroids: *In vivo* Bioequivalence[32], in which it was stated that any investigations initiated after the issue date, 2 June 1995, should generally conform to the recommendations of the guidance. The use of an instrumental method involving a chromameter to measure skin blanching was one of the recommendations of the guidance. The reasoning being that the use of an instrumental method for the assessment of blanching is supposed to be objective, more reproducible/reliable than visual assessment and can readily be validated through calibration and other appropriate procedures. Policies adopted by the FDA tend to have regulatory implications in countries other than the US. Consequently, many regulatory agencies have also adopted this procedure and approach for the bioequivalence assessment of topical corticosteroid dosage forms.

## 2.2 Blanching Assessment Methods

### 2.2.1 Visual Assessment

Quantitative assessment of induced skin blanching in the past has generally been carried out subjectively by visual observation and grading.[28,43,45,46] Ordinal data scales are used in these assessments such as:

0 – Normal skin

1 – Slight blanching of indistinct outline;

2 – More intense blanching

3 – Even blanching with a clear outline of the application site;

4 – Intense blanching.

Visual assessment of the degree of skin blanching varies among investigators, environments and subject populations and requires the use of experienced observers or assessors. A minimum of two blanching trials (12 subjects) using at least 10 observation

times[28] has been suggested as the minimum experience needed for one to be an acceptable/reliable visual assessor. The surrounding vascular skin colour and degree of pigmentation interfere considerably with the interpretation of results.[47] In addition, the use of ordinal data limits the power of the statistical analyses which can be carried out on the resultant data.[43]

Many factors influence human perception of colour such as age, mood, certain medications, source of light and background. There is no physical scale for measuring colour hence no two people can describe colour in the same way. Scientifically, it is preferable to have an objective method for the measurement of skin blanching. Human vision cannot recognize colour as precisely as reflectance spectrometry [48]. However, visual assessment has been proven to be reproducible in spite of the subjectiveness of the method [28,45], hence it provides a standard against which an objective technique must equate or surpass prior to being replaced with any alternative such method.

#### 2.2.1.1 Scores

Total possible score percent (TPS %) against time was used to assess visually obtained results.[29,30] The following equation was used:

$$\%TPS = \frac{Actualscore}{TPS} \times 100\% \qquad \text{(Equation 2.1)}$$

TPS is calculated using the formula:

$$TPS = 4 \times n \times S \times V \qquad \text{(Equation 2.2)}$$

Where,

4 = the maximum blanching score per site,

$n$ = Number of independent observers

$S$ = Number of sites per dose duration and

$V$ = Number of volunteers

The use of a %TPS versus time profile allows computation of the area under curve (AUC) using the trapezoidal rule.

### 2.2.2 Chromameter Assessment

A chromameter is a reflectance colorimeter that uses sensors that simulate the way the human eye sees colour and quantifies colour differences between a standard and a sample.

**Figure 2.1 Minolta® chromameter CR-400**

The Minolta® chromameter (Model CR-400, Minolta, Osaka, Japan) comprises a measuring head and a data processor as shown in Figure 2.1. In the measuring head, a pulsed xenon arc lamp inside the mixing chamber provides diffuse, even lighting over an 8 mm diameter measuring area. This light shines on a specimen area of about 201 $mm^2$. Only the light reflected perpendicular to the specimen surface is collected by the optical fibre cable for analysis. The photocells convert the light energy they receive into a current proportional in strength to the brightness of the light. This current is further changed into a proportional analogue voltage, then into a digital signal that the microcomputer uses to determine the values of the measured surface.[49]

Before any measurements are taken, the white plate provided by the manufacturer is used to calibrate the chromameter to baseline. This serves as a basis for comparison of subsequent readings. In addition to similar sensitivities to the human eye, the chromameter measurements are effected using the same light source and illumination

method. As a result, the measurements generated are objective and reproducible provided a planar hard surface is measured.

The *Commission Internationale de l'Eclairage*, CIE (L a*b*) 1976 standardized colour space was used to quantify skin colour. This colour space simulates the sensitivity of the human eye and has been used by a number of researchers[43,44,46,48,50-52] including the FDA.[32] This system is now widely accepted. CIE (L a*b*) 1976 has been described as an approximately uniform colour space produced by plotting, in rectangular coordinates (scale readings), L* (lightness 0–100), a* (+60 redness/–60 greenness) and b* (+60 yellowness/–60 blueness). It has been recommended for use in the guidance, hence its use in this research project.

#### 2.2.2.1 a* Scale Readings

The FDA recommended method of assessment of blanching intensity utilizes the a* coordinate only, disregarding L and b* coordinates.[32] However, both L and a* coordinates have been shown to have highly significant correlations with % TPS.[44] These findings have resulted in almost all researchers using a* coordinates only for the assessment of skin blanching.

#### 2.2.2.2 Euclidean Distances

Colour vision is trichromatic meaning that it is described by three values. Hunter and Harold[46] suggested that a single perceived colour results from the effect of three separate stimuli on the visual cortex. These separate stimuli can be regarded as a mixture of red, green and blue, the primary colours. As such describing colour is like giving it a position in 3 dimensions. A change in one direction results in a new position which can only be adequately expressed by three values. Hence each colour can only be fully described in terms of the 3 unique coordinates, Euclidean distances.[46] When the chromameter is calibrated according to the white plate, a point of reference is defined in 3 dimensions described by L*, a* and b*.

The Euclidean distance (ED) representing skin colour is calculated as the vector of the three points using Equation 2.3:

$$ED = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2} \qquad \text{(Equation 2.3)}$$

All readings following the application of topical corticosteroid formulation were baseline adjusted by subtracting the reading for each site taken at the beginning of the experiment before the formulation was applied (Equation 2.4).

*Raw reading – Site reading before drug application = Baseline adjusted value* (Equation 2.4)

The baseline-adjusted sites were also corrected by subtracting the average of the 2 blank sites on each forearm to take away the effect of the endogenous cortisol and other effects on the absolute colour coordinate (Equation 2.5). These corrections allow us to obtain the effect on skin colour due to blanching.

*Baseline adjusted reading – Untreated control, baseline adjusted reading = Treated site, baseline adjusted value* (Equation 2.5)

Analysis of chromameter results was made using both a* scale values[2,28,32,45] and the EDs.[43,46] Area under effective curve for each subject was calculated using the trapezoidal rule for both sets of data.[53]

### 2.2.2.3 Application of the Chromameter

The chromameter has been employed by several workers for quantitative analysis of blanching but less than satisfactory results have been obtained. Consequently, the precision and reproducibility of the chromameter in measuring human skin blanching has been questioned.[28,43,46,50] Hand-held chromameters have been developed for measuring planar, homogenous surfaces. When measuring skin colour using the chromameter, reproducibility problems have been encountered due to the varying pressure applied at each site and the change in the angle at which the chromameter is presented to the skin.

As a result, skin colour measurements obtained are not very reproducible, accurate or precise.[43] These problems make inter-laboratory comparisons impossible. The current FDA Guidance does not have any recommendations relating to the handling of the chromameter in order to ensure an acceptable degree of reproducibility. Skin blanching assessment using the chromameter requires the operator to make sure that constant pressure is applied through out the experimental duration. Skin pallor changes with pressure, variable amounts of which would introduce inevitable skin colour changes.[54] Measuring skin colour at a focal point above the skin, as put forward by some researchers,[28] contradicts with the *modus operandi* of the chromameter. The site to be measured must be sealed off; the measuring head must be in contact with the site so that neither incident light nor light reflected from the site is lost. It is also difficult for an investigator to hold the measuring head stationary and motionless with the same uniform pressure applied at each site and consequently reproduce these conditions each time measurements are undertaken.[28,55] At each observation time each subject usually has about 8 application sites on each arm. When using at least 12 subjects for each study, 192 readings have to be made at each sitting. Hand holding the chromameter measuring head introduces human error. Such problems have resulted in chromameter measurements not surpassing visual assessment.[56] This has been further explained by Schwarb *et al*[43] who concluded that the use of the hand-held chromameter was still subjective as the reading depended on the operator of the chromameter.

Because of these problems, modifications to the Minolta® chromameter CR–400 were made to attempt to improve the reproducibility and precision of measuring skin colour by controlling pressure and the angle at which the chromameter was applied to the skin site. Studies were conducted to assess any differences between the hand-held and the modified mounted chromameter. Information on reproducibility is a prerequisite before a method can be used in comparative studies.[54] Reproducibility within and between operators of the measuring instrument should be demonstrated. Inter-instrument reproducibility and day-to-day variation should be established.[54]

## 2.3 Objectives

Experiments were conducted to assess the effect of the following:

- Mounting the chromameter compared to the hand held chromameter.
- Change in spatial orientation and alignment of the chromameter.
- Differences of skin colour between Caucasian, Indian and black ethnic groups.
- Effect of pressure on skin colour.
- Inter-operator variation.
- Intra-operator reproducibility.

## 2.4 Methods and Procedures

### 2.4.1 Modifications to the Chromameter

The Minolta® chromameter CR–400 was mounted onto a movable horizontal beam connected to a system of swivels which allowed free movement in 3 dimensions (Figure 2.2). A movable balancing mass was coupled to the beam and was used to control the pressure exerted onto the skin site during measurements. Four pressure sensors were attached to the base of the measuring head of the chromameter, equally spaced from each other. These were in turn connected to a light system that comprised 8 small light sensors that were arranged in 2 concentric circles of 4 each. The inner circle of green light sensors illuminate when light pressure is applied to the skin during measurements. Adding more pressure illuminates the outer red light sensors to indicate excess unacceptable pressure. Opposite sides of the demarcated application sites and the corresponding sites on the chromameter measuring head base were marked with reference marks to ensure the exact placement of the chromameter for successive readings.

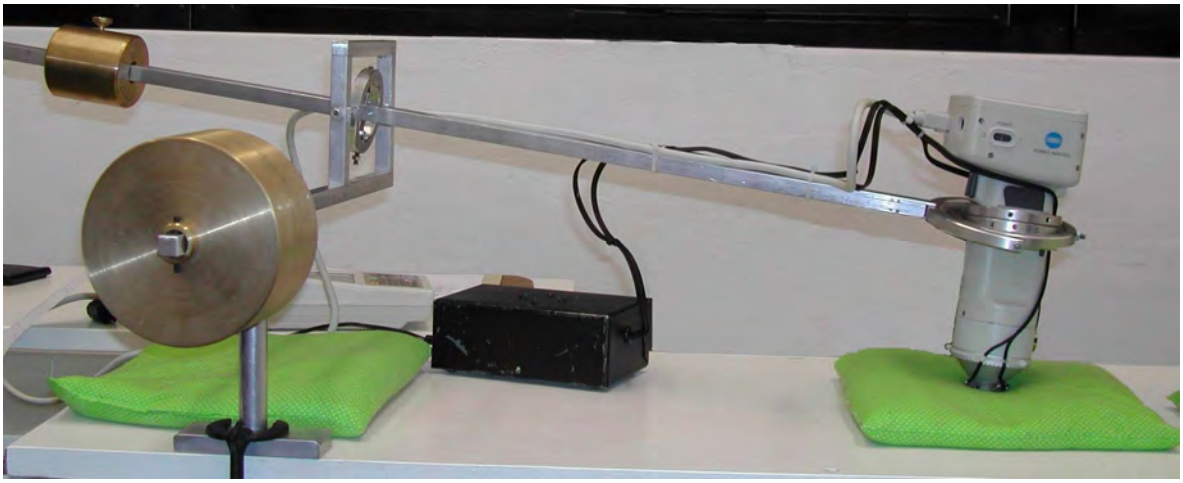


**Figure 2.2 Patented mounted chromameter**

Care was taken to place the measuring head of the chromameter flat onto the skin site, which is indicated when all the 4 light sensors of the inner concentric circle become illuminated. Unbalanced positioning of the chromameter on the skin site results in one or more of the green light sensors not being illuminated. Application of excess pressure results in the illumination of one or more of the red light sensors. Once the optimum positioning of the measuring head had been established with the aid of the light sensors, the balancing mass was set to a predetermined position for the duration of the studies to control the degree of pressure applied by the measuring head on the skin. The system of mounting was subsequently patented (Patent number 2006/04964).

### 2.4.2 Preparation of Volunteers

The ventral forearms of volunteers were washed and patted dry prior to the start of the studies. Sites, 2.5 cm centre-to-centre and singly stacked on the flat flexor aspect of the left forearm between the antecubital fossa and the wrist of the volunteer, 3-4 cm from either end, were demarcated and numbered (Figure 2.3). The opposite sides of the demarcated application sites and the corresponding sites on the chromameter measuring head base were marked to ensure the exact placement of the chromameter for successive readings.

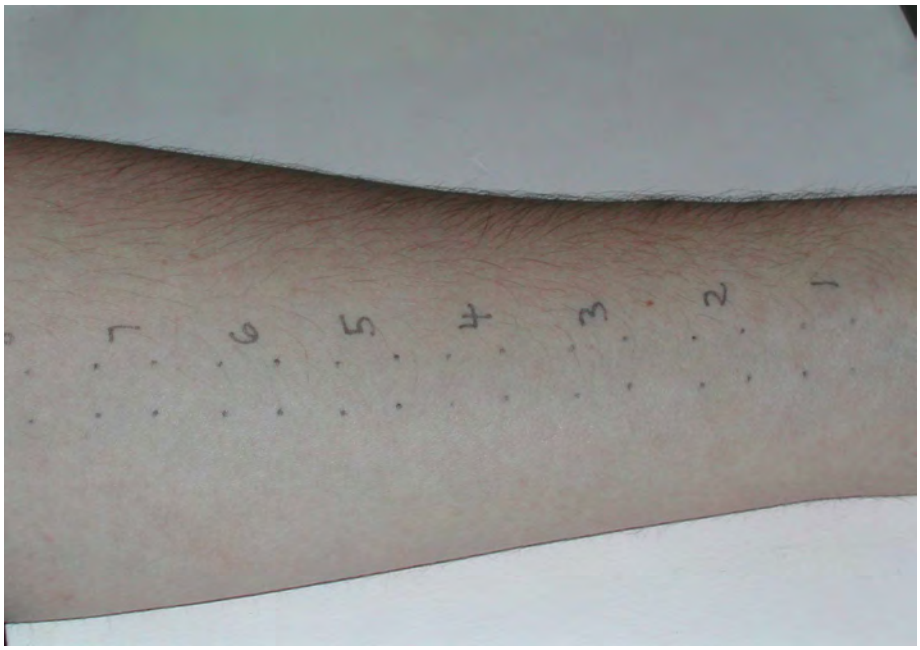



**Figure 2.3 Numbered skin sites on the flexor aspect of the forearm showing the placement marks for each site**

Topical corticosteroid formulations were not used in this set of studies. The volunteers enrolled were not suffering from any dermatological conditions and had not used any topical corticosteroids in the last two months. The study population comprised of the following volunteers shown in Table 2.1.

**Table 2.1 Chromametric method validation**

| Study | Description | Chromameter used | Volunteers |
|---|---|---|---|
| **1** | Static chromameter | Hand-held and Mounted | 1 black<br>1 Caucasian |
| **2** | Realigning chromameter | Hand-held and Mounted | 1 black<br>1 Caucasian |
| **3** | Non-aligned chromameter | Hand-held and Mounted | 1 black |
| **4** | Effect of pressure | Mounted (10, 30, 50, 100 g pressure) | 1 Caucasian |
| **5** | Skin colour rhythm variation | Mounted (30 g pressure) | 2 blacks<br>2 Caucasians<br>2 Indians |

## 2.4.3 Assessment of Instrument Measurement Variables

### 2.4.3.1 Positioning and Application Pressure

In study **1**, the chromameter was positioned with the reference marks on the measuring head corresponding to those on the demarcated skin site. Three successive readings were made at each site without lifting the chromameter off the skin, i.e. statically, and 3 second intervals were allowed between readings at the same site. Both the hand-held and mounted chromameter setting were used to take static readings. By statically holding the chromameter in position, the variation due to the inexact placement of the measuring head over a skin site could be eliminated. Using the hand-held chromameter, the variation in measurements due to variable pressure applied by the operator could be elucidated. Using the mounted instrument, the appropriate pressure could be kept constant.

In study **2**, one reading per site was taken from site 1 through to site 7. This procedure was repeated twice, ensuring exact realignment to the marks each time, to give three readings per site. Both, the hand-held and mounted chromameter configurations were used to assess the effect of realignment. This experiment mimicked the FDA designed chromametric study[32] where readings are taken at intervals e.g. at times 0, 2, 4, 6, 19 and 24 hours after drug product removal. The variability of the measurements due to realigning was used to assess precision.

Study **3** differed from study **2** in that the spatial orientation of the measuring head of the chromameter was not kept constant but the same skin site was repeatedly measured (n=3). The change in spatial orientation was accomplished by rotating the chromameter through 180˚. Reference marks were used in reverse to ensure that readings were made on the same site each time. Readings were taken using both the mounted and the hand-held configurations. As in study **2**, one reading per site was taken from site 1 through to site 7 and the procedure repeated to give three sets of measurements per site.

The effect of pressure on the skin of both forearms was explored in one Caucasian by two different chromameter operators in study **4**. In this study only the mounted chromameter was used. The balancing mass on the horizontal beam holding the chromameter head was

adjusted so as to apply a predetermined pressure on the arm. The following average masses were used: 12.04 ± 1.49 g, 28.10 ± 4.02 g, 49.96 ± 1.56 g and 101.80 ± 1.77 g (n=5). For each pressure application, at each site, triplicate readings were made in the manner described in study **2**, realigning the chromameter. The same chromameter was used by both operators.

#### 2.4.3.2 Assessment of Skin Colour

In study **5**, the forearms of six human volunteers were gently washed and dried 30 minutes prior to chromameter measurements being done sequentially on each of the volunteers in order to allow the skin to equilibrate with respect to its natural colour. Each volunteer was processed sequentially at 10-minute intervals to minimize environmental variables. Sites were demarcated and numbered (Figure 2.3). During this time interval subjects were confined in an air conditioned room where the study was conducted allow to stabilization of blood flow. The mounted chromameter was used and when the measuring head of the chromameter was properly aligned over the skin site, (all green pressure light sensors on), the chromameter was left in position and the shutter button on the chromameter data processor depressed to measure the skin colour. One reading per site at various time intervals was taken as follows: Subject 1: 0800, 1200, 1400, 1500, 1700, 2000, 2100, and 2200 and continued at 0830 and at 1130 hours the following day.

## 2.5 Results and Discussion

### 2.5.1 Positioning and Application Pressure

The readings obtained from the hand-held chromameter were generally higher than those from the mounted chromameter (Figure 2.4). The precision, calculated as percent relative standard deviation (% RSD) was however, comparable between the mounted (0.3–0.6%) and hand-held configurations (0.1–0.8%). The higher a* readings obtained with the hand-held chromameter could be due to higher, unregulated pressure applied to the skin on measuring[44] (Figure 2.4).

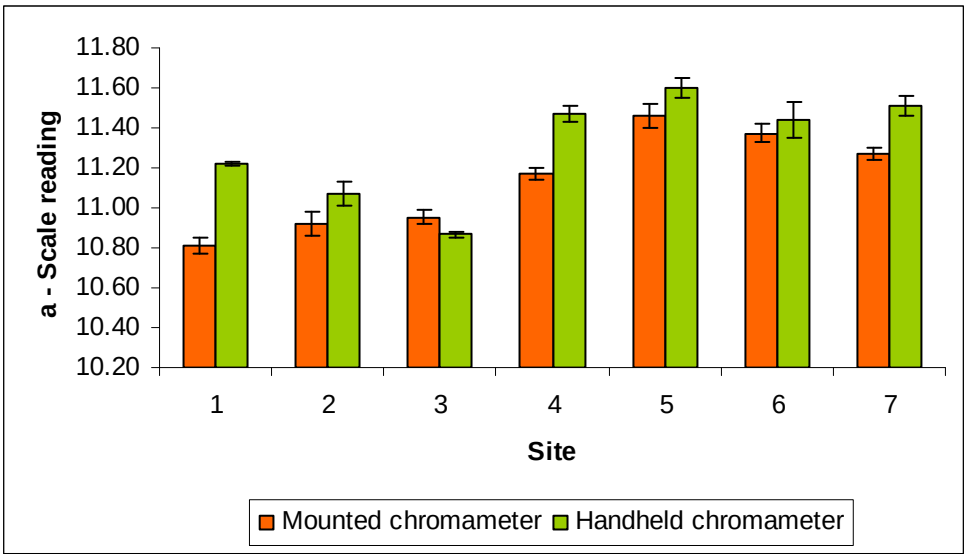



**Figure 2.4 Effect of application pressure**

Significantly different means were observed between the hand-held and the mounted chromameter in the static position ($p < 0.0001$) using the paired t-test. This indicates that there is a significant contribution from uncontrolled application pressure when the hand-held configuration is used to make the measurements.

Both hand-held and mounted chromameter configurations were realigned to the exact skin site for repeat measurements. Variation was found to be from 0.2 to 2.0% for the hand-held chromameter whereas the mounted chromameter showed percent relative standard deviations of 0.5% to 1.5%. Wide variations in the %RSD obtained per site implies a variation in the applied force. Such variations are likely to produce erroneous results when using a hand-held chromameter since the application force is neither known nor constant. Although the hand-held chromameter may be comparably precise, it may not be reliable as evidenced by the chromameter assessment studies that have been previously been reported.[28,43,46,50]

A comparison between the realigned and the non-aligned mounted chromameters show that no discernible differences were observed due to the spatial orientation of the chromameter (Figure 2.5). A change in spatial orientation of the chromameter which could occur between measurements may result in a change in the sealing off of the skin

site hence producing significantly different results. However, if the measurement is appropriately controlled by completely sealing off the site, as was achieved in this study, then significant differences between the different alignment positions can be avoided.

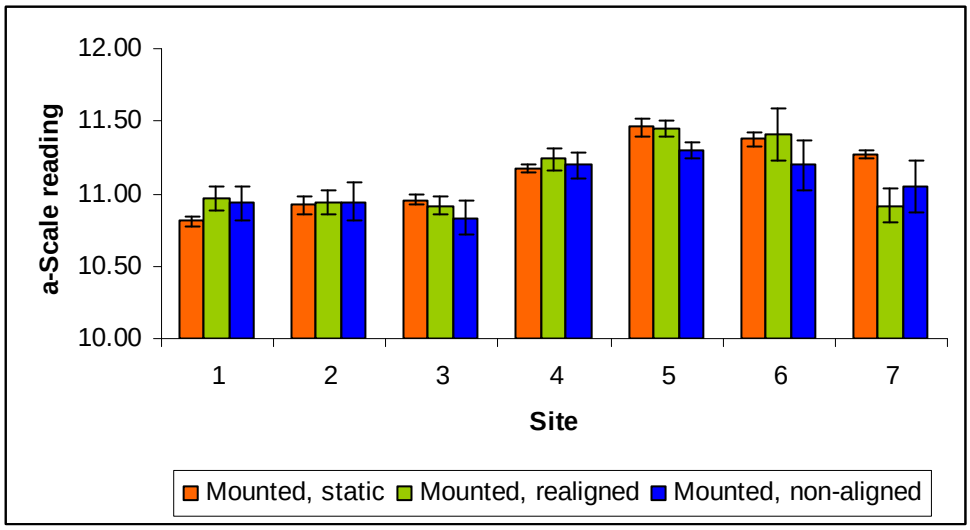



**Figure 2.5 Effect of chromameter placement**

## 2.5.2 Effect of Skin Colour

It was observed that generally Caucasian skin gives lower a* readings compared to that of black skin (Figure 2.6). The fluctuation of readings taken consecutively from a particular site is wider for Caucasian skin as compared to black skin. From these preliminary results it would seem better to use blacks in blanching experiments as there is potential for consistent, less variable results, however, this would depend on the detection of an adequate blanching response in such individuals.

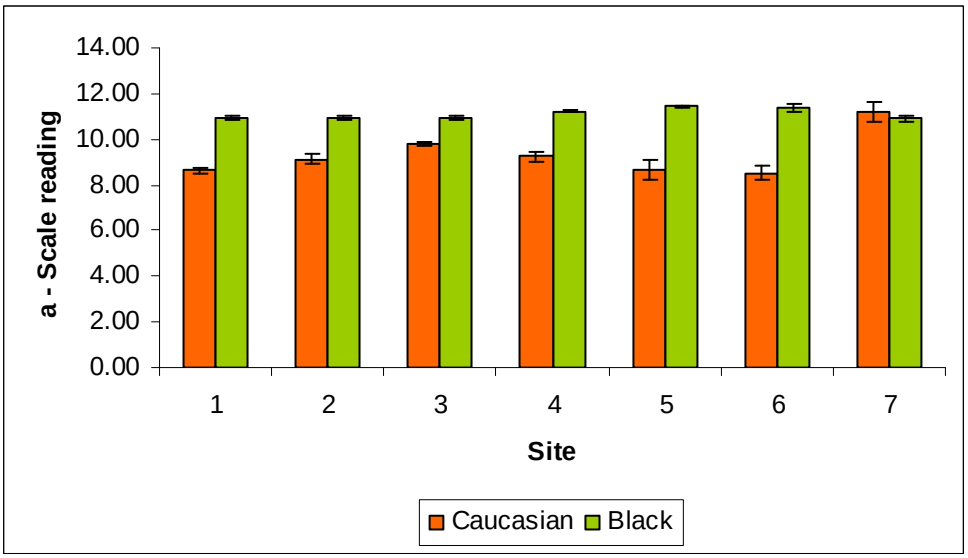



**Figure 2.6 Effect of Skin Colour – a* readings from Caucasian and black skin**

### 2.5.3 Control of Application Pressure

In order to control the application pressure and placement of the chromameter on the skin, a mounted configuration was used.

It was found that as the pressure applied to skin site increased, higher a* readings were obtained. Highest a* readings were observed when a 100 g weight was applied to the respective sites, whilst the lowest readings were seen with a minimum weight of 10 g. These observations were consistent between operators (Figure 2.7 and 2.8). Readings obtained by both operators were very reproducible and inter-operator variability was low. The percent relative standard deviation (%RSD) for operator 1 was from 0.5% to 6.8% while for operator 2 it was 0.9% to 9.6%. There were no significant differences between the readings by the two operators. The benefits of using a mounted chromameter configuration are thus apparent.

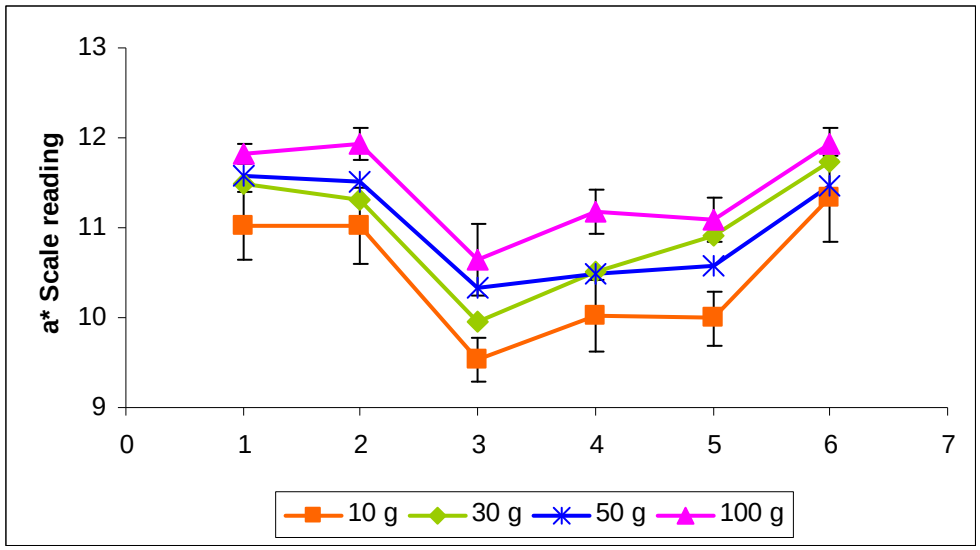



**Figure 2.7 Effect of pressure on a* scale readings – Operator 1**

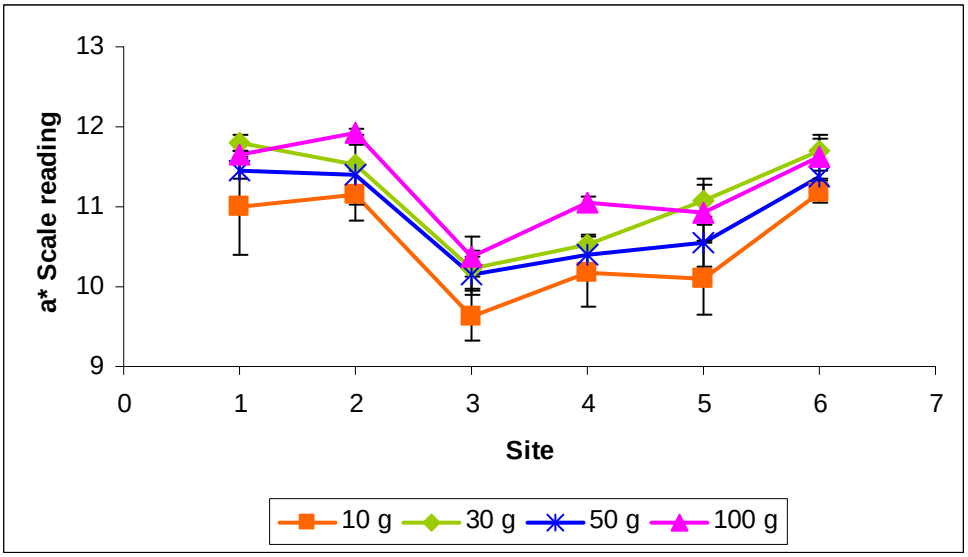



**Figure 2.8 Effect of pressure on a* scale – Operator 2**

Application of weight to the chromameter measuring head resulted in differences in L*, a* and b* values related to the particular weight. The a* values which have been recommended for use by the FDA[32] actually show the most marked change with pressure.[44] About 11% increase, from 10 g to 100 g, on the a* scale reading was observed at sites 3, 4 and 5.

In this research it was found that L* readings also increased with pressure (Figure 2.9) but not as much as a* readings. No obvious pattern was seen with b* readings when pressure applied to the site was increased (Figure 2.10). Waring *et al*[44] found contradicting results in terms of L* value assessment where L* values were found to decrease with pressure.

Graphical results for operator 1 are given to show comparisons between L*, b* scales and EDs.

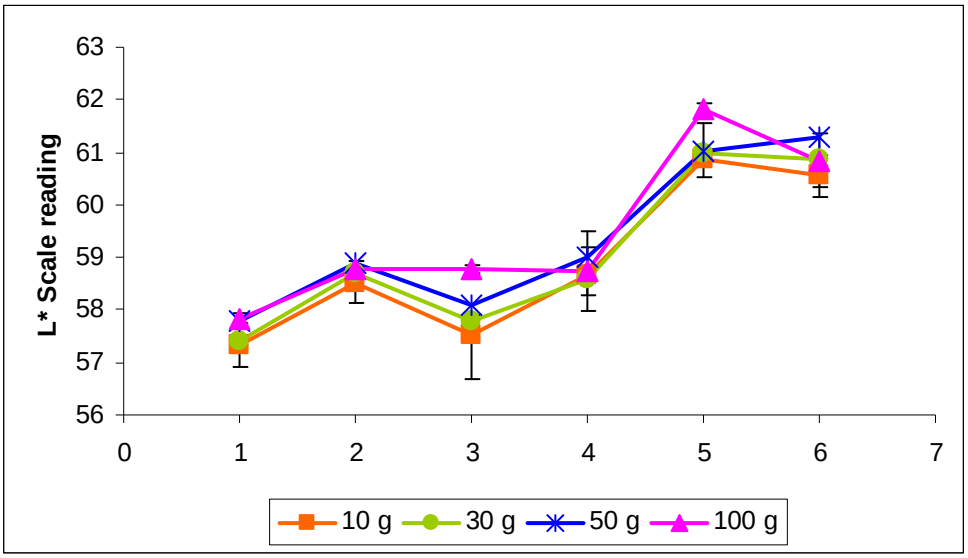



**Figure 2.9 Effect of pressure on L* reading – Operator 1**

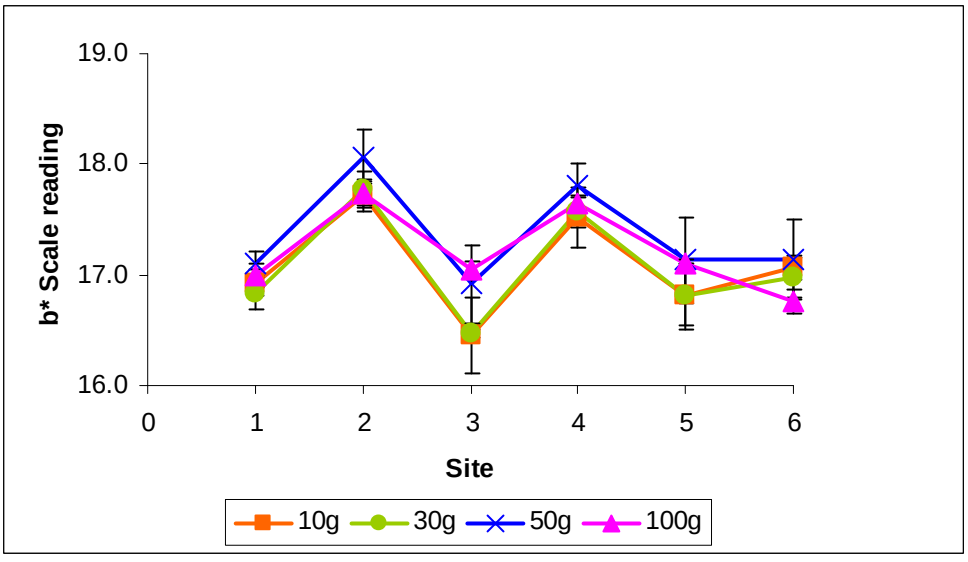



**Figure 2.10 Effect of pressure on b* reading – Operator 1**

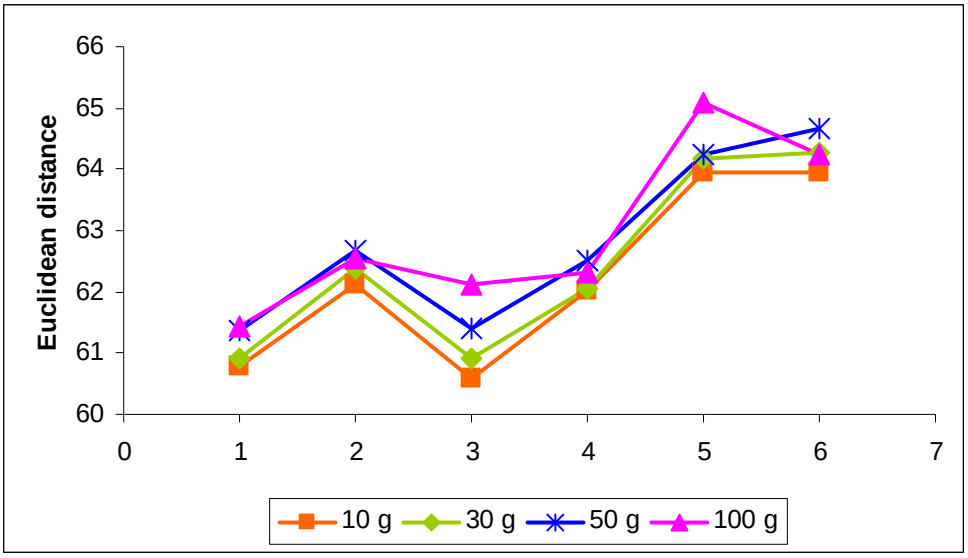



**Figure 2.11 Effect of pressure on Euclidean distance – Operator 1**

Like a* values, EDs increase with increasing pressure (Figure 2.11). Euclidean distances describe colour more precisely than the use of any one of the components of the CIE L*, a*, b* system. Since pressure plays a role in the colour change of the skin,[57] it should therefore always be controlled when assessing blanching.

When using a minimum mass of about 10 g the likelihood that that the measuring head base of the chromameter did not completely seal off the skin site was high. The measuring head "bounced" off the site and required more time before constant placement was possible. The chromameter assesses surface colour based on the tristimulus analysis, i.e. the analysis of the three colours; red, green and blue, of the reflected xenon light pulse. Incomplete sealing off of the site to be measured results in the loss of some of the xenon incident light or that reflected from the site leading to erroneous data. An average mass of 100 g results in excess pressure being applied to the site. Excess pressure changes skin colour possibly by occluding blood from the region being measured.[57] Since skin colour varies due to variability in factors such as perfusion and vasomotion,[54] a constant pressure should be applied for consistent readings. This pressure should not be in excess such that it changes perfusion and results in skin colour changes. A minimum pressure that allows the measuring head to be in appropriate contact with the surface of the skin permitting the site to be completely sealed off thereby preventing any light

escaping is necessary. This minimum pressure needed was found to be approximately 30 g. Maintenance of a consistent minimum pressure when taking chromameter measurements on the skin surface permits acquisition of results that are both accurate and precise. The aperture diameter of the measuring head is 8 mm (surface area of 0.50 $cm^2$); the pressure applied to the skin by the mounted chromameter is thus only 60 $g/cm^2$. If the hand-held chromameter is applied to the skin by weight alone, it applies a pressure of 1060 $g/cm^2$ since the chromameter head weighs about 530 g. In addition, the pressure is increased and varied by the operator's hand. Application of pressure greater than 1 kg per $cm^2$ definitely skews data resulting in erroneous results. This pressure on its own results in a change in perfusion in the forearm hence a true representation of the blanching profile is not obtained. Some workers have increased the surface area of the chromameter measuring head base so as to control the pressure applied to the skin during measurements.[58] Manual handling of the chromameter by an operator may add a variable amount of pressure between individual operators.[43] The results obtained from the patented mounted chromameter are thus more reliable since important variables can be well-controlled and are independent of the operator.

An average mass of 30 g was set on the patented mounted chromameter and used for subsequent blanching studies.

### 2.5.4 Circadian skin colour changes

On plotting a* scale values versus time, the amplitudes associated with the changes in a* values per unit time for each site on Caucasian skin were much more pronounced than those of either blacks or Indians. The a* values for blacks and Indians ranged from about 8 to 10 yet those for Caucasians were in the range 3 to 9. This shows that Caucasians respond much more to the factors that result in a change in skin colour. Racial differences in skin colour are due to the activity of the melanocytes rather than the number of melanocytes present.[59] Melanocytes are found in the basal epidermal layer of the skin below the squamous keratinocytes that make up the outermost layer of the skin. Melanin production takes place in melanosomes which are organelles found in melanocytes.

Melanin is distributed to the keratinocytes via dendrites. Darker skinned individuals have higher concentrations of melanin in the epidermal layer of the skin and this probably camouflages the change in skin colour that is otherwise detected in their lighter skinned counterparts. In addition, racial variations as a response to topical corticosteroid application have been found to exist.[29]

Increases in a* readings were observed periodically in each of the subjects following meals (meal times are shown by the arrows). It was shown in 1971 by Krieger *et al*[60] that plasma corticosteroid and ACTH levels increased after meals and were high between 0400 and 0800 hours whilst nadirs occurred during late evenings. Maximum plasma corticosteroid levels occurred ½ to 2 hours after awakening. The pattern of change in skin colour as observed in this research follows closely to the pattern described for the release of endogenously secreted cortisol. Cortisol may thus have an effect on skin colour as it is an endogenous corticosteroid. It can thus be deduced that skin colour follows a circadian rhythm governed by the secretion of endogenous corticosteroids.

Euclidean distances also showed a similar pattern to a* readings, but the pattern was not as prominent. Whereas a* scale readings are the most affected by skin colour changes inclusion of the L* and b* readings in the calculation of EDs results in the dampening of the effect otherwise observed when only a* scale data are used. Based on these results it is more appropriate to use a* readings only for assessment of blanching. However, both a* readings and EDs were assessed after topical corticosteroid application (*vide infra* Chapter 3).

The assessment of skin colour without the application of topical corticosteroids reveals why the chromameter data must be doubly corrected at each site before this data can be used for analysis. The manipulation of chromameter data is shown in Section 2.2.2. Skin colour changes due to endogenous cortisol secretion in addition to other factors during an experiment, hence to analyse only the effect due to blanching there is a need to adjust the baseline and to further correct the measurements using untreated site controls. Figures

2.12 to 2.17 show the skin's circadian response to endogenous cortisol in the different races. The response was calculated using both a* scale readings and EDs.

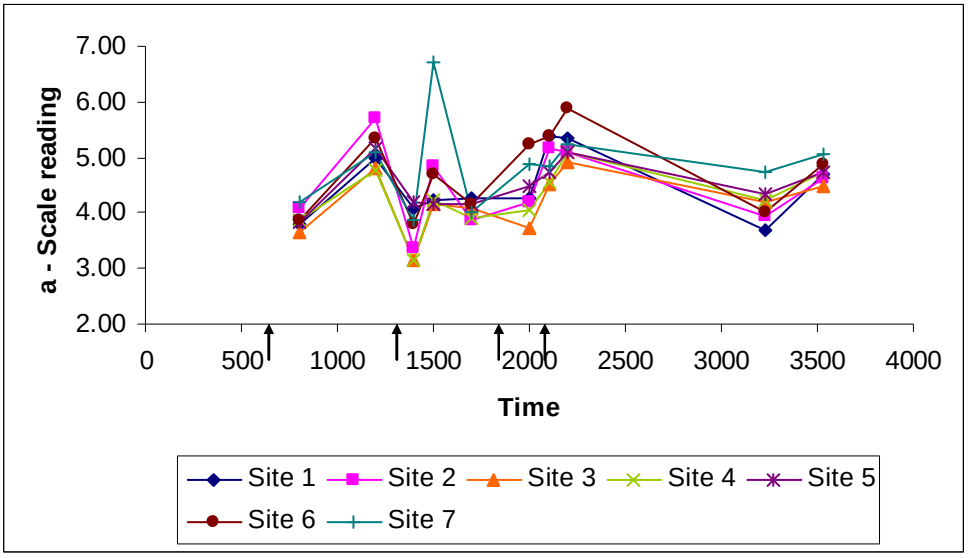



**Figure 2.12 Skin colour readings (a*) in volunteer 5 (Caucasian)**

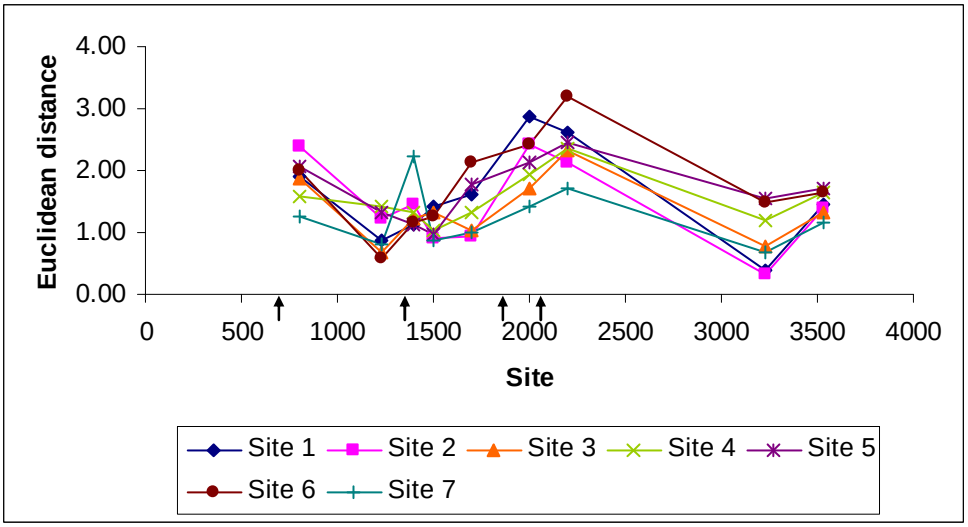



**Figure 2.13 Skin colour changes (ED) in volunteer 5 (Caucasian)**

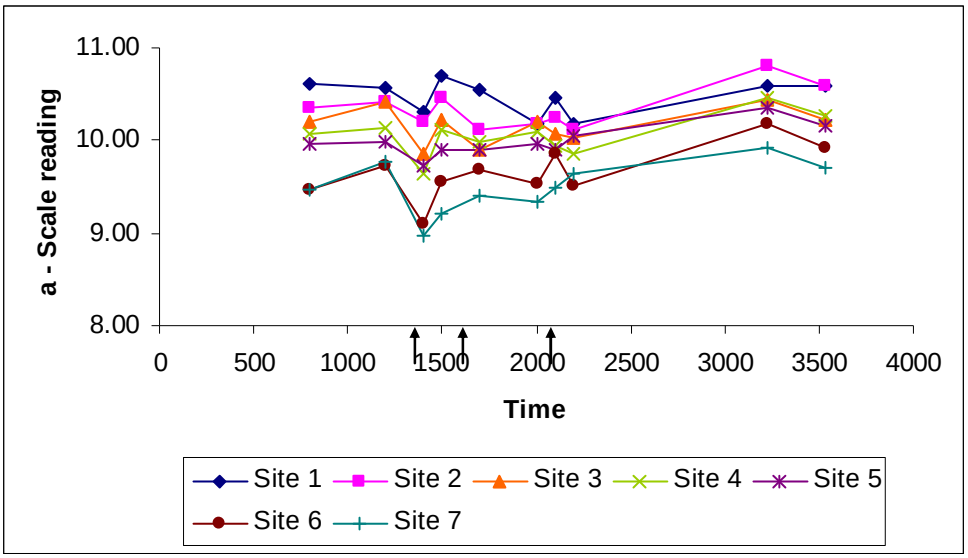



**Figure 2.14 Skin colour changes (a*) in volunteer 3 (black)**

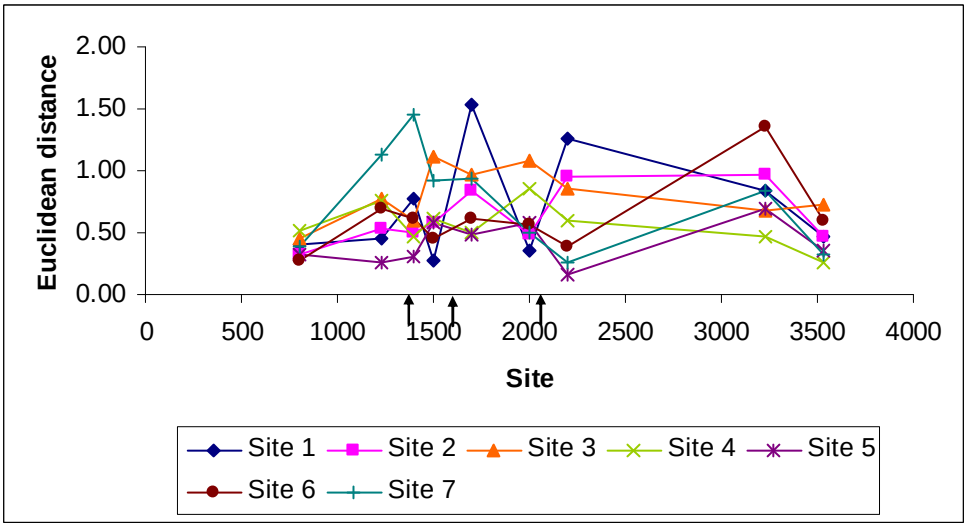



**Figure 2.15 Skin colour changes (ED) in volunteer 3 (black)**

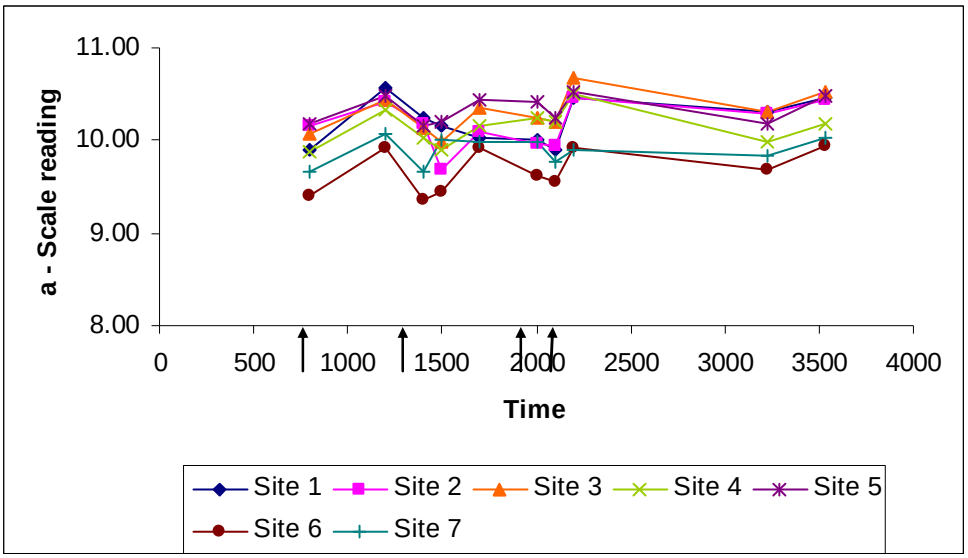



**Figure 2.16 Skin colour changes (a*) in volunteer 6 (Indian)**

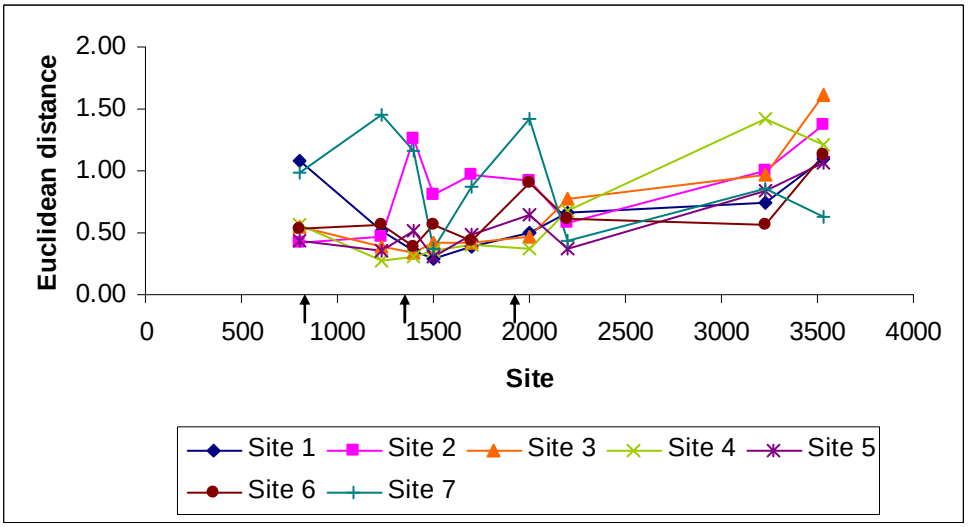



**Figure 2.17 Skin colour changes (ED) in volunteer 6 (Indian)**

## 2.6 Conclusions

When using the chromameter, it is imperative that the pressure applied to the skin site be closely controlled in order to get precise, reproducible results. Whilst the use of a mounted chromameter configuration facilitated the assessment of blanching as well as providing better control with respect to pressure on the skin and placement of the measuring head of the instrument, the data obtained do not provide convincing evidence of superior reproducibility. Generally, higher L* and a* values were observed with the

hand-held chromameter as opposed to the patented mounted chromameter. Spatial orientation does not significantly change the results obtained. It is important that placement of the chromameter ensures that no loss in incident or reflected light occurs during measurements which is facilitated by the use of a mounted chromameter configuration. It is however emphasized that the above described investigations were performed on untreated skin in order to establish skin colour variability using a chromameter. It is thus quite likely that a well-controlled procedure for assessing skin blanching on corticosteroid treated skin by using a mounted chromameter, should provide an advantage over the commonly used hand-held system.

Furthermore, during a HSBA, measurements are taken at 2 to 3 hour-intervals for about 30 hours which can result in operator fatigue using the hand-held configuration with a deleterious effect on precision and reproducibility. The advantage of the mounted chromameter configuration is that it provides more consistency of measurement as readily observed in this investigation where no significant differences were observed between operators using the mounted chromameter configuration.

The pressure applied to a site when measuring skin colour results in a distortion of the readings obtained. As the pressure applied increases, L*, a* and ED values also increase. The b* values are the least affected by pressure. Application of a constant and adequate pressure which allows the complete sealing off of the skin site to be measured is required to ensure accurate, precise measurements and can be readily achieved using a mounted chromameter configuration.

The patented mounted chromameter was successfully used to assess baseline skin colour changes in different races. Caucasians showed the largest amplitudes in the response versus time plots. There was no significant difference between the respective amplitudes seen on Indian and black skin. Larger amplitudes imply better response to factors causing skin colour changes and the results obtained indicate that Caucasian skin has a potentially better blanching response than either Indian or black skin.

In the studies conducted, it has become evident that the patented mounted chromameter can measure skin colour precisely and reproducibly compared to the commonly used hand-held chromameter configuration. The patented mounted chromameter has therefore been chosen for use in all the subsequent skin blanching assessment studies.

# CHAPTER 3

# ASSESSMENT OF BIOEQUIVALENCE OF MF FORMULATIONS USING THE CHROMAMETER

## 3.1 Introduction

Bioavailability of a drug is the rate and extent to which the active pharmaceutical ingredient (API) or therapeutic moiety is absorbed from a pharmaceutical product and becomes available at the site of drug action.[61] If a comparative bioavailability study is done on different formulations of the same drug and they are claimed to be bioequivalent, it is assumed that they will be equivalent with respect to safety and efficacy. Bioequivalence is thus an indirect or surrogate measure of safety and efficacy, and can be defined as the absence of a significant difference in bioavailability between two pharmaceutically equivalent products under similar conditions in an appropriately designed study. For registration of generic formulations, proof of bioequivalence when compared with the innovator product is required. Innovator companies are required to go through all the phases of clinical testing to ensure that the drug formulation is safe and effective for clinical use. The costs accrued by the innovator company are huge. This is reflected in the cost price of the innovator product as the company tries to recover the money expended on drug development. Companies manufacturing generic products do not have to repeat the initial clinical trials performed by the innovator on their product; instead they undertake a bioequivalence assessment to compare the bioavailability between the generic (test) product with the innovator or brand (reference) product. Circumventing clinical trials results in less expenditure in that component of drug development, thereby permitting the cost of a generic product to be considerably reduced.

The innovator company usually holds a patent on their product for a certain number of years (usually 20 years in most countries) which provides marketing exclusivity for the

period of the patent. Only at the end of the patent period can generic companies enter the market with their generic product.

The bioequivalence test procedure for topical corticosteroids is known as the HSBA. Topical corticosteroid formulation is applied to healthy skin, penetrates it and elicits a blanching response. The response is quantitated chromametrically or visually and compared.

Different ethnic groups respond to topical corticosteroids in different ways. The actual reason why this occurs is not known but several postulates have been put forward. In addition, variation in blanching response even may exist among individuals within the same ethnic group. Skin structure and the factors that affect the percutaneous absorption of topically applied products have been summarized below.

## 3.2 Skin Structure

The skin consists of three main histological layers, the epidermis, dermis and the underlying subcutaneous layer (Figure 3.1).

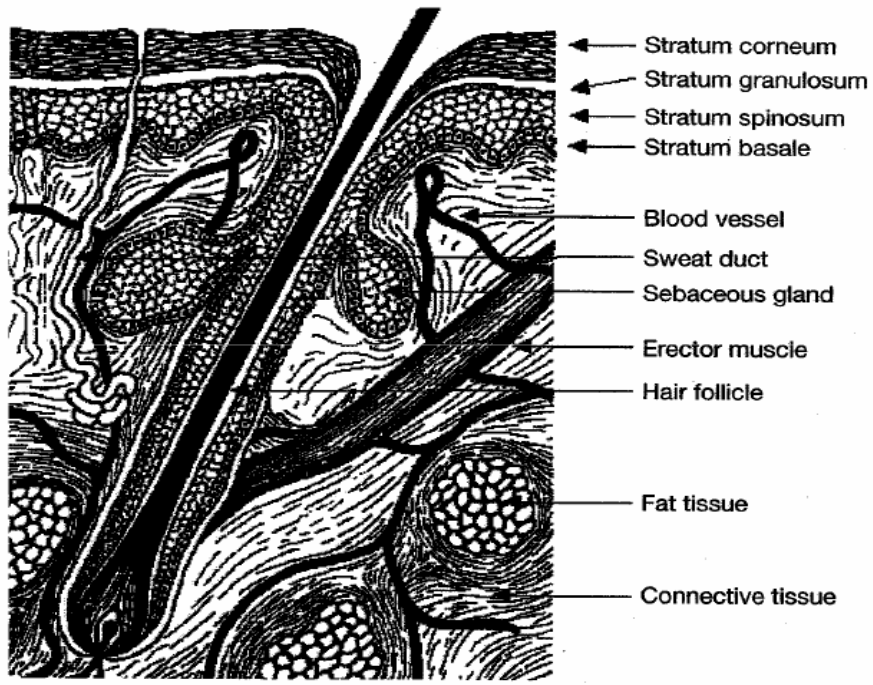



**Figure 3.1 The components of the skin[62]**

### 3.2.1 Epidermis

The epidermis, the uppermost layer consists of two main parts, the *stratum corneum*, a dry keratinized outer layer of dead and anucleate, flattened cells and the inner moist viable *stratum basale*. Two minor layers, the *stratum granulosum* and *stratum spinosum* lie between the two main layers. The epidermis consists of stratified squamous cells. The overall epidermis is about 0.06–0.1 mm thick and where it is thickest, on the palms of hands and soles of feet, it is about 0.6 mm.[47,63]

In the *stratum corneum*, keratinocytes produced in the viable basal layer of the skin differentiate and move successively towards the surface. At the surface they alter morphologically and histochemically, and flatten and shrink into a stratified squamous type of epithelium. In addition, these cells extrude lipid components which end up in between the keratinocytes arranged in bilayers. This bilayer of intercellular cement has a major function in skin barrier properties. The *stratum corneum* has 15-20 layers of flat keratinized, cornified dead cells, which are vertically stacked in a highly organized structure.[64] The cell edges interdigitate with the other adjacent cells. An integral factor in the final stages of formation of keratin is the oxidation of the sulfhydryl containing substances. The strong disulphide bond of keratin accounts for the great resistance of keratinized structures. These structures resist the effects of acids and many enzymes.[47,63] This horny layer provides an almost impermeable layer which controls the percutaneous absorption of compounds.[47,65,66] The *stratum corneum* is the rate limiting step of drug absorption via intact skin.

Although the *stratum corneum* is the principal barrier to the penetration of substances in the skin, lipid soluble substances transverse this layer more readily than water and water soluble substances. Amphipathic substances penetrate the *stratum corneum* more easily than either of the above.[47] Permeation of the *stratum corneum* can be described by the laws of passive diffusion.[27]

### 3.2.2 Dermis

The dermis is a layer of dense connective tissue which functions as a supportive vascular stroma for the epithelial structures of the skin. The connective tissue is composed of collagen fibres and elastic fibres embedded in an amorphous ground substance of glycosaminoglycans, salt and water. The dermis forms papillae towards the epidermis which project into and are enclosed by the overlying epidermis. The papillae are composed of cellular connective tissue and each papilla contains a tortuous loop of capillary blood vessels or tactile corpuscle.[66] Where it is thickest the dermis is almost 4 mm thick. The dermis is divided into two main layers; the *pars papillaris* and the *pars reticularis*. In contrast to the *stratum corneum*, the dermis is a freely permeable.[27,64]

### 3.2.3 Subcutaneous layer

The fatty subcutaneous layer, *stratum subcutaneum* is situated below the dermis but is inseparable from it. It contains many blood vessels that supply the skin and also because of the lipophilic nature it acts as a sink, absorbing lipophilic substances that pass through the dermis.[27] This layer is variable in thickness but generally the deeper layers of the subcutaneous tissue are continuous.[64]

## 3.3 Percutaneous Absorption

### 3.3.1 Via *Stratum Corneum*

The main pathway for drug penetration is via the *stratum corneum.*[28,31] The extent of absorption through the *stratum corneum* is determined mainly by the composition of the delivery vehicle, concentration of the active substance in contact with the barrier,[27] the integrity of the *stratum corneum* and the degree of hydration of the skin. The *stratum corneum* is accepted as the rate-limiting step in the process of percutaneous absorption when it is continuous and unperturbed.[26] If the *stratum corneum* is damaged, as in certain diseases or physical or chemical trauma, leaving the aqueous sub-tissues exposed to the preparation, the rate limiting step becomes the diffusion of the drug in the preparation.[31]

Absorption of materials through the epidermis follows some general rules. Molecules pass through the epidermis more easily than ions; small molecules pass more easily than larger ones and materials soluble in both lipid and water pass more easily than those only soluble in either water or lipid.

The *stratum corneum* acts as a passive, but not an inert diffusion medium. Application of preparations to the skin results in changes in the hydration of the skin due to the water contained in the preparation or to the occlusive nature of the preparation. Other components of topical dosage forms such as penetration enhancers result in a change in the barrier nature of the *stratum corneum*. Exposure to such treatment results in the interruption of the protein framework and alteration of the distribution coefficient between the skin and the preparation of the drug, changes in the keratin adsorption and desorption of the drug and other physical parameters. Together with these changes the sink function of the skin is enhanced thus increasing the transport rate of the dermatological preparation.[39]

Topically applied drugs which have an effect on living tissue cells penetrate the non-living *stratum corneum* until they get to the regions where they exert their effects. No energy is required but transfer across the skin requires a concentration gradient. Diffusion of the drug molecules occurs from a site of high concentration to that of lower concentration until equilibrium is obtained. Drug molecules diffuse intra-cellularly, inter-cellularly and via the appendageal route, equilibrating laterally until they emerge from the distal surface of the *stratum corneum*.

### 3.3.2 Appendageal Route

Sweat glands and hair follicles have a potential role in the absorption of drugs from the skin surface. Topically applied dyestuffs showed that the vicinity of follicles was stained more intensely than the rest of the epithelium.[47] The appendages act as short-circuit diffusion pathways or diffusion shunts. Such pathways can be important for some substances but not all. Ions and large polar molecules that struggle to cross the intact

*stratum corneum* use this pathway.[31,67] In the human forearm the density of sweat glands is around 220 $cm^{-2}$ and that of hair follicles is about 100.[47] This pathway is about 0.1% available for drug transport.[67]

### 3.3.3 Hydration of the Skin

Skin hydration decreases the diffusional resistance of the barrier and results in an increased percutaneous absorption rate.[68] This has been shown by the better clinical response obtained after occlusive dressings were applied to the application site.[8] On such sites, it was observed that equivalent vasoconstriction appeared with topical corticosteroid concentration 100 times less than that needed for a non-occluded arm. It was also noted that plasma cortisol levels decreased when dermatitic patches of a patient were occluded with glucocorticosteroids and the pituitary adrenal axis was suppressed by the percutaneously absorbed synthetic steroids.[47] Absorption in the well hydrated nappy areas, perianal, flexures and other moist sites results in greater permeation of drug molecules.

### 3.3.4 Vehicle Composition

For the drug to be available for absorption it has to be released from the vehicle in which it is formulated. A vehicle with greater affinity for the drug substance than the horny layer of the skin results in less drug partitioning into *stratum corneum* per unit time. Less affinity for the vehicle results in maximized thermodynamic leaving potential of the drug for the *stratum corneum*.[34,55,69-72] The greatest thermodynamic potential of a system would be one that contains a saturated solution of the drug in the vehicle.[15] The release from a vehicle can be influenced by a number of factors which include: lipophilicity, particle size of active pharmaceutical ingredient (API) and selection of solvents and the drug concentration that provides for maximal thermodynamic activity of the drug.[27] Thermodynamic activity of water in the vehicle and the *stratum corneum* also affect percutaneous absorption. The multi-step process of percutaneous absorption requires drug dissolution within the vehicle first before it is released for partitioning into the *stratum corneum*.[73] The pH of the vehicle has an effect on drug release.[74] By varying the vehicle

only, betamethasone dipropionate has been formulated into four potency classes.[2] The therapeutic effect depends on the rate of steroid release from the vehicle as well as the rate at which the steroid passes through the s*tratum corneum*.[75] Intra-vehicle diffusion of a drug is affected by the viscosity of the formulation vehicle.

The inclusion of penetration enhancers results in increased percutaneous absorption. Chemical penetration enhancers improve the thermodynamic properties of the drug and/or alter the skin integrity increasing its permeability. The penetration enhancer is purported to replace water bound in the *stratum corneum* forming a much looser structure through which drug molecules penetrate easily or lead to swelling of the *stratum corneum* due to its hygroscopicity.[37]

### 3.3.5 Chemical Structure of the Active Pharmaceutical Ingredient

A study using steroids inferred that the more polar molecules penetrated relatively slower as a result of stronger chemical binding with the *stratum corneum*.[47] This inference could form the basis of the explanation for the reservoir function of the skin towards corticosteroids. Chemical structure also influences the drug partition coefficient. The rate of absorption of substances increases with an increase in lipid solubility. However, there is a delicate balance between lipophilicity and water solubility. The water soluble compounds find it the most difficult to traverse the *stratum corneum*. Lipophilic compounds have more difficulty penetrating the *stratum corneum* than an amphipathic compound with bipolar moieties. As such, optimal percutaneous absorption occurs when the drug substance combines lipid solubility with moderate water solubility. Sufficient lipophilicity allows for partitioning into the *stratum corneum* and moderate hydrophilicity enables second partitioning step into the highly aqueous viable epidermis.[73]

### 3.3.6 Particle Size

Macromolecules are not readily taken up if they are in an aqueous medium. However, if in a solvent that has high lipid solubility these macromolecules penetrate the barrier layer.[47] The effect of particle size on percutaneous absorption has been further

investigated using topical corticosteroids as the blanching response they elicit can be quantitated. A formulation containing micronized fluocinolone acetonide was found to be superior to another containing coarse particles of the same compound.[37]

### 3.3.7 Drug Concentration

Diffusion of drug molecules across the *stratum corneum* is driven by a diffusion gradient. The rate of diffusion is directly proportional to the concentration of drug at the *stratum corneum*. The effect of concentration is demonstrated by Fick's law[37] (Equation 3.1).

$$J_s = K_p \Delta C_s \qquad \text{(Equation 3.1)}$$

Where,

$J_s$ = steady state flux of solute

$K_p$ = permeability coefficient

$C_s$ = concentration difference of solute across the membrane

The degree of the blanching response is greater when higher amounts of drug are applied to the skin. In addition, increasing the time allowed for drug contact with skin and the frequency of application results in a corresponding increase in drug absorption.[55,76]

### 3.3.8 Temperature

An increase in skin temperature has been shown to result in a corresponding increase in percutaneous absorption (Table 3.1).

**Table 3.1 Effect of temperature**

| Drug | Temperature change | Increase in permeability constant | Reference |
|---|---|---|---|
| Acetylsalicylic acid | 10 ˚C to 40 ˚C | 8-fold | [77] |
| Alcohols (C8 – C10) | 10 ˚C to 50 ˚C | 10-fold | [78] |
| Salicylic acid and carbinoxamine | 20 ˚C to 38 ˚C | 5-fold | [79] |

## 3.4 The USA FDA's Procedure for the Bioequivalence Assessment of Topical Corticosteroid Products

Whereas for most dosage forms, the dose delivered is usually known to a great degree of certainty, in dermatology, the amount of drug that is absorbed through the skin is not easily quantifiable. A number of methods have been put forward to assess the dose responses that use concentration, surface area, frequency and time of exposure. As the concentration, surface area and frequency of application increase a corresponding increase in the dose response is expected. In addition, the dose response increases with time of exposure (dose duration).[80] However, dose response increases until saturation of the receptors. After saturation of the receptors, continued increase in the time of exposure, frequency or concentration does not result in a change in the dose response elicited, thus a plateau is reached. Comparative studies must be done at doses that are not in this plateau region. To figure out the dose duration at which the comparative studies are to be conducted, the FDA recommends the use of a pilot study.

### 3.4.1 Pilot Study

The relationship between a ligand (drug molecule) and its receptor is a simple, reversible, bimolecular relationship (Equation 3.2). This relationship can be explained in terms of the law of mass action which states that the velocity of a chemical reaction is proportional to the product of the concentrations of the reactants.[81]

$$\text{Ligand} + \text{Receptor} \rightleftharpoons \text{Ligand–Receptor} \qquad \text{(Equation 3.2)}$$

The association rate constant for the forward reaction depends on the concentration of both free ligand and free receptor. The number of binding events per unit time is calculated using Equation 3.3.

$$N_b = k_{on} \times [Ligand] \times [Receptor] \quad \text{(Equation 3.3)}$$

Where,

$N_b$ = number of binding events per unit time

$k_{on}$ = association rate constant

[Ligand] = concentration of free ligand

[Receptor] = concentration of free receptor

On the other hand the dissociation rate constant, depends upon the concentration of ligand-receptor complex.

$$N_d = [Ligand\text{-}Receptor] \times k_{off} \quad \text{(Equation 3.4)}$$

Where,

$N_d$ = number of dissociation events per unit time

[Ligand-Receptor] = concentration of ligand-receptor complex

$k_{off}$ = dissociation rate constant

The law of mass action is followed if dissociation does not result in a change in either the ligand or the receptor.

With time, equilibrium is reached, and the rate at which new ligand-receptor complexes are formed is equal to the rate at which the ligand-receptor complexes dissociate. The equilibrium dissociation constant, $K_d$, is thus calculated as the ratio between the dissociation rate constant and the association rate constant as shown in Equation 3.5.

$$\frac{[Ligand] \times [\mathrm{Re}ceptor]}{[Ligand - \mathrm{Re}ceptor]} = \frac{k_{off}}{k_{on}} = K_d \quad \text{(Equation 3.5)}$$

From the above equation, if ligand concentration is equal to $K_d$ at equilibrium, the ratio between the concentrations of the receptor and ligand-receptor complex is 1, meaning that ligand occupies half of the receptors whilst the other half is unoccupied. If a receptor has a high affinity for a ligand, a low concentration of the ligand results in substantial

binding, hence $K_d$ is low. On the contrary, if a receptor shows low affinity for the ligand then $K_d$ is high and a higher concentration of ligand is needed for binding to occur.
The relationship between ligand concentration and the fraction of receptors binding the ligand is given by Equation 3.6.

$$\text{Fractional occupancy} = \frac{[Ligand]}{[Ligand + K_d]} \qquad \text{(Equation 3.6)}$$

The dose response elicited increases as the concentration of the ligand increases until saturation of receptors occurs. Above saturation no increase in dose response may be observed (Figure 3.2).

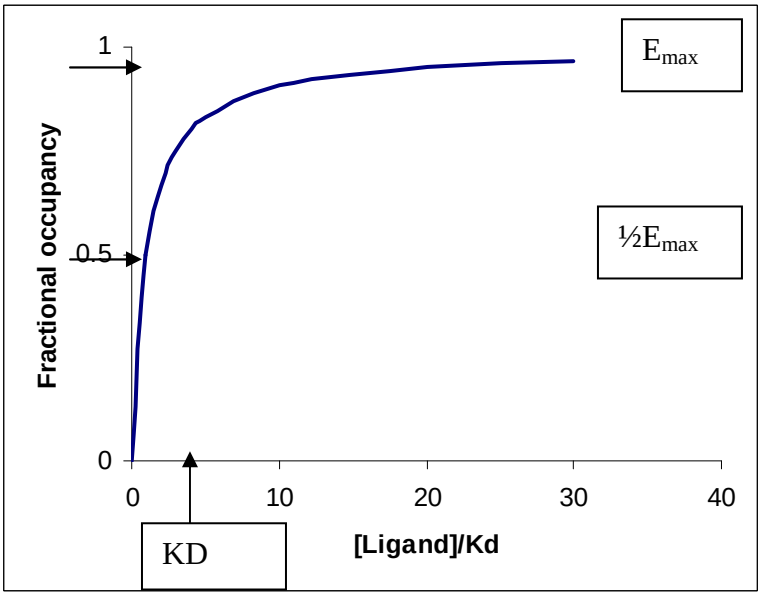



**Figure 3.2 Fractional occupancy of ligand on receptors[81]**

The FDA suggests the use of the $E_{max}$ model to analyse pilot study results (Equation 3.7). The $E_{max}$ model describes binding of a ligand to a receptor that follows the law of mass action. Although the law of mass action is a simple one, it provides a useful approximation.

$$E = E_0 + (\frac{E_{max} \times D}{ED_{50} + D}) \quad \text{(Equation 3.7)}$$

Where,

E = effect elicited

$E_0$ = baseline effect in the absence of ligand

$E_{max}$ = maximum effect elicited

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

The $E_{max}$ model is most commonly used as it has the ability to predict baseline effect in the absence of drug and the maximum achievable effect at increasing doses. This quantitative assessment of the dose-response relationship of a formulation results in the development of a sensitive pharmacodynamic assay to assess bioequivalence.

The reference drug formulation is applied topically for different time durations after which it is washed off completely from the skin and assessment of the response checked at intervals. Long dose durations result in a greater blanching response being observed compared to short dose durations. A plot of area under the curve(AUC) at each dose duration is plotted against the dose duration. AUC represents the blanching response to the drug after a particular dose duration (time of exposure). $ED_{50}$ and KD are calculated in the same manner shown in Figure 3.2. At $ED_{50}$ the response is half maximal. In addition to $ED_{50}$ the FDA guidance defines two other dose durations of importance $D_1$ (lowest dose calibrator) and $D_2$ (highest dose calibrator). $D_1$ and $D_2$ are defined as ½ and twice $ED_{50}$ respectively. $D_1$ and $D_2$ correspond to 33 and 67% of the maximal response which represents the sensitive portion of the dose-duration response curve.[32] The $ED_{50}$ lies between $D_1$ and $D_2$, where the response falls within a sensitive log-linear region (20% to 80% of $E_{max}$).[9] Bioequivalence assessment should therefore be undertaken at the $ED_{50}$.[80,82] The pharmacodynamic response (blanching) and confidence intervals depend on the size of the dose.[9] Close to the plateau response (4 times $ED_{50}$), the confidence interval is reduced greatly and the response is dose independent. Therefore, the use of dose durations greater than $ED_{50}$ indicates decreasing sensitivity to detect a potential difference between the test and the reference products, should a difference exist.

The pilot study determines the dose duration - response relationship of the topical corticosteroid under study. This is analogous to developing a standard curve in the assay of a drug in a biological matrix. The pilot study utilizes “responders” only for its subjects. Responders are subjects with the ability to blanch after application of the topical corticosteroid under set conditions, e.g. 2 hours after the after application of 7 mg of formulation to a 1 × 1 cm site for 4–6 hours a responder must show at least a single unit based on visual assessment. At each dose duration the AUC of a* scale, baseline adjusted and untreated site control corrected, against time after removal of application is calculated for each subject. Data from all the subjects are used in the $E_{max}$ model to compute $ED_{50}$, $D_1$ and $D_2$ using non-linear least squares regression. The objective of the pilot study therefore is to determine $ED_{50}$, $D_1$ and $D_2$.

### 3.4.2 Pivotal Study

The purpose of the pivotal study is to document *in vivo* bioequivalence of the test product to the reference product.[32] The pivotal study should be carried out using the parameters obtained from the pilot study. The drug is applied to the demarcated skin area for $ED_{50}$, $D_1$ and $D_2$ dose durations calculated from the pilot study. For the inclusion of a volunteer’s data in the analysis of bioequivalence, the FDA specifies a minimum dose duration-response ratio between AUC of $D_2$/AUC of $D_1$ to be greater or equal to 1.25 such subjects are called detectors.[32] Only the data from detectors is used for analysis. Data analysis using Locke’s method is described later in Section 3.7.2.

Detectors have the ability to sufficiently discriminate between high ($D_2$) and low ($D_1$) dose durations hence the $ED_{50}$ falls within the required range. Selection of only those volunteers who can discriminate between $D_1$ and $D_2$ enriches the study design and increases the sensitivity of the study to detect potential differences between test and reference products. The HSBA sensitivity is greatest at dose durations that produce response in the rapidly rising region of the dose-response curve based on the $E_{max}$ model.[9]

## 3.5 Objectives

To assess the following after application of a topical corticosteroid product:

- Inter-operator variability during the assessment of skin blanching - preliminary study.
- Differences between blanching assessment using visual grading results compared to Euclidean distances and a* readings using the patented mounted chromameter – preliminary study.
- Bioequivalence assessment of Elocon® cream 0.1% (Isando, South Africa) as a reference product and Elocom™ cream 0.1% (Quebec, Canada), as the test product using the patented mounted chromameter in accordance with FDA requirements.

## 3.6 Methods and Procedures

### 3.6.1 Selection Criteria

The following criteria were used for the selection of the study populations as recommended by the FDA.

#### 3.6.1.1 Inclusion Criteria

Only those subjects meeting the following criteria were included in the study:

- Healthy and normal subjects in terms of physical and dermatological examination at the pre-study screening who were available for the entire study period.
- Willingness to adhere to the protocol requirements, follow study restrictions.
- Able to give an informed consent (in relation to age and mental well-being).
- Between 18 and 50 years of age.

#### 3.6.1.2 Exclusion Criteria

- Clinically significant hypertension or circulatory disease.
- Individuals smoking within one week of study.

- Caffeine intake of more than 5 cups per day prior to or during the study.
- Clinically significant history of alcoholism or drug abuse.
- Use of dermatologic drug therapy on ventral forearms, including prior dosing of a topical corticosteroid in a pharmacodynamic study to a particular skin site, within one month prior to the study.
- Adverse reactions to topical or systemic corticosteroids.
- Any current or past medical condition, including active dermatitis or any other dermatologic condition, which might significantly affect pharmacodynamic response to the administered drug. Use of any vasoactive medication, constrictor or dilator, prescription or non-prescription medicine that could modulate blood flow. (Examples: nitroglycerin, anti-hypertensives, anti-histamines, aspirin, NSAIDs and OTC cough/cold products containing anti-histamines and/or either phenylpropanolamine or phentolamine.
- Any obvious difference in skin colour between arms.

#### 3.6.1.3 General Study Restrictions

- Subjects were not restricted with respect to posture during the study, however, no exercise with both arms and no strenuous exercise overall, was to be maintained for the study duration.
- No bathing or showering during the periods of drug application and assessment of skin blanching.
- No prescription preparations, vitamins, natural products used for therapeutic benefits, or antacids were allowed for at least one week prior to the study.
- No alcohol would be taken by the subjects from 24 hours prior to the start of the studies until the assessment was complete.
- No strenuous physical activity was to be undertaken by subjects from 12 hours before starting time until assessment was completed.
- Subjects were not allowed to apply emollients or skin conditioning creams to their forearms for a period of 24 hours prior to the scheduled time of the start of the study.

- Subjects were confined to the clinic but were allowed to leave in between measurements of skin blanching.
- From the time at which the formulations were applied, custom-made, non-occlusive guards were left in position to avoid smudging of the formulation into the surrounding skin other than at the marked skin sites. The guards were removed when synchronized washing took place for each individual.
- Food and fluid were taken *ad libitum*.

### 3.6.1.4 Criteria for Removal from the Study

- Any subject may be withdrawn from the study at any time due to the following:
- Voluntary withdrawal by the subject due to any reason.
- Illness or injury if regarded as clinically significant.
- Any adverse events or toxicity if regarded as clinically significant.
- Failure of the subject to comply with, or who is uncooperative towards, any study requirements or restrictions if regarded as clinically significant by the study investigator.

### 3.6.1.5 Pre- and Post-study Medical Check Up

The following check-up was conducted on each volunteer that was used in the preliminary, pilot and pivotal studies.

**Table 3.2 Screening tests**

| | Pre-study | Post-study |
|---|---|---|
| Medical history | √ | |
| Physical examination | | √ |
| Dermatological assessment | √ | |
| Blanching response | √ | |
| Adhesive sensitivity | √ | |

- Medical history – demographic data (date of birth, sex, origin), emotional (psychiatry), alcohol consumption, smoking habits, dietary habits, sporting commitments.

- Dermatological – general assessment of skin and any dermatological condition that may influence the barrier function of the skin and impact on the absorption of mometasone furoate.
- Blanching response – assessment of subject's ability to blanch after topical application of the reference product. Selected subjects showed distinguishable visual skin blanching following exposure to 7.5 mg of formulation to an area 1.1 cm × 1.1 cm, for 6 hours. The visual reading was made 2 hours post exposure.
- Adhesive sensitivity – assessment of subject's sensitivity to adhesive on application site demarcation tape.
- Physical examination – examination of forearms and application sites.

### 3.6.2 Study Products

The test and reference products were kept in a dark cupboard, away from direct light and below 25˚C.

**Table 3.3 Description of study products**

| | Test product | Reference product |
|---|---|---|
| Trade name | Elocom™ | Elocon® Lot 2 |
| Dosage form | Cream | Cream |
| Drug/Strength | Mometasone furoate 0.1% | Mometasone furoate 0.1% |
| Manufacturer | Schering Canada Inc | Schering – Plough (Pty) Ltd, |
| City, Country | Quebec, Canada | Isando, South Africa |
| Batch/Lot No. | 6NGFA17 | 4NGFA07 |
| Expiry Date | AL 08 | October 2006 |

### 3.6.3 Study Populations

Three healthy Caucasian volunteers, two males and one female (aged from 35 to45 years) who had been pre-screened for positive blanching were selected for use in the preliminary blanching study. In the pilot study, six volunteers (4 Caucasians, 1 Chinese and 1 black) with no history of dermatologic disease were chosen to participate. The pivotal study incorporated 24 Caucasian volunteers. Only the results from detectors were used to analyse bioequivalence data.

Approval to conduct the studies was obtained from the Rhodes University Ethical Standards Committee in compliance with the 1964 Declaration of Helsinki and its subsequent amendments.

### 3.6.4 Method of Application and Subject Monitoring

Using the flexor aspect of the forearm eight 1.1 × 1.1 cm sites were demarcated on each arm. Squares 1.1 × 1.1 cm were cut out of adhesive labels (Redfern labels, Johannesburg, South Africa) to make templates which were placed over the demarcated sites on the volunteers' arms. The templates were secured in position using Micropore™ 1530 surgical tape (3M, St Paul, Minnesota, USA). The appropriate topical formulation was dispensed from a 2 µL Eppendorf® combitip from (Eppendorf Ag, Hamburg, Germany) set to extrude approximately 7.5 mg.

The extruded formulations were evenly spread on each designated area using a glass rod. Polyvinyl protective covers were used to avoid inadvertent contamination of other test sites, accidental smearing of untreated sites or loss of the formulation from the treated site. Care was taken to avoid contact of the protective guard with the formulation. These protective covers allowed free air circulation and did not occlude the sites.

In the preliminary and pilot studies only the reference product was used in the application of the following dose durations; 0.25, 0.5, 1, 2, 4 and 6 hours. The chromameter baseline readings were taken one hour prior to formulation application of the longest duration, which was 6 hours. Staggered application with synchronized removal was used.Two untreated sites per forearm were used as controls. After the required dose durations, the adhesive tape was removed together with the templates and the drug was washed off and the forearm dried as previously described in Chapter 2. Readings were started 1 hour post removal of the formulation and continued as follows: 2, 3, 4, 6, 8, 18, 22 and 26 hours post removal.

All readings were taken in an air conditioned room of about22°C which provided a comfortable temperature. The standard overhead fluorescent lights were used as the light source in all studies. The volunteers placed their hands horizontally on the table directly in front of the observer for both visual and chromameter assessments. This position was used for the assessment of blanching for the duration of the study.

In the pivotal study, 8 sites per arm were randomized according to the following specifications:

T: Test product at the dose duration corresponding to approximately $ED_{50}$ (2 sites per arm).
R: Reference product at dose duration corresponding to approximately $ED_{50}$ (2 sites per arm).
$D_1$: The low dose calibrator, $D_1 = 0.5 \times ED_{50}$, (1 site per arm).
$D_2$: The high dose calibrator, $D_2 = 2 \times ED_{50}$, (1 site per arm).
UNT: The untreated control (2 sites per arm).

For each volunteer the application pattern on one forearm was complementary to the other as per FDA requirements[32] as shown in Figure 3.3. Readings were taken one hour prior to time of drug application to active sites and skin blanching readings at the following times after drug product application: 0, 2, 4, 6, 8, 10, 12, 18, 22, 24 and 26 hours.

| ANTECUBITAL FOSSA | |
|---|---|
| Left Arm | Right Arm |
| D1 | D2 |
| T | R |
| UNT | UNT |
| R | T |
| UNT | UNT |
| T | R |
| D2 | D1 |
| R | T |
| **WRIST** | |

**Figure 3.3 Application sequence for a particular subject**

## 3.7 Assessment Methods

### 3.7.1 Visual Assessment

Visual assessment was conducted for the preliminary study by one trained observer. The grading of the response was done using a five-point scale as described earlier (Section 2.2.1). The grading was made under a standard overhead fluorescent light with the volunteers' forearms placed horizontally on a table at about 22˚C.

### 3.7.2 Chromameter Assessment

The chromameter was calibrated at the beginning of the experiment. Two different operators assessed the blanching response chromametrically in the preliminary study. Readings of the same volunteer by one operator were made within 10 minutes of the other. In the pilot and pivotal studies the blanching response was assessed using the mounted chromameter only.

Locke's method is recommended by the FDA for the analysis of chromameter generated skin blanching data that has been corrected for baseline and untreated site responses. The

use of Locke's method for the analysis of chromameter data generates an exact confidence interval without the need to transform the data.[35]

Locke's method was utilized to calculate the exact 90% confidence interval (CI) for the pivotal study using Equations 3.8 to 3.10.

$$CI = \frac{(\frac{\overline{X}_T}{\overline{X}_R} - G\frac{\hat{\sigma}_{TR}}{\hat{\sigma}_{RR}}) \mp \frac{t}{\overline{X}_R}\sqrt{\frac{\hat{\sigma}_{RR}}{n}K}}{1-G} \qquad \text{(Equation 3.8)}$$

Where G is calculated as follows:

$$G = \frac{t^2 \hat{\sigma}_{RR}}{n\overline{X}_R^2} \qquad \text{(Equation 3.9)}$$

And K is calculated using Equation 3.10:

$$K = (\frac{\overline{X}_T}{\overline{X}_R})^2 + \frac{\hat{\sigma}_{TT}}{\hat{\sigma}_{RR}}(1-G) + \frac{\hat{\sigma}_{TR}}{\hat{\sigma}_{RR}}(G\frac{\hat{\sigma}_{TR}}{\hat{\sigma}_{RR}} - 2\frac{\overline{X}_T}{\overline{X}_R}) \qquad \text{(Equation 3.10)}$$

Where,

$\overline{X}_T, \overline{X}_R$ = sample means of test and reference

$\hat{\sigma}_{TT}, \hat{\sigma}_{RR}$ = sample variances of test and reference

$\hat{\sigma}_{TR}$ = sample covariance

t = the 95$^{th}$ percentile of the t-distribution for n-1 degrees of freedom

## 3.8 Data Analysis

Data analysis is the backbone to all studies. First the most appropriate method for collecting data must be utilized. Experimental designs range from parallel group design through to crossover study design and balanced incomplete block design.[36] In addition, an appropriate mathematical model must be selected to infer what the generated data means.

### 3.8.1 Study Designs

In parallel group design, subjects are randomly assigned to one of the 2 groups where one of the groups acts as the control. One group receives a test product whilst the other group receives a reference product. Such a design is not appropriate for most bioequivalence studies as the inter-subject variability is not accounted for. The crossover study design is more rigorous and eliminates the inter-subject variability by allowing the same subject to be tested for both the reference and the test product with a wash out period, 5 times the half life (t½), in between the experiments.[36] Each subject serves as their own control increasing the sensitivity of the method hence this design can be used with fewer subjects but with the same statistical power as the parallel group design.

Another experimental design method is the balanced incomplete block design. This is used when a number of treatments, usually more than two are required and the crossover design would take too long. The design is incomplete if the number of treatments received by each volunteer is less than the total number of treatments to be evaluated in the study.[36] If balanced, the number of subjects receiving each treatment is equal.

The study design recommended by the FDA involves a balanced complete block design hence each volunteer is its own control.

### 3.8.2 Statistical Analysis

#### 3.8.2.1 Comparison of Visual and Chromameter Methods

The selection of the appropriate statistical procedure to rank the blanching responses associated with the respective dose durations in both the visual and chromameter data was done using the statistical tree in Figure 3.4.

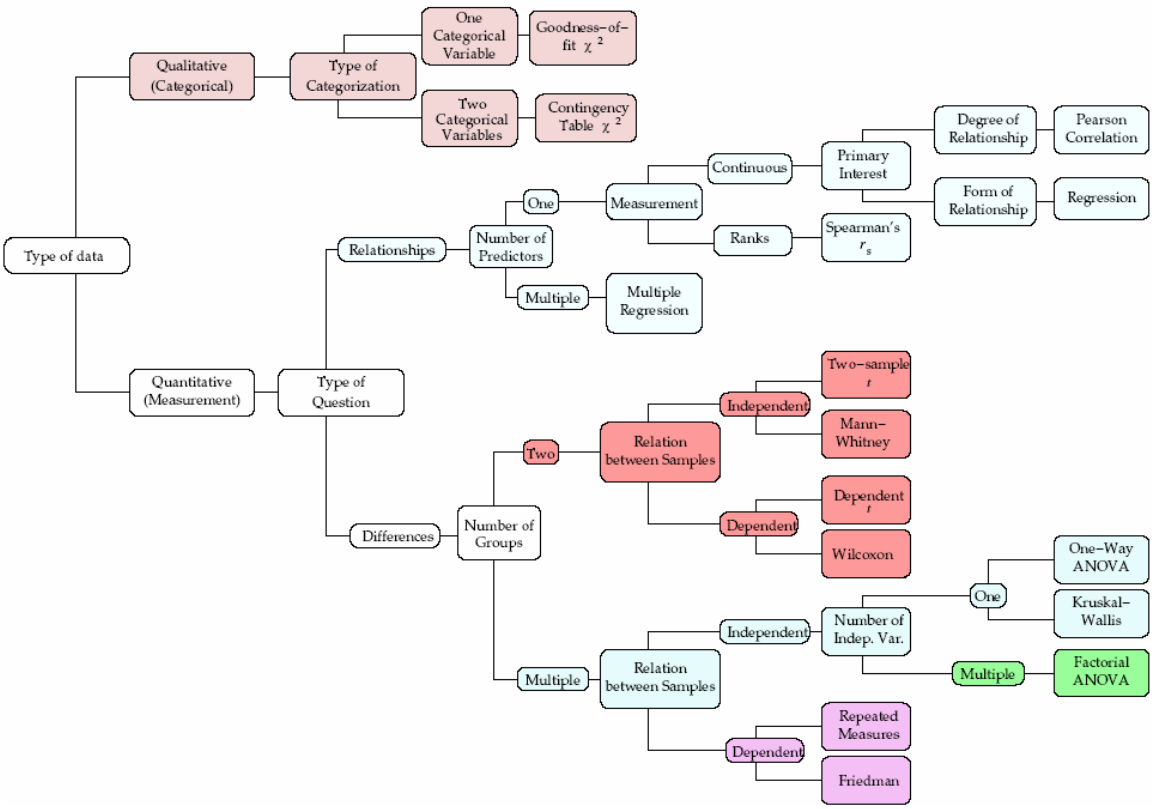



**Figure 3.4 Statistical method decision tree[83]**

Statistical analysis of data requires defined parameters for the selection of an appropriate method. One needs to know the type of data being used, quantitative or qualitative. Different statistical methods are employed depending on whether one is looking for a relationship or differences between measurements. Statistical procedures used for the comparison of two groups of data are different from those used for three or more groups. Selection of the appropriate test also depends upon the relation between the samples, whether there is a dependent or independent relation as depicted in the statistical method decision tree.

Furthermore, different statistical procedures are used for data following Gaussian as opposed to non-Gaussian distributions. However, small samples sizes do not have enough power to discriminate between Gaussian and non-Gaussian distributions.[84] Testing for deviations from Gaussian distribution (test for normality), in small sample sizes, using the Kolmogorov–Smirnov test is thus inadequate. Statistical approaches that cater for non-Gaussian data are non-parametric tests. Non-parametric tests consider the relative

ranks of the values and not the absolute values, hence the suitability of such tests in data analysis from small sample sets (n=3, preliminary study). No assumptions about the distribution of data are made hence both visual assessment and chromametric data can be assessed using non-parametric tests.

Data obtained from the preliminary study was assessed for the rank order between dose durations. It may seem that the Wilcoxon matched pair test is the best to use for data analysis. However, the Wilcoxon matched pair test is a non-parametric test that compares two paired groups. There are six groups of data corresponding to the dose duration used in this study, matched according to the time at which the readings were taken. Hence in this study, the Wilcoxon matched pair test cannot be used since more than two groups need to be concurrently assessed. An appropriate method is required to analyse all the groups at one time.

The Friedman 2-way ANOVA by ranks test is more appropriate and has been used by a number of researchers[85-88] for similar types of analyses. This test is appropriate when differences between quantitative data of more than two groups need to be analysed and in which there are dependent relationships between samples. A Friedman p-value greater than $\alpha$ (0.05) shows that there is no significant difference between the groups and any differences observed in the data are due to chance alone. If the Friedman p-value is less than 0.05 then there is at least a dose duration that significantly differs from the rest of the others. The Friedman test is followed by a *post hoc* test to pinpoint exactly where such differences lie, i.e. which dose durations are significantly different from each other.

Various other methods of statistical analysis can be used such as the chi-square test and Fisher's test.[84] However, for small samples (<100), application of the chi-square test needs to be used in conjunction with Yates' continuity correction in order obtain acceptable results.

In this research the Friedman test followed by Dunn's *post hoc* test was used for the statistical analysis to compare chromameter versus visual data.

The correlation between visual and chromameter data was assessed using the Spearman rank-order correlation, ρ. This test was chosen because other workers have reported reliable results using the same test when comparing visual to chromameter data.[28,89] Spearman's rank-order correlation is used to quantify the correlation between 2 sets of data, *A* and *B*. A correlation of zero implies that *A* and *B* do not vary together hence there is no correlation. If $0 > \rho > 1$, it means that *A* and *B* increase or decrease together whereas when $-1 > \rho > 0$ it implies that as *A* increases, *B* decreases. When $\rho = 1$, a perfect positive correlation exists but when $\rho = -1$ a perfect negative or inverse correlation exists.[84] A p-value is also calculated from the correlation. If the p-value is large (>0.05), the data dose not give a reason to conclude that the correlation is real hence the probability of correlation is insignificant. When the p-value is smaller (<0.05) the hypothesis that correlation is due to random sampling is rejected.

## 3.9 Results and Discussion

All volunteers completed the studies. No adverse events occurred during the treatment period. Figure 3.5 shows the typical blanching response observed at $T_{max}$ (8 hours) in both the preliminary and pilot studies. (Raw data of these studies and application sequences can be found in the appendices filed on the compact disc).

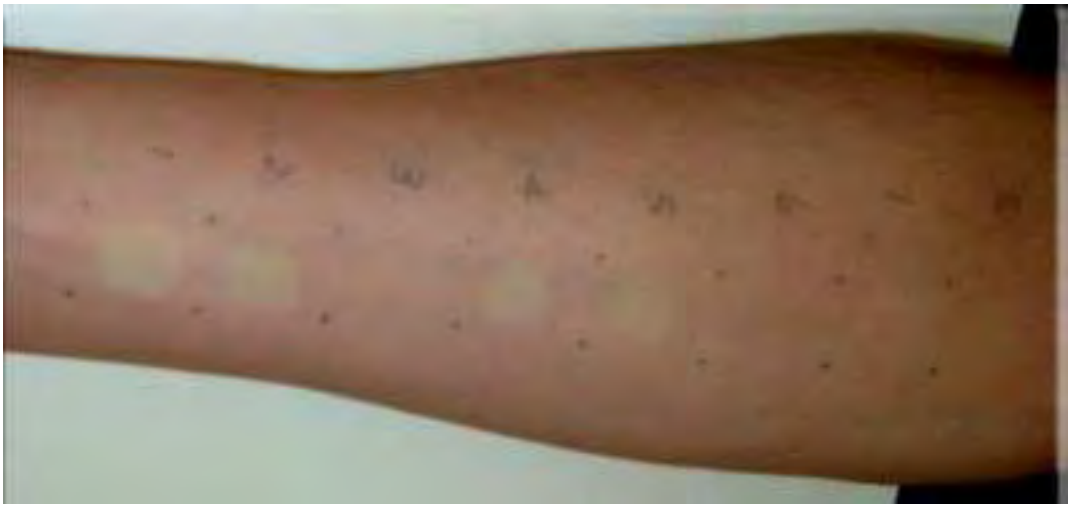
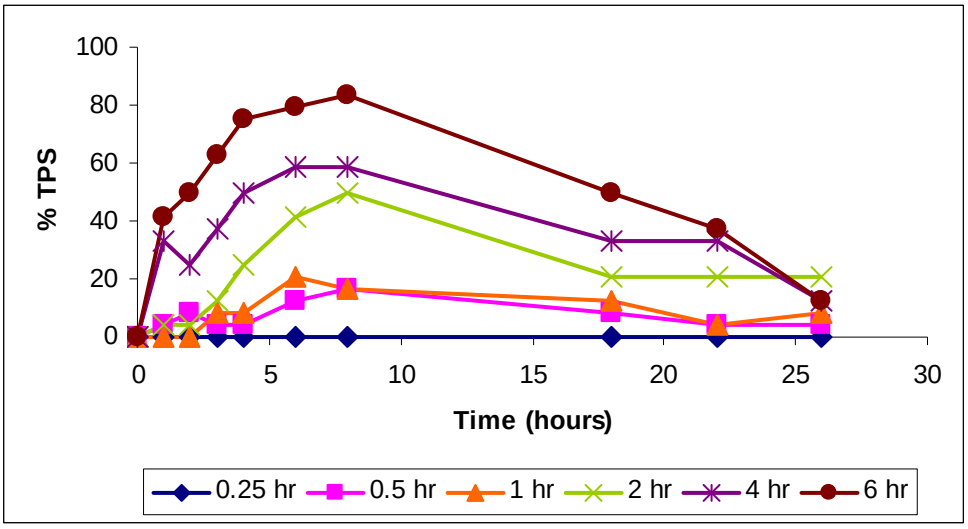


**Figure 3.5 Skin blanching at 8 hours**

### 3.9.1 Visual data analysis

Visual and chromameter (a* and EDs) generated data from the preliminary study were compared by ranking the blanching responses at the various dose durations used. Although the assessment of visual data usually necessitates at least three trained observers, it has been shown that there is no significant difference in the grading of the response to blanching between trained observers hence only one trained observer was used in this study.[28,88]

The maximum blanching response observed visually occurred at approximately 8 hours post removal of the excess topical corticosteroid formulation (Figure 3.6).




*The symbols represent the dose durations*

**Figure 3.6 %TPS versus time profile (n=3)**

The percent total possible score (%TPS) versus time profile shows that the greatest degree of blanching occurred at the longest dose duration. It was noted that no discernible skin blanching was observed at sites to which the drug formulation had been applied for only 0.25 hours. These sites show no difference to untreated sites.

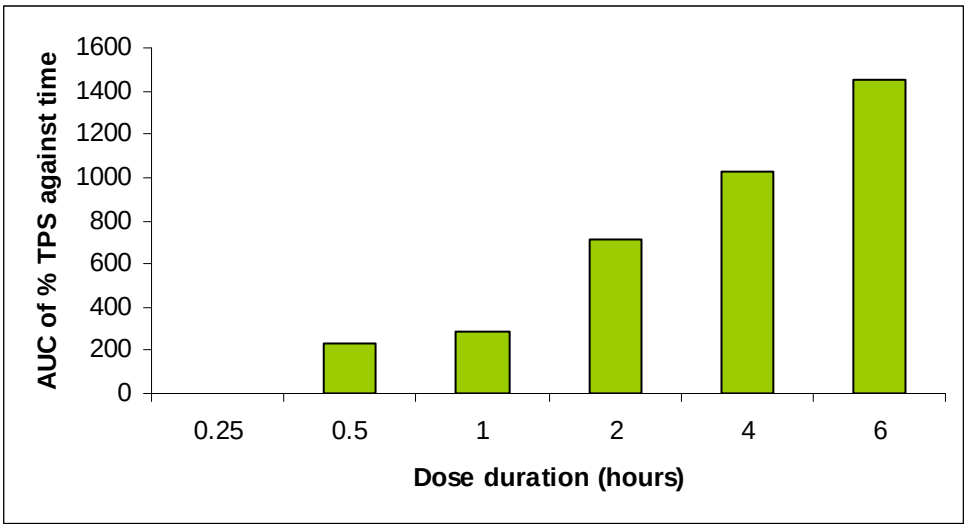



**Figure 3.7 AUC of % TPS – time profile**

AUC increases as the dose duration increases (Figure 3.7). From this graph one may conclude that the maximum blanching response was not achieved as no plateau is evident from the plot. Analyses of visual data were performed to give the rank order of the blanching responses associated with the different dose durations. Statistical analyses were performed at the 95% level of significance using the Friedman test followed by Dunn's *post hoc* test using GraphPad Prism Version 4.0 (2003) (GraphPad Software Inc., San Diego, California, USA). A Friedman statistic of 40.47 was obtained ($p<0.0001$) which indicated that the blanching response of at least one of the dose durations differed significantly from the rest. Dunn's *post hoc* tests were carried out and the results obtained are shown in Table 3.4.

**Table 3.4 Dunn's *post hoc* test – on visual data**

| Dose duration (hour) | 0.5 | 1 | 2 | 4 | 6 |
|---|---|---|---|---|---|
| 0.25 | NS | NS | S* | S*** | S*** |
| 0.5 | - | NS | NS | NS | S** |
| 1 | - | - | NS | NS | S** |
| 2 | - | - | - | NS | NS |
| 4 | - | - | - | - | NS |

*Key:* ****P < 0.001, ** P < 0.01, NS > 0.05, NS = not significant, S = significant*

The rank order of the responses at the associated dose duration could be classified as 0.25 < 2 < 4 < 6 hours. However, blanching responses at dose durations of 0.5 and 1 hour did not show significantly different results from the response observed at dose duration 0.25

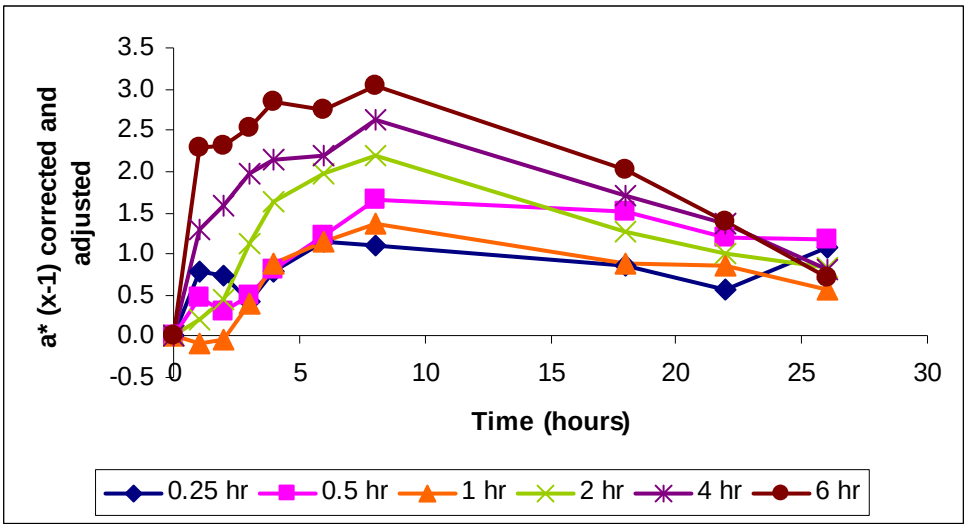
hours. Even though visual assessment was able to distinguish between the responses at the various dose durations in this study, previous studies have indicated that such differences have not always been distinguishable. In some cases 'equivalent' product formulations, according to visual assessment, were shown to be inequivalent using the chromameter.[49]

## 3.9.2 Chromameter Data Analysis

### 3.9.2.1 Effect of Blanching on a* Readings

Chromameter a* readings that were baseline and control site adjusted were used to plot Figure 3.8. Since visual measurements are made by comparing blanched skin to the surrounding untreated skin, chromameter data likewise need to be adjusted accordingly to take into account any inherent underlying contribution to the readings at each site. Comparisons between visual and chromameter data can thus be carried out only when chromameter data have been adjusted and corrected for the baseline and untreated site control. The adjustment of values to baseline ensures that only the effect of the drug is reflected in the obtained value.[2,90]

The a* scale reading versus time profile was similar to the %TPS versus time profile (Figure 3.6). However, whereas no blanching could be detected visually following a dose duration of 0.25 hours, blanching was detected with the chromameter at that dose duration. This suggests that the chromameter is better at detecting much smaller responses/changes in blanching compared to the eye. Notwithstanding, the time at which maximum blanching occurs, $T_{max}$, was 8 hours in both cases.




*The symbols represent the dose durations*

**Figure 3.8 Average a*(x-1) adjusted and corrected**

Significant differences were observed between the six groups of data (Friedman statistic = 34.78, $p < 0.0001$). Dunn's *post hoc* test results are shown in Table 3.5

**Table 3.5 Dunn's *post hoc* test - a* scale readings**

| Dose duration (hour) | 0.5 | 1 | 2 | 4 | 6 |
|---|---|---|---|---|---|
| 0.25 | NS | NS | NS | S* | S*** |
| 0.5 | - | NS | NS | NS | NS |
| 1 | - | - | NS | S** | S*** |
| 2 | - | - | - | NS | NS |
| 4 | - | - | - | - | NS |

*Key* *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, NS > 0.05, NS = not significant, S = significant*

Significant differences were observed between responses at 0.25 hours compared to those at dose durations of 4 and 6 hours. No significant differences in responses were observed between 0.5 hours and at the other dose durations. However, the blanching responses at dose durations of 4 and 6 hours showed significant differences compared to those seen at dose duration of 1 hour. These anomalies indicated that Friedman's test could not be used to compare the various methods (visual and chromameter). Further attempts were made in order to establish whether a correlation exists between the two methods. Spearman's rank-order correlation was therefore used to assess the correlation between visual data and a* readings and EDs (*vide infra* Section 3.9.3).

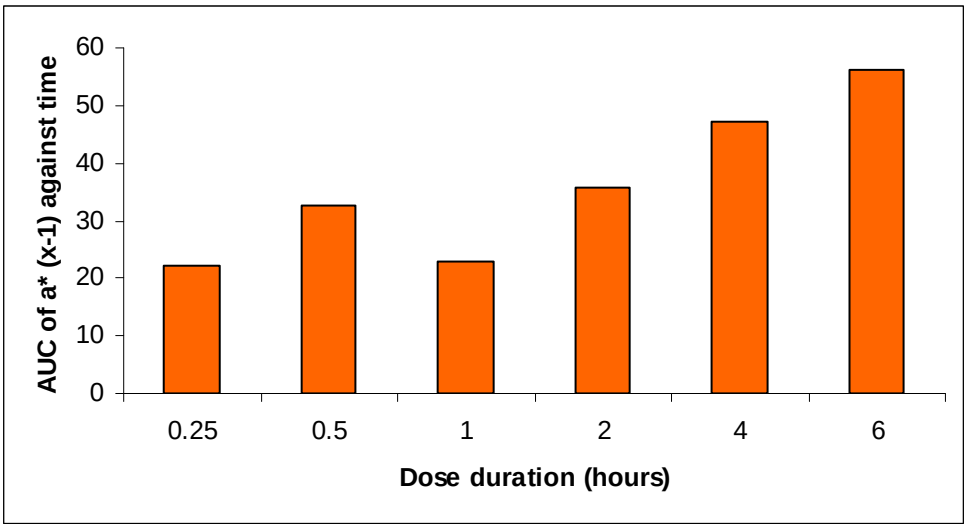


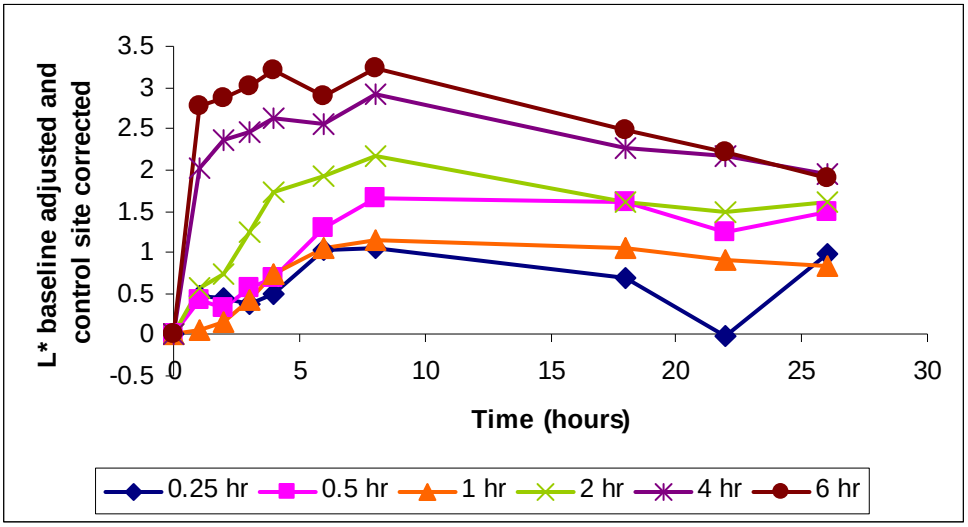

**Figure 3.9 $AUC_{0\text{-}26}$ of a*(x-1) adjusted and corrected**

Area under the curve is the calculation of total amount of drug that passes into the skin. As shown in Figure 3.9 above, the greater the exposure of the drug to the skin, the greater the penetration that occurs. AUC at dose duration 0.5 hours was greater than AUC at dose duration 1 hour. No differences in AUCs were observed between dose durations of 0.25 and 1 hour. In view of large inter-individual variability in the blanching response, the FDA recommends that a pilot study be done in order to establish the optimum conditions for a subsequent pivotal study. Since dose-response varies from individual to individual, the data obtained from a pilot study is pooled and used to approximate a population's dose-response curve. To further reduce this inter-subject variability a subset of volunteer data is selected for analysis in a pivotal study depending on whether it adheres to the set criteria (Section 3.4.2).

### 3.9.2.2 Effect of Blanching on L* and b* Readings

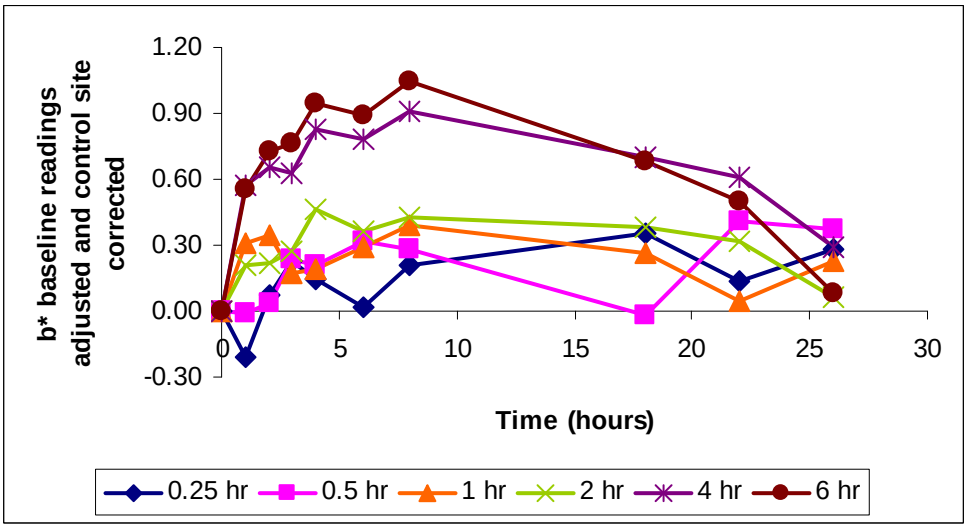
It was also observed that both L* and b* readings change with time in a blanching study (Figure 3.10 and 3.11). L* and b* readings increase to a maximum and fall off after peaking at $T_{max}$ = 8 hours. Since all the components of the CIE L*, a*, b* system are affected by blanching, this suggests that the use of a* readings only in skin blanching assessment is questionable. Hence L*, a* and b* readings should all be used to fully describe the colour change as each of them contributes in the quantification of colour.

The results obtained show that only using a* readings, will not give a true indication of the change in skin colour at the application site.




*The symbols represent the dose durations*

**Figure 3.10 Averaged L* scale results (n=3)**




*The symbols represent the dose durations*

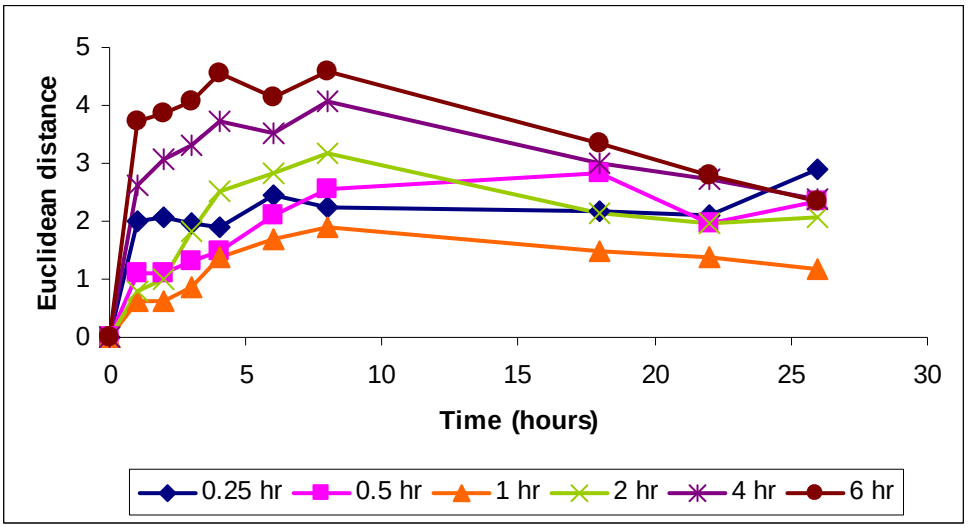
**Figure 3.11 Averaged b* scale results (n=3)**

Changes of L* and b* readings in response to skin blanching have also been observed,[91] yet they are not included in the skin colour assessment. The use of all three indices in the formula cannot be overstated; the use gives a true representation of skin colour due to blanching.

### 3.9.2.3 Effect of Blanching on Euclidean Distances

It has been shown that ED plots show greater similarity to the visually obtained blanching profiles in contrast to the individual profiles of the L*, a* and b* values.[1] The 3-dimensional colour specification technique closely resembles the sensitivity of the human eye to colour[44] hence the similarity of ED plots to visual profiles.

Like both a* and visual data, skin blanching increases to a maximum in 8 hours and then declines with time in the ED-time plot as depicted in Figure 3.12.




*The symbols represent the dose durations*

**Figure 3.12 Average Euclidean distances results (n=3)**

Analyses using the Friedman test (95% level of significance) were performed to give the rank order of the different blanching responses associated with the respective dose durations. Significant differences were observed between the groups (Friedman statistic = 38.40, $p < 0.0001$).

**Table 3.6 Dunn's *post hoc* test results - Euclidean distances**

| Dose duration (hour) | 0.5 | 1 | 2 | 4 | 6 |
|---|---|---|---|---|---|
| 0.25 | NS | NS | NS | NS | NS |
| 0.5 | - | NS | NS | NS | S* |
| 1 | - | - | NS | S*** | S*** |
| 2 | - | - | - | NS | S* |
| 4 | - | - | - | - | NS |

*Key* *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, NS > 0.05, NS = not significant, S = significant*

The results obtained show no discrimination between blanching at dose durations of 0.25, 0.5, 1.0 and 2 hours. Significant differences in response were observed at the 6 hour dose duration compared to the responses at 0.5, 1 and 2 hour dose durations.

The AUC of the ED versus time profile followed the typical blanching response that has been observed with visual blanching. Lower dose durations have correspondingly lower AUC values compared to the higher dose durations.

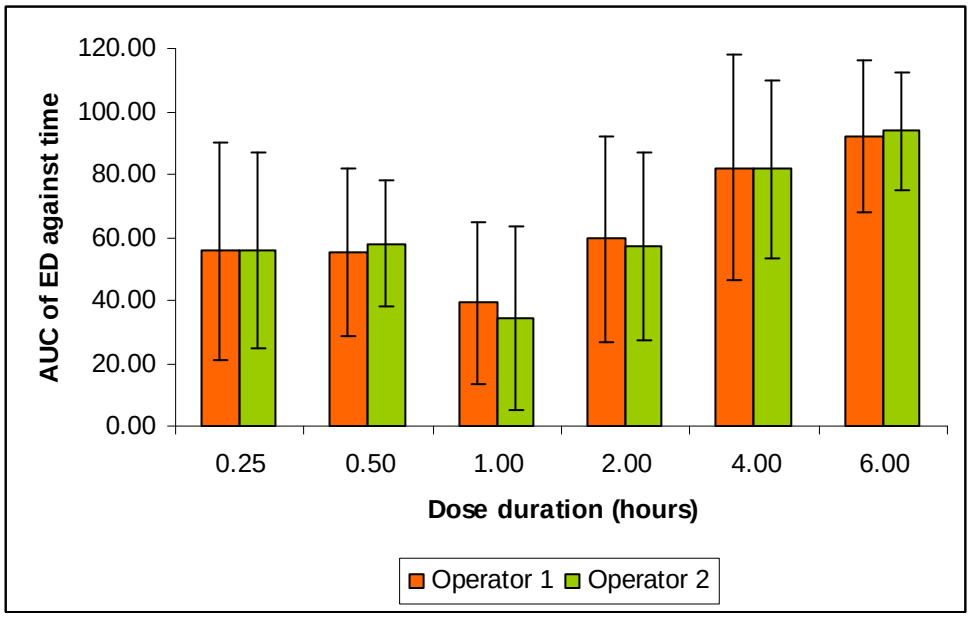



**Figure 3.13 Inter-operator variability (n=3)**

It is seen (Figure 3.13) that no significant differences were observed between the operators of the mounted chromameter. Large variability was typical in this study.

### 3.9.3 Comparison of Visual Assessment to a* Readings and Euclidean Distances

As previously mentioned, the drawback of statistical methods such as the Friedman ANOVA based method in the assessment of dose-response profiles is that each point is tested independently of the others.[92] Such methods result in a myriad of comparisons making it difficult to obtain a clear and meaningful outcome.

The AUC at the different dose durations is tabulated in Table 3.7. The results obtained showed that there was a significant correlation between a* readings and visual data, ($\rho$ = 0.8986, p-value = 0.0333). The Spearman rank-order correlation ($\rho$) between visual and EDs ($\rho$ = 0.8286, p-value = 0.0583) was close to 1. However, a larger sample size is required to confirm such a correlation if it truly exists.

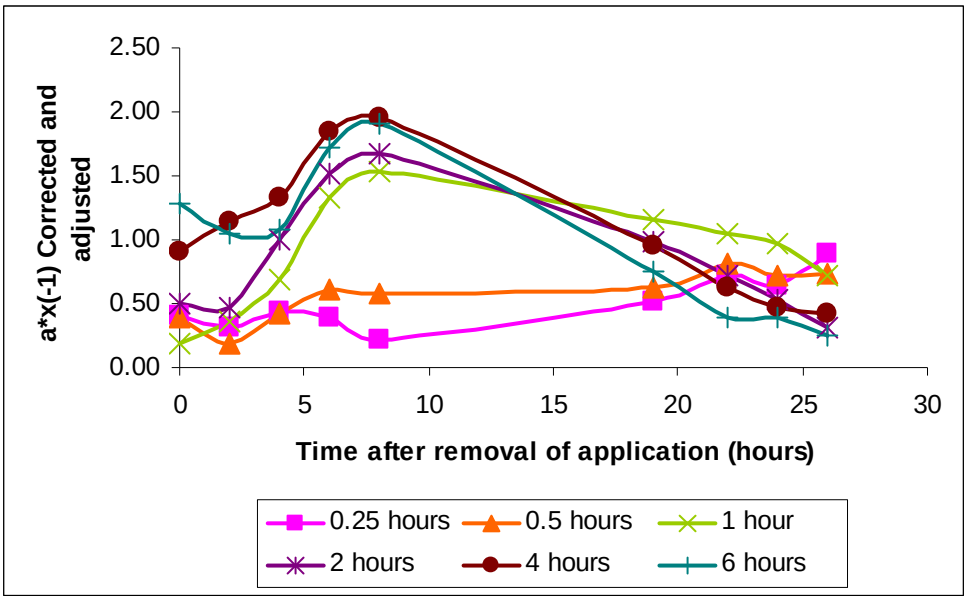
**Table 3.7 AUC at different dose durations – visual and chromameter**

| Dose duration (hour) | Visual assessment | Chromameter | |
|---|---|---|---|
| | | ED | a* scale |
| 0.25 | 1 | 57 | 22 |
| 0.5 | 16 | 58 | 33 |
| 1 | 247 | 37 | 22 |
| 2 | 652 | 60 | 36 |
| 4 | 977 | 83 | 47 |
| 6 | 1310 | 94 | 56 |

Based on these results, subsequent data analysis was performed using a* readings only.

### 3.9.4 Pilot Study

The pilot study blanching-profile was found to be comparable to that obtained visually in the preliminary study. Due to the variability of the human skin blanching data, standard deviation bars were omitted in blanching profiles for clarity sake.




*The symbols represent the dose durations*

**Figure 3.14 Pilot study blanching profile, a* scale**

It can be observed from Figure 3.14 that the curves at 0.25 hours and 0.5 hours fall in a region were the response to blanching is small. At a dose duration of 1 hour the response increases to a peak which is almost the same as that of 2 hours. Increases in response after 1 hour are smaller. From this data it can thus be inferred that the most sensitive part of the graph is between 0.25 and 1 hour. The areas under the curves shown in Figure 3.14 was calculated and used to determine the $ED_{50}$ and subsequently $D_1$ and $D_2$ from the $E_{max}$ model (Figure 3.15).

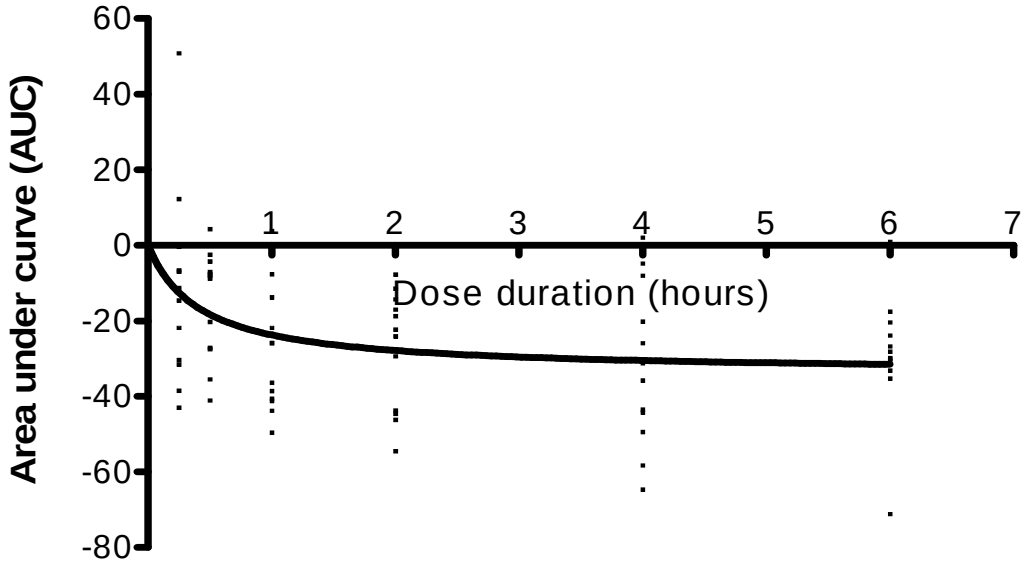



**Figure 3.15 $E_{max}$ model for MF reference product**

GraphPad Prism was used to analyse the results obtained from the pilot study on MF using a one site binding equation which describes binding to a receptor that follows the law of mass action. The best fit values for $E_{max}$ and $ED_{50}$ were -33.73 and 0.4226 hours respectively.

$ED_{50}$ was rounded up to 30 minutes, $D_1$ and $D_2$ used were thus 15 minutes and 1 hour respectively.

From the pilot study 50% of the responders met the detector criterion. As previously discussed, a responder is a subject who shows a response to a single dose duration of the reference drug under the conditions used in the pilot study. The same conditions used in the pilot study are to be used for the pivotal study when testing the test drug against the reference. The FDA requires the use of 40 to 60 detectors per assessment of the bioequivalence. Detectors are subjects whose AUC values at $D_1$ and $D_2$ are both negative. In addition the ratio of the AUC at $D_2$ to that at $D_1$ should be greater or equal to 1.25.This implies that pivotal studies stemming from the described pilot study must have about 80–120 volunteers. The use of such large numbers of volunteers is prohibitive cost-wise hence in this research project only 24 volunteers were used.

### 3.9.5 Pivotal Study

Declaration of bioequivalence requires that the test product meets the acceptance criteria stipulated by the FDA and most other regulatory authorities/agencies. Generally, this is so when the means of the AUC ratios (T/R) fall within the bioequivalence limits of 80 to 125% at a 90% confidence interval. However, for topical corticosteroids the FDA recognizes that wider limits may be necessary,[32] but does not state these limits. The declaration of bioequivalence thus is left to the agency that evaluates the data submitted. In South Africa, the Medicines Control Council (MCC) accepts the registration of generic topical corticosteroids if they do not differ by more than 20% from the innovator product based on visual assessment.[1]

Before calculation of the 90% confidence interval using Locke's method, a parameter G calculated as shown in Equation 3.9 must be less than 1. This parameter is not defined in the guidance except that if G $\geq$1, the study does not meet the bioequivalence requirements.[32] From this statement it can be assumed that if G < 1 then the test product is bioequivalent to the reference. The confidence interval is only calculated when the *in vivo* bioequivalence requirements are met. The 'G' parameter decreases with an increase in the number of evaluable volunteers in the study.

As previously mentioned, the FDA recommends the use of 40 to 60 evaluable volunteers (i.e. detectors), which may be inferred to be the number of subjects needed to provide adequate statistical power. Hence data from a smaller number of volunteers such as used in this project cannot unequivocably be used to declare bioequivalence.[35] Using a small sample set results in very wide confidence intervals such that the bioequivalence of the formulations cannot be readily demonstrated.[93]

From the 24 subjects incorporated into the pivotal study, only seven evaluable subjects were obtained. Replication of the data of the seven evaluable subjects to simulate larger cohorts of detectors based on the same data obtained from the seven were made in an attempt to predict the assessment using adequate numbers as required by the FDA (Table 3.8).

**Table 3.8 Pharmacokinetic data obtained from evaluable volunteers (n=7)**

| Number of iterations | G | Mean T/R (%) | Calculated Limits (%) |
|---|---|---|---|
| 7 | 0.7144 | 74.1 | -13.4 – 107.7 |
| 48 | 0.0710 | 74.6 | 64.2 – 83.2 |
| 120 | 0.0261 | 74.1 | 68.2 – 79.4 |

The width of the bioequivalence interval decreases with an increase in the number of volunteers, n. Using the results from only the seven evaluable volunteers resulted in a wide interval of -13.4% to 107.7%. Hence in spite of the G parameter predicting that *in vivo* bioequivalence requirements were met between the Canadian and the South African MF creams (G<1), the data fall outside the equivalence limits for the declaration of bioequivalence. Once again, it is re-emphasized that in view of the lower number of detectors, confirmation of bioequivalence or otherwise could not be confirmed.

Iterations of the results of detectors to give larger numbers advocated for by the FDA show a significantly reduced calculated limit up to the use of data of 48 detectors. Larger numbers of detectors (beyond 48) do not have a large effect on the upper and lower limits of the intervals. From the iterations (n=120) the calculated limits are 68–79%. Under statistical analysis the FDA guidance refers to the following statement "The Office of Generic Drugs has not determined at this time the equivalence interval for bioequivalence. The Office recognizes that an equivalence interval wider than 80–125%, as a public standard, may be necessary pending evaluation of data submitted to the agency".[32] On the other hand, as described in SUPAC-SS,[94] *in vitro* assessment of drug release from semisolids a confidence interval of 75–133% is used.

In comparison to the published FDA limits, the results obtained show that MF release from the test product, Elocom™ 0.1% cream (Canada) is lower than that of the reference product, Elocon® 0.1% cream (South Africa). The calculated interval of 68–79% is quite close to the 80–125% limits hence if such data is submitted to the office of the FDA it may pass the bioequivalence test.

The data obtained from all the subjects (n=24) used in the pivotal study, disregarding the findings that they did not meet the "detector" criteria, were also processed and are shown in Table 3.9.

**Table 3.9 Pharmacokinetic data obtained from all volunteers (n=24)**

| Number of iterations | G | Mean T/R (%) | Equivalence Limits (%) |
|---|---|---|---|
| 24 | 0.2118 | 86.1 | 57.7 – 121.6 |
| 48 | 0.0994 | 86.1 | 67.2 – 107.9 |
| 120 | 0.0383 | 86.1 | 74.4 – 98.8 |

A blanching profile of the pivotal study using data from seven evaluable subjects is depicted in Figure 3.16.

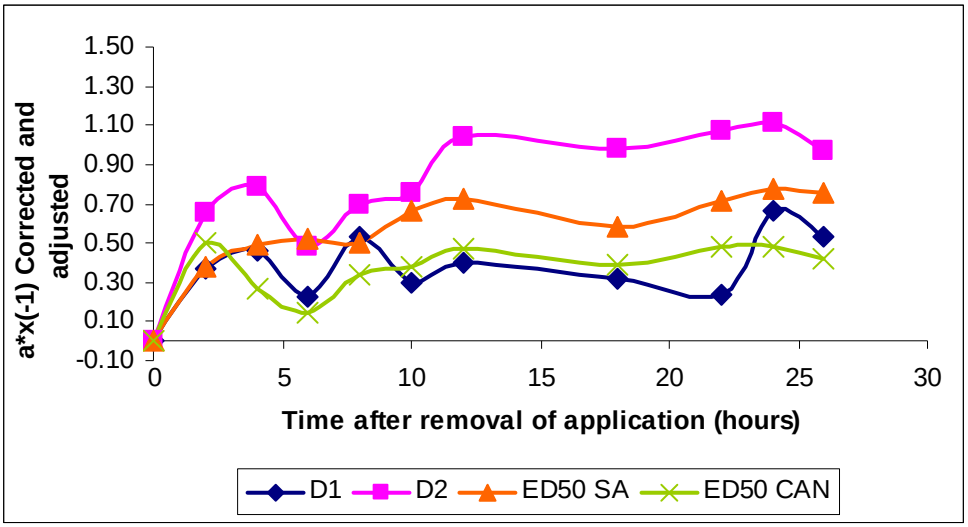



**Figure 3.16 Pivotal study – Skin blanching profile (n=7)**

The data shown in Figure 3.16 is comparable to that obtained from the pilot study. The AUC of $ED_{50}$ for the reference drug is between AUC of $D_1$ and that of $D_2$ confirming that the pivotal study was done at dose durations within the sensitive part of the graph. However, large standard deviations from the means were observed and are summarized in Table 3.10 below:

**Table 3.10 Pharmacokinetic parameters derived from area under curve of $D_1$, $D_2$ and $ED_{50}$**

| (n=7) | $D_1$ | $D_2$ | $ED_{50}$ Reference | $ED_{50}$ Test |
|---|---|---|---|---|
| Mean | -8.6 | -24.6 | -14.9 | -11.1 |
| Standard deviation | 5.1 | 21.5 | 17.1 | 15.5 |
| % RSD | -59.4 | -87.3 | -115.1 | -138.9 |

The data obtained were quite erratic. The deviations from the mean were between 59 and 138%. Large deviations are typical of the human skin blanching studies as shown in the example provided in the FDA guidance[32] and which are due to inter-subject variability. An increase in the number of evaluable subjects, therefore, is necessary in order to declare bioequivalence, if equivalence truly exists.

## 3.10 Conclusions

Using a highly precise and accurate method has been shown to increase the power of the statistical analysis.[95] Furthermore; the use of a mounted chromameter as opposed to the hand-held mode also reduces variability thereby contributing to the increased reliability of data. The extent and justification to reduce the number of subjects in such studies, however, remains to be elucidated.

Visual assessment by a trained observer showed that the blanching response at a 0.25 hour dose duration was virtually not detectable i.e. a reading of zero was observed throughout this study whereas blanching using the a* scale readings showed a curve that peaks at about 8 hours. This demonstrated the distinct advantage of using a chromameter compared to visual assessment. Clearly, the chromameter readings were found to be more sensitive to the colour changes than the eye.

Even though the blanching response based on L* and b* readings were readily measured subsequent to topical corticosteroid application, incorporation of that data into the statistical analysis as EDs did not show a significant correlation between visual and EDs. A larger number of subjects may be necessary for conclusive results since it was found that the value of $p > 0.05$ was obtained using such data. On the other hand, a* readings

showed a strong correlation with the visual data. Since visual assessment of blanching has on numerous previous occasions been shown to be reliable in the hands of experienced observers[45,96] the visual method has been considered as the yardstick with which any method to be adopted should be compared. In this study, since a* readings correlated well with the visual data, a* values were subsequently used for bioequivalence assessment by chromameter in both the pilot and pivotal studies.

From the pilot study, it was estimated that about 50% of the volunteers recruited for the pivotal study would be evaluable subjects. However, out of the 24, only seven were evaluable. This ratio implied that to get the required 40 to 60 evaluable subjects, more than 200 volunteers would be needed for the particular MF products used in this research.

Some researchers have also obtained small numbers of detectors from pivotal studies they conducted.[9,28] Both studies enrolled 40 subjects each for the pivotal studies and one obtained seven evaluable subjects whilst the other obtained 16. Definitive conclusions could not be drawn from these studies as a result of the lack of explanation of the parameters 'G' and 'K' used in Locke's method. As seen in this and other studies,[28] bioequivalence is met using the 'G' parameter alone for evaluation. However, at times the confidence interval limits generated are not in the acceptable range (80 to 125%). The use of 'G' in the determination of bioequivalence requires further investigations.

# CHAPTER 4

# ANALYTICAL HPLC METHOD DEVELOPMENT AND VALIDATION

## 4.1 Literature Review Including Background

The development of an *in vitro* method for the analysis of MF was based on previous methods found in literature. Most of the methods are based on high performance liquid chromatography (HPLC) with various detection methods. Ultraviolet light (UV) has been used as the main detection method by most researchers whilst mass spectrometry has also been successfully applied. Other methods used, which include spectrophotometry,[97] competitive enzyme immunoassay[98] and a TLC densiometric method,[99] have been described for determination of MF. TLC methods have been used for purity determination and qualitative identification of MF.[11,97] Table 4.1 summarizes the methods and conditions for the analysis of MF as published in literature.

## 4.2 High Performance Liquid Chromatography (HPLC)

The emergence of liquid chromatography as an alternative to gas chromatography in the 1960s provided a rapid separation method. Greater potential was realised with liquid chromatography in terms of column efficiency over gas chromatography.[100,101] Consequently, liquid chromatography gained favour over other methods used and has now become the main analytical technique for the analysis of a large variety of compounds. Liquid chromatography has been referred to as high speed (HSLC), high efficiency (HELC) and high performance liquid chromatography (HPLC) due to its attributes. HPLC is now the generally accepted term. The HPLC column can be re-used without need for regeneration of the column. HPLC provides great resolution and is less operator-dependent, hence reproducibility is increased. In addition, the sample can be readily recovered after use in HPLC.[100] HPLC has been widely applied to an enormous

variety of compounds and has the capability to effect separation with great speed, sensitivity and precision.[101,102]

**Table 4.1 Review of the analytical methods used for the determination of MF**

| Column | Matrix | Mobile phase composition | Detection | Reference |
|---|---|---|---|---|
| Supelco LC-8<br>250 × 4.6 mm | API | methanol/water (35/65)<br>1 ml/min | Radioactivity,<br>UV 254 nm | [103] |
| Partisil 10<br>Normal phase<br>a)250 × 4.6 mm<br>b)500 × 9.4 mm | API | dichloromethane-methyl/tert.-butyl ether (93/7)<br>a)1 ml/min<br>b)4 ml/min | Radioactivity,<br>UV 254 nm | [103] |
| Symmetry RP-18<br>150 × 4.6 mm | plasma | methanol/water (65/35)<br>1 ml/min | UV 254 nm | [104] |
| Luna $C_{18}$<br>50 × 2.1 mm | API | 0.1% formic acid (v/v) in water and 0.1% formic acid (v/v) in acetonitrile, (gradient elution) | MS/MRM-positive ion mode, source temperature 550 ˚C | [105] |
| Ultrasphere octyl<br>150 × 4.6 mm | biological fluids | methanol/water (59/41)<br>1.5 ml/min | UV 248 nm | [106] |
| Ultrasphere<br>750 × 4.6 mm | API | 10 mM sodium phosphate buffer (pH 6.5)/methanol (41/59) | UV 247 nm | [107] |
| Octylsilane<br>250 × 4.6 mm | API | methanol/water (65/35)<br>1.7 ml/min | UV 254 nm | [11] |
| LC-18-DB<br>33 × 4.6 mm | plasma | methanol/ammonium acetate 25 mM (80/20)<br>1 ml/min | APCI/MS/MS (positive ion mode) | [108] |
| Symmetry $C_{18}$<br>150 × 4.6 mm | plasma | 0.2% acetic acid (v/v) in water/ acetonitrile in water from 60/40 to 29/71 (water/acetonitrile) in gradient elution,<br>1 ml/min | UV 254 nm | [109] |

HPLC is an analytical procedure where components of a complex mixture can be separated, identified and quantitatively determined. The separation of sample constituents is facilitated by differences in the partition coefficients of solutes between a stationary and a mobile phase. In HPLC, the sample is injected onto the column (stationary phase) whilst mobile phase is permeating through the column. The average rate at which a solute migrates depends upon the average time it spends in the mobile phase. This rate will be less for solutes with partition ratios that favour retention on the stationary phase, and *vice versa* for solutes with partition ratios that favour partition in the mobile phase. Ideally,

the differences in affinity of solutes between stationary and mobile phases cause the components in a sample mixture to separate into separate bands located along the length of the column, which then move along and out of the column with the mobile phase.

The object of HPLC is thus to separate sample components within a reasonable time. This separation depends on three main factors: column efficiency, column selectivity and retention.

### 4.2.1 Column Efficiency

The plate theory, the original theory of chromatography, describes migration rates in quantitative terms. This theory envisages the column to be composed of a series of narrow discrete theoretical plates, the movement of the solvent and solute then being viewed as a series of stepwise transfers from each step to the next. At each step, the equilibration of the sample between the mobile and stationary phase is assumed to take place. The efficiency of a chromatographic column as a separation device can then be said to improve as the number of equilibrations or "steps" increase. Thus, the number of theoretical plates, N, is used as a measure of column efficiency. A second term, the height equivalent of a theoretical plate, H, also serves this purpose. The relationship between these two parameters is described by Equation 4.1.

$$N = \frac{L}{H} \qquad \text{(Equation 4.1)}$$

Where,

L = Length of column packing (cm)

N = Number of theoretical plates

H = Height equivalent of theoretical plate (cm)

The use of the plate theory was limited as it failed to describe the effects of numerous variables responsible for zone broadening. This theory was then replaced by the kinetic or rate theory, which is capable of accounting for these variables; however N and H are still used as criteria for the description of column efficiency. N can be experimentally evaluated from a chromatogram by the substitution into Equation 4.2 or 4.3 of the various

parameters depicted in Figure 4.1. The theoretical plate number (N) should ideally be greater than 2000.

$$N = 16(\frac{t_R}{W})^2 \text{ Or} \qquad \text{(Equation 4.2)}$$

$$N = 5.54(\frac{t_R}{W_{h/2}}) \qquad \text{(Equations 4.3)}$$

Where,

$t_R$ = Retention time of solute

W = Width of peak at base

$W_{h/2}$ = Peak width at half –height

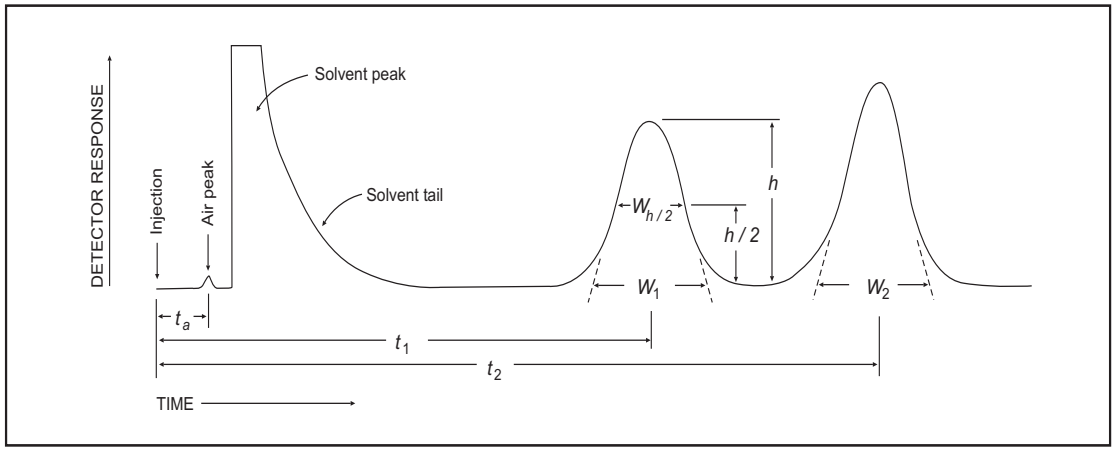



**Figure 4.1 Chromatographic separation of two substances[11]**

## 4.2.2 Capacity Factor

The capacity factor (k') is used to describe the migration rate of the solute. The capacity factor simply describes where the solute peak of interest elutes relative to the solvent front or void volume. Equation 4.4 illustrates how k' can be derived from a chromatogram.

$$k' = \frac{t_R - t_M}{t_M} \qquad \text{(Equation 4.4)}$$

Where,

$t_R$ = time taken before elution by substances not retained on column (dead time)

$t_M$ = retention time of solute

Ideal capacity factors range between 1 and 5. Capacity factors less than unity result in rapid elution and those greater than 20 give long retention times.[101]

### 4.2.3 Selectivity

The selectivity factor ($\alpha$), is a measure of how well the column separates two solutes. It is defined as:

$$\alpha = \frac{k'_B}{k'_A} \qquad \text{(Equation 4.5)}$$

Where,

$k'_B$ = capacity factor for the strongly retained species

$k'_A$ = capacity factor for the more rapidly eluting solute

The selectivity factor, α is therefore always greater than unity. There are two primary ways to improve resolution as illustrated in Figure 4.2. The first is by decreasing peak width (zone width) whilst maintaining the zone centre constant, and the second by increasing the distance between the zone centres between the two peaks, whilst holding the peak width constant. The first method involves the efficiency of the column, and the second, the selectivity.

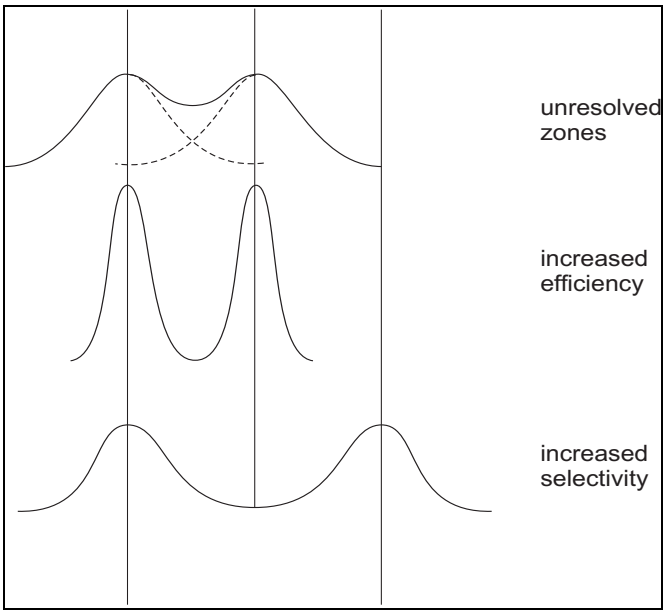



**Figure 4.2 Illustration of the difference between increasing efficiency and increasing selectivity[11]**

### 4.2.4 Resolution

The ability of a column to resolve any two peaks of interest is of primary importance in HPLC. Resolution ($R_s$) is dependent upon both selectivity and efficiency. Resolution can be calculated from Equation 4.6.

$$R_s = (\frac{\sqrt{N}}{4})(\frac{\alpha - 1}{\alpha})(\frac{k'_B}{1 + k'_B}) \qquad \text{(Equation 4.6)}$$

Resolution less than 1.5 means that there is incomplete separation of peaks.[101]

### 4.2.5 Liquid Chromatography Modes

Liquid chromatography has several operational modes (Table 4.2). The liquid chromatography modes use a liquid mobile phase and differ from each other by the stationary phases employed.

**Table 4.2 Different liquid chromatography modes, stationary phases employed and the potential analyte to which the method is applicable**[100,101]

| Liquid chromatography mode | Stationary phase | Analyte to which method is applicable |
|---|---|---|
| Liquid – liquid | Solvent adsorbed on packing material | MW<10000, non-ionic, polar |
| Liquid – bonded phase | Solvent covalently bonded to packing material | MW<10000, non-ionic, polar |
| Liquid – solid | Solid particles | MW<10000, non-polar |
| Ion exchange | Ion exchange resin | MW<10000, ionic |
| Gel permeation | Liquid in polymeric solid | MW>10000, non-polar |
| Gel filtration | Liquid in polymeric solid | MW>10000, polar or ionic |

The major disadvantage of using liquid-liquid chromatography is the lack of stability when compared to liquid bonded phase chromatography.[101] The stability and versatility of liquid phase bonded chromatography has resulted in its use in several different fields - clinical medicine, forensics, food industry and in the pharmaceutical industry. Liquid bonded phase chromatography can be divided into two further operational modes; normal phase and reversed phase.

The above differ from each other based on the polarity of the stationary and mobile phases. The stationary phase in NP-HPLC is polar and the mobile phase is relatively non-polar and is used to separate polar compounds which are preferably retained on the polar stationary phase. The use of NP-HPLC for the analysis of topical corticosteroids would result in ultra-short retention times. Topical corticosteroids are relatively non-polar due to the fused ring system which is beneficial for local application. Hence such corticosteroids would not be retained on a polar stationary phase making separation of the relevant corticosteroid drug difficult. RP-HPLC stationary phase is non-polar and relatively polar solvents are used as the mobile phase. Corticosteroids are retained on the stationary phase of RP-HPLC and by changing the composition of the mobile phase, chromatography of the corticosteroid drug can be readily achieved. The retention time can also be readily adjusted by changing the composition mobile phase.

Reverse phase liquid chromatography was chosen as it appeared to be the method of choice as gleaned from the reviewed literature.[11,103-109] RP-HPLC is more rugged and

provides better separation than other liquid chromatography methods. Furthermore, the solvents employed in RP-HPLC are generally compatible with UV detectors.

## 4.3 Methods

### 4.3.1 Method Development

#### 4.3.1.1 Reagents and Chemicals

Mometasone furoate (MF) and clobetasol 17-propionate (CP) were obtained from Symbiotec Pharmalab (Indore, India). HPLC grade acetonitrile 200 UV ROMIL - SpS™ Super Purity Solvent was obtained from Romil Ltd (Waterbeach, Cambridge, UK). The water used for chromatography was initially purified by reverse osmosis followed by filtration through a Milli-Q system (Millipore, Bedford, MA, USA). The water purification system consisted of a Milli-Q® Academic A10 with a Quantum™ EX Ultrapure Organex Cartridge equipped with Q-Gard® 1 Progard pre-treatment packs.

#### 4.3.1.2 Instrumentation and Chromatographic Conditions

All experiments were performed using a Waters Alliance HPLC system equipped with a separation module (Model 2690), a photodiode (PDA) detector (Model 2996), an online degasser, and an auto-sampler (Waters Corporation, Milford, MA, USA). The column temperature was maintained at 25 ± 1 ˚C and the injection volume was 10 µl.

#### 4.3.1.3 Preparation of Standards Solutions

Standard methanolic stock solutions (1 mg/ml) were made by accurately dissolving about 20 mg of MF and CP in 20 ml volumetric flasks using a top loading analytical balance (Mettler Model AE163, Zurich, Switzerland). Serial dilutions of the relevant stock solutions were made to prepare seven concentrations over the concentration range of 0.2–15 µg/ml. Stock solutions and serial dilutions thereof were sonicated prior to use using a Branson B12 sonicator (Shelton, Connecticut, USA). These solutions were prepared on

three different days for use in linearity, precision and accuracy experiments and further to assess the limits of detection and quantitation.

#### 4.3.1.4 Mobile Phase Preparation

The mobile phase consisted of acetonitrile and HPLC - grade water (46/54 v/v). Prior to use, the mobile phase was filtered under reduced pressure through a 0.45 µm Durapore (PVDF) filter (Millipore, Bedford, MA, USA) and degassed using an Eyela Aspirator A-2S (Tokyo Rikakikai Co. Ltd, Tokyo, Japan).

#### 4.3.1.5 Column Selection

The solvents used in RP-HPLC are generally polar, water-miscible organic solvents and percolate over the stationary phase which allows separation of a mixture of compounds into its various components. The molecular structure determines the chromatographic behaviour of the analyte molecules.

MF is a lipophilic (log P = 4.725) basic (pKa of 13.08)[10] compound with a chemical structure consisting of a fused ring system which imparts the drug's hydrophobic nature (refer to Figure 1.1 in Chapter 1) and thus poor water solubility. The lipophilicity of MF facilitates retention on reversed-phase material due to preferential partitioning of MF from the predominantly hydrophilic mobile phase onto the hydrophobic stationary phase surface.

A reversed phase Luna $C_8$ (2) (Phenomenex, Macclesfield, Cheshire, England) column was used for the HPLC analysis of MF. The column contains 5 µm particles with a pore diameter of 100 Å, an internal diameter of 2 mm and a length of 150 mm.

#### 4.3.1.6 Mobile Phase Selection

Typical mobile phases used in RP-HPLC are water with either/or acetonitrile and/or methanol and other alcohols, and organic modifiers such as tetrahydrofuran, dioxane and

acetone,[110] amongst others. The choice of mobile phase was based, initially, on the separation of MF and clobetasol 17-propionate (CP) as illustrated in Figure 4.4 using CP as internal standard. CP has comparable characteristics to MF and the elution of MF and CP is thus conveniently similar. The mobile phase can be manipulated to give relatively short retention times that are economically advantageous as use of expensive HPLC-grade solvents is minimized.

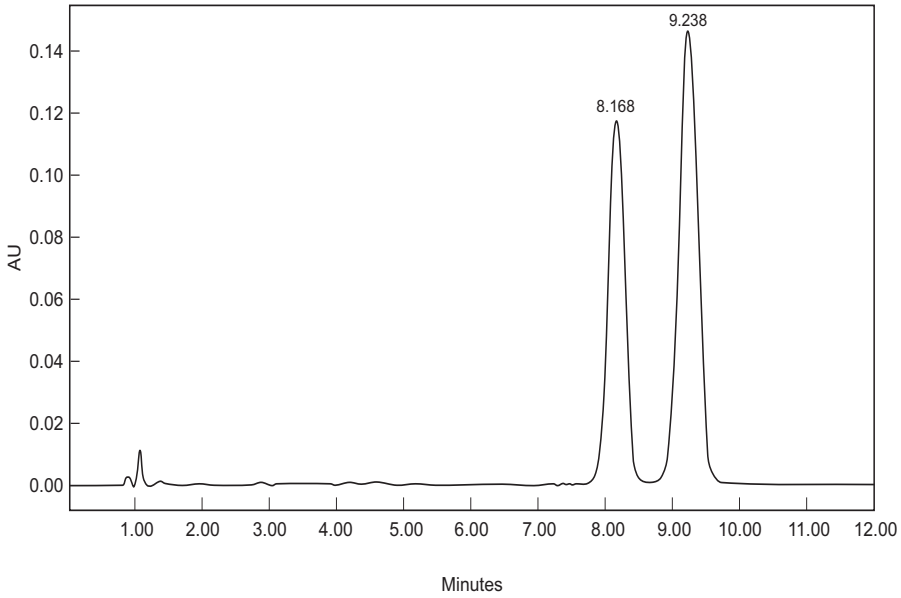



**Figure 4.3 Chromatogram of MF ($t_R$ ~ 9.2 min) and CP ($t_R$ ~ 8.2 min) at 238 nm**

A mobile phase composition of acetonitrile/water (45/55) yielded no separation as shown in Figure 4.4. Increasing the acetonitrile content of the mobile phase resulted in slightly longer retention times. However at a mobile phase composition of 48/52 acetonitrile/water, the retention times increased to about 10 and 12 minutes for CP and MF, respectively which was considered too long. Above 48/52 acetonitrile/water the retention times decrease with a loss in resolution. Adequate separation was thus achieved at a mobile phase mixture of 46/54 acetonitrile/water which resulted in acceptable retention times of 8.2 and 9.2 minutes for CP and MF, respectively.

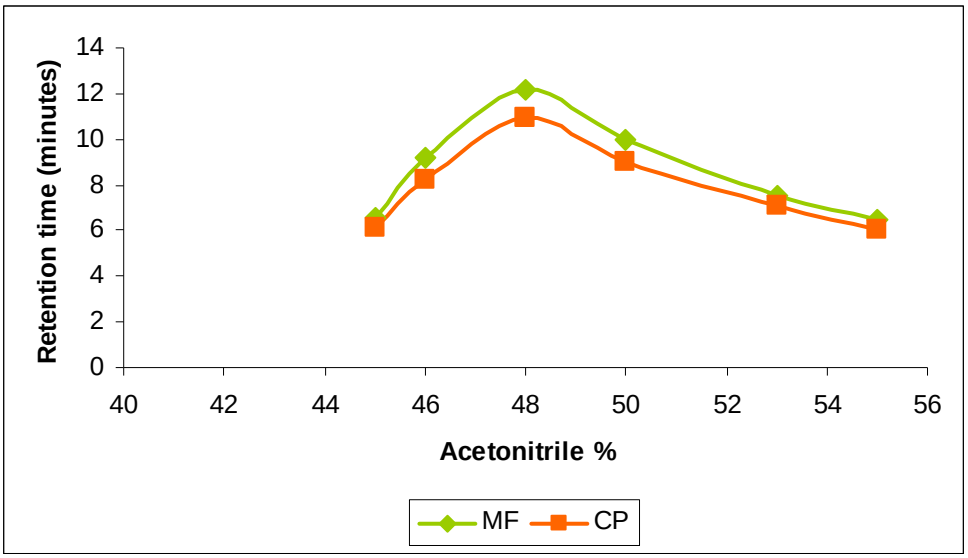



**Figure 4.4 Retention times versus percent acetonitrile in mobile phase**

### 4.3.1.7 Detection Method

A PDA detector was used in view of its scanning facility and associated advantages.[111] The PDA detector permitted the simultaneous collection of chromatographs at different wavelengths with spectral scanning between 200 and 400 nm. The chromatograms at the different wavelengths were then analysed in order to optimize the detection wavelength required to provide the best peak shape and requisite sensitivity. HPLC analysis of the samples was performed at 238 nm where MF and CP showed optimum absorption.

#### 4.3.1.8 Chromatographic Conditions

**Table 4.3 Chromatographic conditions**

| | |
|---|---|
| **Column** | Phenomenex ® Luna $C_8$ (2) column<br>Length 150 mm<br>Internal diameter 2 mm<br>Particle size 5 µm<br>Pore diameter 100 Å |
| **Detector** | Waters 2996 PDA |
| **Pump and injector** | Waters 2690 Separations Module, Alliance |
| **Recorder** | Waters Empower™ Software |
| **Wavelength** | 238 nm |
| **Flow rate** | 0.5 ml/min |
| **Injection volume** | 10 µl |
| **Temperature** | 25 ± 1 ˚C |
| **Mobile phase** | Acetonitrile: Water (46: 54) |
| **MF retention time** | ca 9.2 minutes |
| **CP retention time** | ca 8.2 minutes |
| **Column pressure** | 2500 psi |

### 4.3.2 Stability Studies

The stability of MF in 70/30 PG/water and methanol was assessed one month after storage at 4˚C in a refrigerator and protected from light. Drug samples initially containing between 1.5 and 18 µg/ml of MF were used in this assessment.

## 4.4 Results and Discussion

### 4.4.1 Assay Validation

Assay validation is the process that establishes that the performance characteristics of the method meet the requirements for the intended analytical application. Validation provides the yardstick from which results obtained by the use of that method are compared. These results play a essential role in the evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetic data.[112]

Typical characteristics used in method validation are accuracy, precision, specificity, detection limit, quantitation limit, linearity and range according to compendial methods.[11]

In addition, ruggedness and robustness are specified according to the Federal Food, Drug and Cosmetic Act.[15]

### 4.4.2 Accuracy and Bias

Accuracy is the closeness of the obtained results to the true value[112] and should be established across the whole range. Bias is the difference between the calculated value determined for the analyte of interest and the theoretical or known level of analyte actually present.[113] The Tripartite International Conference on Harmonization (ICH) suggests a minimum of nine determinations over a minimum of three concentration levels covering the specified range.[114] Percent bias was calculated using the equation:

(Theoretical value – Calculated value)/Theoretical value × 100% (Equation 4.7)

**Table 4.4 Accuracy data**

| Theoretical concentration (µg/ml) | Calculated mean concentration (µg/ml) (n=3) | % RSD | % Bias |
|---|---|---|---|
| 0.9 | 0.9 | 3.0 | -2.1 |
| 19.8 | 20.7 | 1.8 | -4.3 |
| 102.6 | 101.5 | 1.2 | 1.1 |
| 180.4 | 182.0 | 1.0 | -0.9 |

The bias is less than ± 5% which meets the requirements for the maximum allowable deviation and thus complies with the validation requirements for accuracy.

### 4.4.3 Precision

Precision is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogenous sample.[11,115] Precision can be divided into three types: repeatability, intermediate precision and reproducibility.[113]

#### 4.4.3.1 Repeatability

Repeatability is the intra-assay (with-in day) precision over a short period of time using the same operating conditions.[11] The percent relative standard deviation (%RSD) was

calculated using Equation 4.8 below and found to be less than 5% indicating full compliance with the validation criteria for repeatability.

$$\% \text{ RSD} = \text{Standard deviation} / \text{Calculated mean} \times 100\% \qquad \text{(Equation 4.8)}$$

#### 4.4.3.2 Intermediate Precision

Intermediate precision refers to data obtained on different days (inter-day). Intermediate precision was thus assessed over three days. Triplicates of three concentrations covering the specified range were used. Results depicted in Table 4.5 show that all the % RSD values were less than the 5% limit set in our laboratory.

**Table 4.5 Precision data**

| | Theoretical concentration (µg/ml) | Mean calculated concentration (n=3) | Standard deviation | Precision % RSD |
|---|---|---|---|---|
| Day 1 | 0.9 | 0.9 | 0.0 | 1.6 |
| | 19.8 | 20.7 | 0.4 | 1.8 |
| | 102.6 | 101.5 | 1.2 | 1.2 |
| | 180.4 | 182.0 | 1.8 | 1.0 |
| Day 2 | 0.9 | 1.0 | 0.0 | 4.3 |
| | 19.8 | 20.0 | 0.1 | 0.4 |
| | 102.6 | 100.1 | 0.1 | 0.1 |
| | 180.4 | 180.8 | 0.8 | 0.5 |
| Day 3 | 0.9 | 0.9 | 0.0 | 2.1 |
| | 19.8 | 20.7 | 0.3 | 1.3 |
| | 102.6 | 101.1 | 1.6 | 1.6 |
| | 180.4 | 180.4 | 1.0 | 0.6 |

#### 4.4.3.3 Reproducibility

Reproducibility refers to use of the same analytical procedure in different laboratories as in collaborative studies[11] or method transfer experiments.[110] Reproducibility was not assessed in these studies.

### 4.4.4 Linearity

Linearity is a measure of how well a calibration plot of response versus concentration approximates the straight line. The calibration curve was constructed from 0.5 to 200 µg/ml. Triplicate determinations of the nine concentrations were used and the calibration curve showed a high degree of linearity as demonstrated by the $r^2$ values that were obtained, 0.9997. $R^2$ is the correlation coefficient obtained from the least squares of linear regression analysis of the data. The equation for the regression line was found to be $y$ = 50 063$x$–21328. Such linearity (Figure 4.5) confirms the suitability of the assay for the studies as these calibration curves can be used directly to ascertain the concentration of analyte in sample in the tested range.

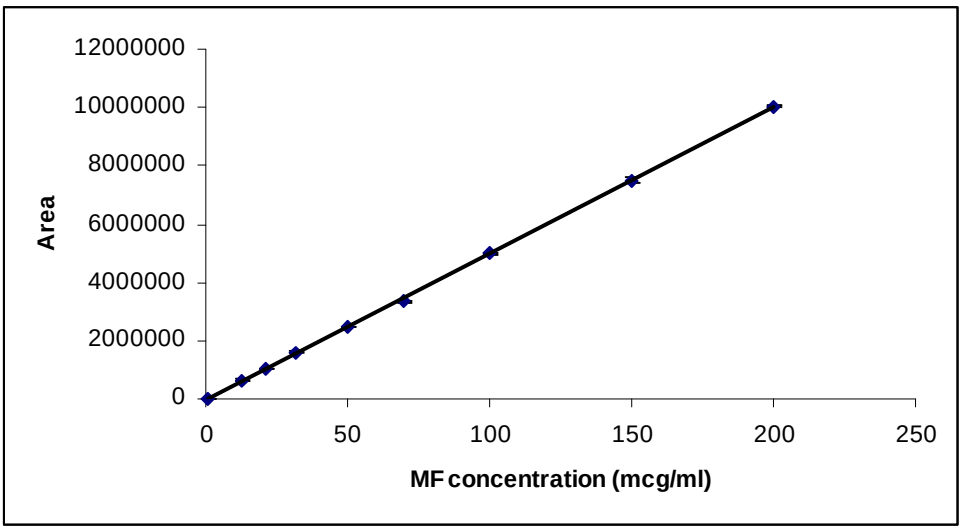



**Figure 4.5 Calibration line of the plot of area under curve versus MF concentration**

### 4.4.5 Limit of Quantification and Detection

The limit of quantitation (LOQ) is the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions[11] or the lowest concentration of an analyte that can be measured with a stated level of significance.[112] The limit of detection (LOD) is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated under the stated experimental conditions.[11] It has also been described as the lowest concentration of an analyte that the analytical process can reliably differentiate from the background noise.[112]

Several methods have been put forward for the determination of the limits of quantification and detection. According to ICH regulations in the case of instrumental analytical procedures that exhibit background noise, a comparison of the signal from a low concentration of analyte with that of a blank sample is made. The LOD is described as the concentration of the analyte at which the signal to noise ratio is either 3:1 or 2:1.[110,115] For HPLC methods however, the ratio 3:1 is applicable.[110] Another method for the calculation of LOD is based on the standard deviation (SD) of the response and the slope of the curve at levels approximately equal to the LOD. This method uses the formula: LOD = 3.3 (SD/slope of curve).[116] The LOD can also be calculated using the equation: LOD = intercept + 3 Sy. The intercept is determined from linear regression and Sy is the standard deviation of the y estimate from linear regression.[117] Another method qualifies LOD as the mean of blank value plus 2 or 3 standard deviations.[118] Visual approach sets LOD as the minimum level of analyte detection.[115]

LOQ is defined in a number of ways. LOQ can be estimated as the concentration at which the signal to noise ratio (S/N) is 10 to 1.[110,115] Another method involves the injection of different sample concentrations of different S/N. The different chromatograms are then assessed for precision. Depending on the definition of precision, LOQ is defined. A third method calculates LOQ using the formula LOQ = 10 (SD/slope of curve)[116] and the fourth one: LOQ = intercept + 10 Sy.[117] Statistically determined LOQ is set at 10 times the standard deviation above the mean blank value.[118] The quantitation limit is the minimum concentration of analyte that is quantifiable.[115]

The LOD and LOQ quoted in this research were determined using the Tripartite ICH[115] guidelines on method validation based on the use of S/N. LOD = 0.02 µg/ml (S/N = 3.4) and 0.2 µg/ml (S/N = 10.9).

### 4.4.6 Stability of MF in Methanolic Solutions

MF was found to be unstable in methanol at low concentrations when stored at 4˚C in the refrigerator for a month.

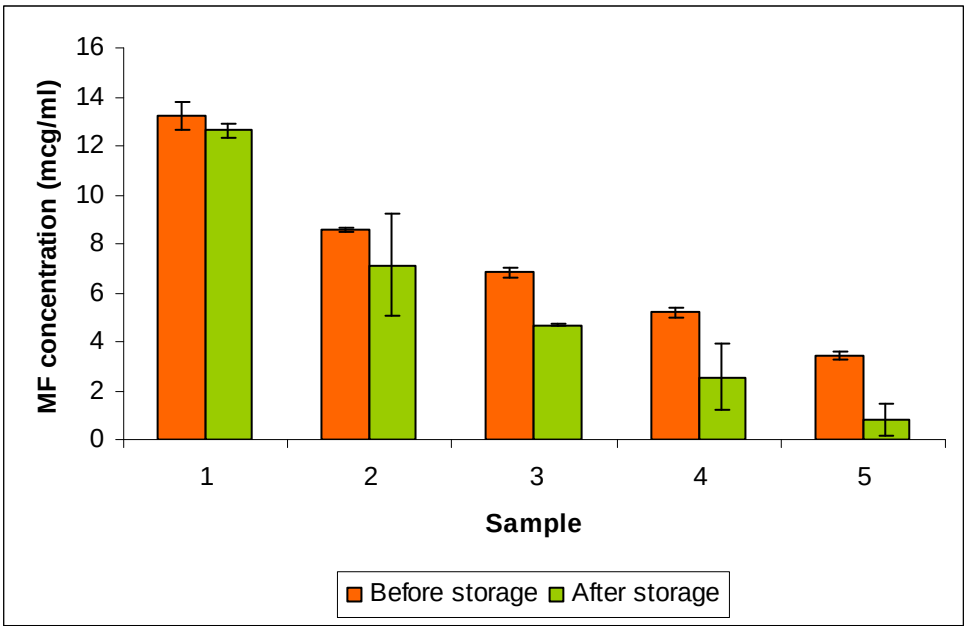



**Figure 4.6 MF stability after 1 month of storage in methanol at 4˚C**

C-17 and C-21 steroids undergo aqueous degradation. The ester group of C-17 and C-21 steroids is hydrolyzed resulting in a steroid base remnant. The steroid base undergoes further degradation via C-17 side chain alteration or ring A degradation.[119] MF is a C-17 furoate ester hence susceptible to hydrolysis and other degradation reactions. Like most steroids, MF is stable in acidic conditions and degrades in alkaline solution. MF degrades in phosphate buffer of pH greater than 6.73 via base catalysis. In phosphate buffer at pH 7.38 almost 25% of MF may be degraded to epoxides. Some degradation may occur at lower pH values but is relatively insignificant.[119]

MF undergoes a rearrangement following loss of water to form the C-21 steroidal ester. Both the C-21 steroidal ester and MF may undergo epoxide formation with concomitant loss of hydrochloride resulting in 3-11 epoxides. The C-17 epoxide may lose the ester to give another degradation product.[119]

## 4.5 Conclusions

The HPLC method described is simple, rapid, reproducible, precise and accurate and thus applicable for the quantitative analysis of MF. Baseline separation was achieved within

suitable retention times using a reverse phase $C_8$ minibore column. Optimisation of the mobile phase and detection wavelength resulted in well resolved peaks. Performance characteristics of the optimised method met requirements for the intended analytical applications. Although CP could readily have been incorporated in this assay as an internal standard since it is well-resolved from MF, it was deemed unnecessary. The Waters Alliance Separations system included an auto-injector which was highly precise as can be seen from the precision data thereby precluding the possibility of injection volume errors.

MF was found to be relatively unstable in methanolic solutions. Therefore, all methanolic calibration standards were freshly made on the day of analysis.

# CHAPTER 5

# APPLICATION OF MICRODIALYSIS TO EVALUATE PHARMACEUTICAL AVAILABILITY

## 5.1 Background

Microdialysis is a relatively new bioanalytical sampling technique that enables the measurement of substances in the body's extracellular fluid (ECF), which is 15–20% of the body's tissue volume.[120,121] Microdialysis allows for the quantification of drug content that passes through the *stratum corneum* into the vicinity of the microdialysis probe. It is advantageous as it gives high temporal resolution unlike tape stripping, skin blister formation or skin biopsy which results in a single concentration-time point being determined per administration.

During the development of corticosteroids, their lipophilicity was increased by fluorination. Whilst this feature has several practical advantages with important physiological/biological consequences, the increase in lipophilicity, however, poses a great drawback for studying such compounds by microdialysis because of poor aqueous solubility. When applied topically the drug tends to remain in the corneocytes hence very little passes through to the vicinity of the probe. Consequently, either more sensitive methods of analysis have to be used or the perfusate has to be modified to facilitate adequate recovery.

Modification of the perfusate can pose problems when used *in vivo*. Addition of lipids or propylene glycol (PG) has the potential to increase the osmotic potential of the perfusate resulting in larger dialysate volumes and a profound effect on the microenvironment of the probe. In other cases, extraction from the perfusate may be necessary and specific techniques and resources have to be available. The use of a modified perfusate *in vivo* is restricted to its compatibility and suitability for use at physiological pH and tonicity

whereas when used *in vitro*, the perfusate can be easily modified without such constraints.

### 5.1.1 Components of the Microdialysis System

The microdialysis probes used in this investigation were made from single hollow fibres (210 µm i.d. and 218 µm o.d.) with a molecular weight cut off of 5 000 Daltons as depicted in Figure 5.1. Cut-off is defined as the molecular weight in Daltons of a compound at which 80% of the molecules are rejected by the membrane.[122] The membrane fibre used was obtained from a Haemophan fibre dialyser Alwall GFS plus 12 (Gambro, Leuven, Belgium) which is normally used as haemodialysis membrane. Guide wire (Metalann, Meslin-I-'Eveque, Belgium) 100 µm i.d. and 70 mm long was inserted into the fibre. The use of the guide wire is necessary to stabilize the probe and does not influence recovery at flow rates below 10 µl/min.[123] The length of the membrane accessible to dialysis was 40 mm. On either side of the membrane Portex® nylon tubing of flexible grade, 0.50 mm i.d. 0.63 mm o.d., tubing (Scientific Laboratory Suppliers Ltd, Nottingham, UK) 10 cm long, was attached using Loctite® Super glue gel, a cyanoacrylate glue (Scientific Laboratory Suppliers Ltd, Nottingham, UK) unless otherwise stated, to provide linear microdialysis probes as shown in Figure 5.1. The outlet was placed into a 300 µl micro-insert (Waters Cooperation, Milford, Massachusetts, USA) through the pierced vial cap. Samples were collected every 30 minutes and analysed using the validated HPLC method described in Chapter 4.

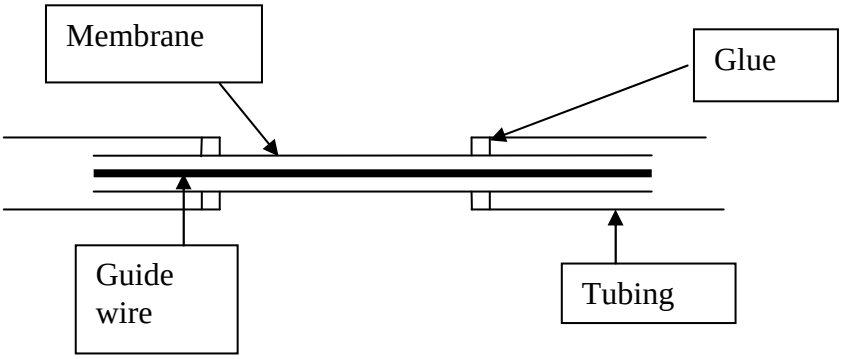



**Figure 5.1 Diagrammatic scheme of a linear microdialysis probe**

The probes were connected to the glass syringes of CMA 400 Syringe microinjection pump, (CMA/Microdialysis AB, Solna, Sweden) via tubing adapters (Ref 340 9500, CMA/ Microdialysis, Solna, Sweden).

#### 5.1.1.1 Membrane Structure

Dialysis of a drug from the probe occurs through the haemodialysis fibre. These fibres may be made from different materials and each affects drug dialysis in different ways depending on the properties of the drug and membrane. The choice of membrane is critical when dealing with compounds that are lipophilic and which may also be adsorbed onto the probe. Lipophilic compounds permeate the skin slowly and thus in the few hours in which microdialysis can be performed, very little drug may be detectable in the dialysate. If the drug, in addition to being lipophilic, also adsorbs onto the tubing or fibre, then it makes it even more difficult to analyse the resultant dialysate with conventional methods such HPLC. More sensitive methods need to be used in such cases and may be prohibitively expensive and therefore unavailable in most university laboratories. Such methods include radio-labelling of drugs, immunological assays or mass spectrometry.

Fibres used to fabricate linear dialysis probes in-house may be obtained from haemo-dialyser cylinders also known as artificial kidneys. These are cylinders containing semi-permeable bundles of fine hollow fibres that are used in blood dialysis in kidney failure.

The different polymeric materials used for dialysis membranes range from regenerated cellulose e.g. Cuprophan,[124,125] to synthetics such as polycarbonates or polyamides. Synthetically modified cellulose membranes are mid-way between the two extremes and include Haemophan and cellulose acetate.

Regenerated cellulose membranes form hydrogels in the presence of water and the compound diffuses across the gel. Regenerated cellulose membranes such as Cuprophan are effective for the diffusive transport of small solutes but cellulose membranes are naturally brittle.[126] As previously mentioned it is necessary to insert a guide wire into the fibre but such handling may result in damage to the fibre. Cellulose-based membranes contain hydroxyl groups, hence the potential to bind positively charged ions or create dipoles on a molecule. They have a poor biocompatibility profile compared with either synthetics or the modified cellulose membranes.[126]

Haemophan is derivatized cellulose and is modified by the inclusion of diethylaminoethyl (DEAE) groups. By reducing the hydroxyl groups available that are present in cellulose, the undesirable characteristics of cellulose membranes can be minimized.

The synthetics form the last class of haemodialysis membranes that are available for clinical use. These are engineered thermoplastics that include polysulfones, polyamides, polycarbonates, saponified ester cellulose and polyacrylonitrile–polyvinyl chloride copolymers. These materials are generally hydrophobic and are blended with hydrophilic polymers like polyvinylpyrrolidone.

#### 5.1.1.2 Structure of the Connecting Tubing

Portex® tubing consists of nylon a synthetic polyamide. The numbers usually appended to the different versions of nylon refer to the number of ‘CH’ units between the reactive ends of the monomer. Portex® has 11 of these units and is made from flexible surgical grade material. Nylon is tough, lightweight, and resistant to chemicals. The amide linkage is affected by acidity and alkalinity with associated implications for retention of the particular drug perfused through such a Portex® probe.

PEEK (polyetheretherketone) is a polyketone obtained from nucleophilic reaction of aromatic dihalides and bisphenolate salts. PEEK is resistant to both organic and aqueous environments, hence its use as a biomaterial for medical implants.

### 5.1.2 Microdialysis Sampling Principle and Technique

Microdialysis follows the principle of passive diffusion across the semi-permeable microdialysis membrane.[121,127] Two fluid compartments can be described in this setting as separated by the microdialysis membrane and these are the ECF and the perfusate flowing through the microdialysis probe. The microdialysis membrane is permeable to water and small molecules hence exchange of molecules across the membrane occurs in both directions i.e. into or out of the probe.

Due to a concentration gradient the drug either enters the perfusate in the probe or leaves the perfusate and enters the ECF following the concentration gradient according to Fick's law of diffusion. The permeability of the probe is controlled by the molecular weight cut-off of the membrane. Diffusion of the drug is also determined by the physicochemical properties of the drug, i.e. size and charge. The geometry of the extracellular space, active processes such as blood flow and the thickness of the membrane wall which controls the effective diffusion rate of the membrane, all affect the rate at which the drug enters the perfusate.[121] Absolute recovery which is the mass of substance recovered during a defined time period also depends upon the molecular weight cut-off of the dialysis membrane, the length of the membrane (area available for diffusion), the flow rate of the perfusion fluid and the diffusion coefficient of the compound through the ECF.[122]

A linear relationship has been demonstrated between the logarithm of percent recovery and molecular weight of the substance sampled using a commercially manufactured microdialysis probe (CMA/10 microdialysis probe). Therefore, an exponential relationship exists between these two factors. As such a microdialysis probe with a

molecular weight cut-off of 20 kDa has minimum recovery at approximately 5 kDa.[128] An acceptable relative recovery is obtained if the drug/substance of interest has a molar mass lower than approximately one-fourth of the membrane molecular weight cut-off.[129]

In microdialysis a blank fluid is used as the perfusate. The perfusate enters the inlet tubing, flowing at the set flow rate, past the microdialysis membrane where diffusion takes place into the outlet tubing from which the collected fluid is now termed the dialysate as illustrated in Figure 5.2. The reverse process of microdialysis, retrodialysis, differs from microdialysis in that the perfusate contains the drug of interest. Diffusion occurs at the membrane resulting in some of the drug of interest being lost to the ECF. Because of the reversibility of the process endogenous compounds can be collected at the same time as the exogenous compounds are introduced into the tissue.[125]

Proteins and enzymes are excluded from entering into the perfusate because of their size resulting in a relatively clean sample that requires little or no sample preparation prior to analysis.

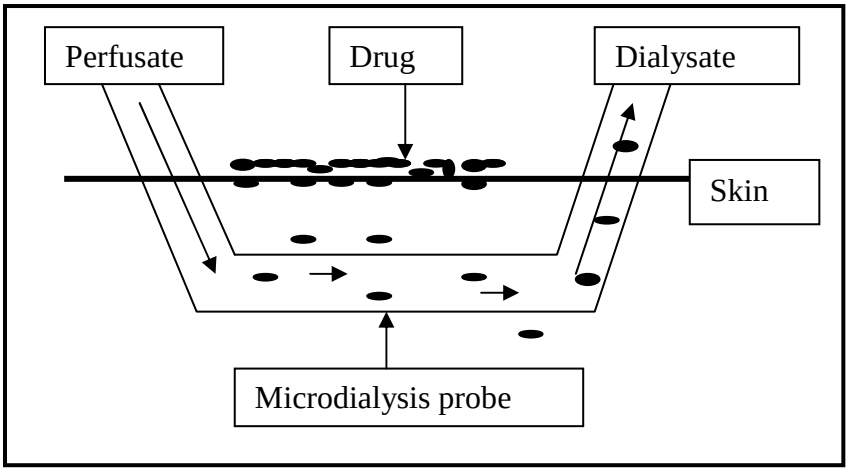



**Figure 5.2 Schematic representation of the principle of dermal microdialysis sampling technique[121]**

When choosing a probe or perfusate, the appropriate choice should result in a good and reproducible analyte recovery. In dermal microdialysis linear probes are useful in order to maximize recovery. As the molecular weight of the analyte increases, the recovery decreases for a particular membrane cut-off limit.[130] Analyte charge in the perfusate or peri-probe fluid affects diffusion across the membrane and the membrane surface may also affect analyte movement depending on whether it is hydrophobic or hydrophilic.

### 5.1.3 Advantages of Microdialysis

Microdialysis sampling is performed from a defined compartment as opposed to skin biopsies, tape stripping and other types of tissue removal. Biopsies undergo homogenization which disrupts all tissue components hence the drug concentration obtained is an average across the whole tissue which may include drug from intracellular fluid, interstitial tissue including that in residual blood entrapped in the tissue and structural tissue components.[131] Assessing the concentration of a drug directly in the target tissue is very useful for the comparison of pharmacokinetic and pharmacodynamic responses between two or more formulations.[120,132] Generally, microdialysis causes minimal tissue damage compared to other methods such as skin biopsies.[131,133] Linear probes used in dermal microdialysis cause even less damage at the insertion site.

Microdialysis allows continuous sampling which can simultaneously be performed at different sites in a single individual.[132] When performed *in vivo,* the perfusion medium is matched as closely as possible, in terms of ion strength, osmotic value and pH, to the ECF of the dialysed tissue.[125] Consequently, there is no net fluid exchange thus no interference with the processes that govern the pharmacokinetic behaviour of the drug.[127,134] Microdialysis thus allows assessment of the linearity of the penetration process in terms of time after application and offers high temporal resolution hence facilitating pharmacokinetic analysis.[127,135] Tissue removal methods have limitations with respect to the number of samples that can be collected per subject therefore data obtained is sparse.[131]

The microdialysis membrane normally used excludes protein and thus direct injection of relatively clean samples into an analytical system is possible.[132] Exclusion of protein prevents enzymatic degradation of sample and usually no or minimal sample preparation is necessary prior to analysis.[134] Microdialysis makes it possible to assess the amount and rate of drug penetration into the skin and consequently that which reaches the site of action.

Experiments may be performed for relatively long periods of time in addition to continuous sampling which is extremely advantageous for use of this method in pharmacokinetic studies. The continuous monitoring of extracellular free drug concentration for metabolic and pharmacokinetic purpose is thus enabled.[121,131]

### 5.1.4 Limitations of Microdialysis

After insertion, blood flow to the skin in the region of the probe increases. The area becomes erythematous and the skin thickens after the insertion. Although skin reaction is minimal, an equilibration period of between 90 and 120 minutes[66,136,137] has been advocated. An equilibration period is required for the vascular reaction to the needle trauma to return to the baseline range.

To further minimize the pain felt on insertion of the probe a local anaesthetic can be applied. Local anaesthesia prior to insertion reduces trauma effects. Topically applied anaesthetics such as EMLA are effective but must be applied approximately an hour prior to guide cannula insertion thus making it impractical in most clinical settings.[138] However, the application of a local anaesthetic affects skin perfusion. An equilibration time of about two hours is generally purported to reduce the effect of the local anaesthetic on the experiment as well.[66] Ice has also been used for pre-treatment of skin prior to needle insertion. Application of ice on the skin numbs the area making the insertion of the needle relatively pain-free.[138] The effect of ice on the skin is quite brief hence the equilibration period required is less than that when local anaesthetic is used.

With time, the microdialysis probe begins to disintegrate as a result of the environment it is exposed to in the skin. Consequently, a true representation of what is happening in the skin may not be possible. Late response allergic reactions can also be expected to occur. However, constant recovery has been shown to be maintained for at least 24 hours following topical application of 5-flourouracil indicating that experiments of 24 hour duration are feasible.[133]

### 5.1.5 Microdialysis of Lipophilic Compounds

Sampling of lipophilic drugs presents one of the greatest challenges in microdialysis due to relatively low recovery of such compounds[132] since lipophilic drugs will not be present in high concentrations compared to hydrophilic drugs in ECF.[139] Topical corticosteroids are intended to remain locally for action in the skin and are therefore largely lipophilic in nature. Lipophilic drugs become highly bound to proteins when they penetrate the skin making it difficult to quantitate them in extracellular fluid. The plasma protein binding of MF is 98.5%.[105] As such only 1.5% of the dose is the fraction unbound available for diffusion and pharmacokinetic and pharmacodynamic processes including receptor binding. In order to increase recovery of lipophilic drugs, the perfusate flow rate may be decreased. Reducing the flow rate is usually accompanied by reduced time-resolution as the adequate dialysate sample takes longer to collect. By making provision for reduced collection intervals,[139] time resolution can be increased. However, an ultra-sensitive analytical method will be necessary to handle the resulting low sample volumes.

Another option would be to alter the composition of the perfusate by adding either a protein or lipid component but this would have a drawback in analysis. The lipid or protein components would first have to be separated from the drug in question and an extraction process will usually be necessary.

### 5.1.6 *In Vitro* Calibration of Microdialysis Probes

Calibration of microdialysis probes is very important and necessary especially with in-house manufactured probes. *In vitro* calibration must be performed to ensure that the type

of probe used provides reproducible sampling. Where work is intended to be done *in vivo* then calibration of the probe should be done *in vivo* as well. *In vitro* recovery may differ significantly from *in vivo* recovery at times due to changes in kinetic processes, physiological parameter changes such as tortuosity and altered volume fraction.[124,140] In addition, *in vitro* drug release does not allow an evaluation of the effects of complex living tissue on the drug and its metabolism.[141]

The assessment of microdialysis probes also allows the investigation of the possibility of adherence to the microdialysis probe components to be investigated. The compound of interest being investigated may adhere to the probe and this must be taken into consideration for the microdialysis data to be reliably used for the estimation of the unbound fraction of the drug being monitored.[142]

Calibration can be assessed in several ways using the "no-net flux" method, the "extrapolation to zero flow" method, extraction efficiency and retrodialysis. During this research, the *in vitro* recovery of MF was determined by extraction efficiency and retrodialysis.

#### 5.1.6.1 "No-Net" Flux Method

The no-net flux method or the zero net flux method[124] is based on diffusion which is a process that occurs in the presence of a concentration gradient.[129,143] Without a concentration gradient, there would be no net flux. In order to obtain the respective recoveries, the concentration of the perfusate is varied.[124] The difference between $C_{dialysate}$ (concentration in dialysate) and $C_{perfusate}$ (concentration in perfusate) is plotted against $C_{perfusate}$. The point where the regression line crosses the x-axis ($C_{perfusate}$) is the concentration in the peri-probe (surrounding medium).[130,139,140,143] The slope of the regression plot represents the drug recovery.[129,130,139,143]

The advantage of this method is that no presumptions about the analyte and the behaviour pattern in the peri-probe fluid has to be made as the relative recovery is interpolated

rather than extrapolated.[129] However, the "no net flux" method is time consuming as a number of measurements have to be made under different conditions.

### 5.1.6.2 Extraction Efficiency

The probe is placed in spiked peri-probe fluid and blank perfusate is pumped through the probe. An equilibration time is allowed for steady state results to be obtained. Subsequently, samples are collected at various intervals of time for analysis. Recovery is calculated using the Equation 5.1:

$$R(\%) = \frac{C_{dialysate}}{C_{peri-probe}} \times 100 \qquad \text{(Equation 5.1)}^{130}$$

Where,

$C_{peri\text{-}probe}$ = average drug concentration in the peri-probe fluid.

Extraction efficiency, which is recovery by gain, mimics the *in vivo* situation in which the drug from a specific site is sampled. *In vivo*, drug from the particular tissue diffuses into the probe. Different levels of the drug concentration should be used to assess recovery to ascertain any differences in recovery that are dependent on concentration.[130]

### 5.1.6.3 Retrodialysis

A spiked perfusate is used and the probe is placed into blank peri-probe fluid. The peri-probe fluid should not contain any drug substance before the actual calibration process. After allowing for equilibration, samples are collected at 30 minute time intervals. Retrodialysis is based on the reversibility of passive diffusion across the dialysis membrane with influx equal to outflux. Loss of the substance through the membrane is thus the same as the recovery. Plock *et al*[129] calculated recovery by Equation 5.2.

$$R(\%) = 100 - (\frac{C_{dialysate}}{C_{perfusate}} \times 100) \qquad \text{(Equation 5.2)}$$

To calculate the percentage relative recovery Schuck *et al*[130] used the Equation 5.3.

$$R(\%) = \frac{C_{perfusate} - C_{dialysate}}{C_{perfusate}} \times 100 \qquad \text{(Equation 5.3)}$$

which is the same as relative recovery as defined by Simonsen.[144]

Unlike the other methods which require the assessment of the probes to be done before the pharmacokinetic experiment, retrodialysis can be done concurrently with the running experiment. This is done by spiking the perfusate with a marker substance. This substance should be well chosen with the diffusion characteristics being close to those of the drug of interest.[139] In such a case, the loss of the retrodialysis marker will be equal to the recovery of the drug of interest by dialysis. During an experiment, air bubbles may form or the membrane pores may be occluded by cells[139] affecting the recovery. With the introduction of a retrodialysis marker into the perfusate, the fluctuations in recovery during the experiment can be taken into account and it can be used to convert analyte microdialysate concentrations into extracellular concentrations.[127]

The use of a retrodialysis marker with different diffusion characteristics from the drug of interest is not recommended even though the two may be structurally similar. Theophylline (1, 3-dimethylxanthine) and caffeine (1, 3, 7-trimethylxanthine) for example, which are structurally close have shown differences in *in vivo* recovery. No interaction should be observed between the retrodialysis marker and drug. To circumvent this aspect of retrodialysis the drug itself may be spiked in the perfusate and a retrodialysis experiment conducted at the start of an *in vivo* study before there is any drug in the tissue.[139]

Percent loss (% loss) may be calculated by using perfusate spiked with the drug of interest. The dialysate concentration and the concentration of drug in the perfusate are used in the calculation. Percent gain (% gain) is obtained by the use of a blank perfusate with a known concentration of drug of interest in the peri-probe fluid. Under these conditions % gain should be equal to % loss.[139] However, this holds true for hydrophilic

compounds only. For lipophilic compounds % loss is not always equal to % gain.[140] *In vivo* the lipophilic compound binds to proteins hence the concentration of unbound compound is greatly reduced affecting the % gain.

## 5.2 Methods

*In vitro* microdialysis was applied to topical formulations for the assessment of release of MF as a measure of pharmaceutical availability. In order to accomplish such an evaluation, several experimental considerations were necessary.

### 5.2.1 Preliminary Investigations and Optimisation of Experimental Conditions

#### 5.2.1.1 Perfusate Selection

Various types of perfusate have been used to recover lipophilic or highly-bound compounds. Perfusate modifiers increase the concentration of the drug by preventing binding to the dialysis equipment.[143] Encapsin® (hydroxylpropyl β-cyclodextrin), Intralipid® (a fatty emulsion containing soybean oil, phospholipids and glycerine)[145] and bovine serum albumin[146] have been used to increase the recovery of certain drugs. On the other hand, solvents such as propylene glycol and other mono- or polyhydric alcohols which are water-miscible could be considered for use as recovery enhancers.

The solubilities of MF in binary compositions of PG and water were tested as described later (*vide infra* Chapter 6). Excess amount of MF was added to each binary composition of PG/water prior to shaking for 24 hours on a Junior Orbit Shaker. The excess undissolved MF was filtered at the end of the shaking period through hydrophilic PVDF syringe filters of pore size 0.45 µm (Millipore Millex-HV, Millipore Co., Billerica, Massachusetts, USA). Stability studies were also conducted to assess how stable MF was in the 70/30 PG/water (*vide infra* Chapter 6).

The samples were analysed after 24 hours of shaking.

### 5.2.1.2 Connection Tubing Selection

Two different types of connecting tubing, Portex® and PEEK, were incorporated with the microdialysis fibres to fabricate linear dialysis probes. Ten centimetres of tubing were used on either side of the microdialysis membrane, as the inlet and outlet tubings. Both the inlet and outlet connection tubing in the one type of the microdialysis probes consisted of Portex®. In the second type, Portex® was used at the inlet whilst the outlet was comprised of PEEK tubing. PEEK (250 µm i.d. and 1600 µm o.d.) could not be used as the inlet tubing because the connection to the glass syringe did not give a snug fit. The tubing adapters were too small to be used to connect PEEK tubing to the glass syringe. Portex® tubing was thus attached to the inlet side of the fibre membrane onto the glass syringe via tubing connectors whilst PEEK tubing was connected to the outlet of the fibre membrane to form a linear microdialysis probe described in Figure 5.1.

Retrodialysis was used to assess the recovery of MF using the two different connection tubings, PEEK and Portex®. A solution of MF in 70/30 PG/water (45 µg/ml) was used as the perfusate. Blank 70/30 PG/water was used as the peri-probe fluid. Samples were collected at 30 minute intervals of time using a microdialysis flow rate of 3 µl/min so that each sample collected would provide a volume of 90 µl.

Drug recovery increases with the length of the dialysis membrane, hence, the longest possible length of membrane would be the best choice. However, the limiting factor in the choice of the length of the membrane is a technical one, i.e. the difficulty of inserting the guide wire through a membrane increases with the length of the membrane. Insertion of a relatively long probe for *in vivo* use would also be impractical and difficult with membrane lengths greater than 3–4 cm. The length of the membrane exposed for diffusion to occur was 40 mm.

### 5.2.1.3 Flow Rate Selection

The effect of flow rate on recovery was investigated using the reference product, Elocon® cream (Lot 2). The microdialysis probes were inserted into glass tubes (4 mm i.d., length

6 cm) with the inlet and outlet tubings protruding from either side of the glass tubes. 700 mg of MF formulation, accurately weighed, and introduced into each glass tube using a 5 µl Eppendorf pipette (Eppendorf Ag, Hamburg, Germany) embedding the microdialysis membrane.

Microdialysis was carried out using 70/30 PG/water as the perfusate at the following flow rates: 3, 6 and 10 µl/min.

### 5.2.2 Assessment of Adsorption Using Retrodialysis in Air

MF was added to binary compositions of PG/water (70/30, 50/50, 30/70, and 20/80) to make 0.5 µg/ml perfusate solutions. The solutions were perfused (3 µl/min) through microdialysis probes suspended in air. The dialysate was collected and weighed every 30 minutes for 4 hours.

### 5.2.3 Assessment of *In Vitro* Retrodialysis Using Different Peri-probe Fluids

Microdialysis probes were perfused at 3 µl/min with a solution of MF in 70/30 PG/water (54.1 µg/ml) whilst immersed in different peri-probe fluids (70/30, PG/water, phosphate buffer at pH 7.4 or water).

### 5.2.4 Assessment of Extraction Efficiency

In order to assess the diffusion of MF, a blank solution of 70/30 PG/water was perfused through the probe that was inserted into a solution of MF (54.5 µg/ml) serving as the peri-probe fluid.

### 5.2.5 Assessment of Proprietary MF Formulations

Drug release from MF creams and ointments from several different countries was assessed using this "*in vitro*" microdialysis technique using 70/30 PG/water as the perfusate. The study products used are described in Tables 5.1 and 5.2.

**Table 5.1 Proprietary creams assessed for pharmaceutical equivalence**

| | Reference | Test | Test | Test |
|---|---|---|---|---|
| Trade name | Elocon® Lot 2 | Elocon® Lot 1 | Elocom™ | Elocom |
| Manufacturer | Schering-Plough (Pty) Ltd | Schering-Plough (Pty) Ltd | Schering Canada Inc | Schering-Plough |
| City, Country | Isando, South Africa | Isando, South Africa | Quebec, Canada | Rio de Janeiro, Brazil |
| Expiry date | October 2006 | October 2007 | AL 08 | December 2007 |
| Batch/Lot No. | 4NGFA07 | 5NGFA99004 | 6NGFA17 | 506 |

**Table 5.2 Proprietary ointments assessed for pharmaceutical equivalence**

| | Reference | Test |
|---|---|---|
| Trade name | Elocon® Lot 2 | Elocom™ |
| Manufacturer | Schering – Plough (Pty) Ltd | Schering Canada Inc |
| City, Country | Isando, South Africa | Quebec, Canada |
| Expiry date | October 2006 | October 2008 |
| Batch/Lot No. | 4NGFA07 | 5UHKA70004 |

## 5.3 Data Analysis - Assessment of Pharmaceutical Equivalence

Comparison of the pharmaceutical availability data for the relevant products obtained from the microdialysis experiments provided information for use to assess pharmaceutical equivalence. This is analogous to using comparative bioavailability between two or more products (usually a test versus a reference) for the assessment of bioequivalence. A number of statistical methods, mathematical models and model-independent models have been used in the study of drug release kinetics.

### 5.3.1 FDA *In Vitro* Release Comparison Test

A test for the pharmaceutical equivalence that is suggested by the FDA is called the *in vitro* comparison test. If a drug release test is conducted for a test product (n=6) and reference product (n=6) the calculations are as follows: Each of the 6 release rates obtained from the test product are divided by the each one of the release rates from the reference product producing 36 individual T/R ratios. The resulting ratios are sorted in order from the smallest to the highest. Once ordered the $8^{th}$ and the $29^{th}$ ratios are the lower and upper limits respectively of the 90% confidence interval for the ratio of the median *in vitro* release rate for test over the median *in vitro* release rate for the reference. Pharmaceutical equivalence limits are between 75 and 133% in this first stage. If the calculated interval for the ratios falls within the equivalence limits then the test product is deemed pharmaceutically equivalent to the reference product.[94]

In the event that the test does not pass the first stage a further four runs are conducted yielding n=18 (including first-stage results) for each product. Ratios are calculated as previously described to generate 324 individual ratios. After ordering the ratios from the smallest to the highest the $110^{th}$ and $215^{th}$ ordered individual ratios are the lower and upper limits respectively. Based on the same equivalence limits as in the first stage, pharmaceutical equivalence is evaluated.

Depending on the number of times an experiment is done, n, the position of the ratios used as the lower and upper limits changes. In cases where some of the data is missing and were the metric system is used (n=5) as opposed to the imperial system (n=6) one may not be able to calculate the lower and upper limits without expert advice.

### 5.3.2 Statistical modelling

ANOVA is assessed at each time point in the drug release profile between the test and the reference products. As such it takes into account the variability in data at the single time point. Differences are elucidated at each time point hence the release mechanism of the formulation can be inferred from ANOVA studies. The disadvantage of ANOVA testing

is that each time point is treated independently from the rest of the profile. A consequence of multiple comparisons is that the overall risk of incorrectly concluding that products are pharmaceutically not equivalent is greater than the nominal 5%.[92] Multiple comparisons may be statistically significant at some points in the profile, but not all, making it difficult to conclude whether differences truly exist. In addition, ANOVA does not rely on curve fitting procedures thus this method of comparing dissolution is tedious to perform, inefficient and ambiguous in interpretation. The use of ANOVA for release profiles other then those from immediate release products at a single time point is inappropriate and not recommended.[92]

### 5.3.3 Model-Dependent Methods

Model-dependent methods are based on different mathematical functions which describe the dissolution profile.[147] A suitable mathematical model is chosen and the drug release profile is evaluated depending on the model parameters. The *in vitro* release comparison test suggested by the FDA[94] is based on Higuchi kinetics which is model dependent. The similarity of model parameters from the different formulations are then compared using statistical analysis such as ANOVA.

#### 5.3.3.1 Zero Order Kinetics

In order to describe the kinetics of drug release from a formulation, various mathematical models have been proposed. A zero order reaction which is independent of the concentration of the reactants is given by:

$$Q_t = k_0 t \qquad \text{(Equation 5.4)}^{148}$$

A change in the concentration of the reactants therefore does not speed up or slow down the rate of reaction.

#### 5.3.3.2 First Order Kinetics

Another model, based on a first order reaction is a unimolecular reaction dependent on the concentration of only one reactant.

$$\ln Q_t = \ln Q_0 - k_1 t \qquad \text{(Equation 5.5)}^{148}$$

#### 5.3.3.3 Higuchi Kinetics

The Higuchi model is derived for a system fulfilling the following three requirements: Firstly, that the drug in the semi-solid formulation is in a fine state such that the particles are smaller than the thickness of the applied layer placed on a surface. Secondly, the amount of drug present per unit volume should be substantially greater than the solubility of the drug per unit volume of the vehicle. Lastly, the surface to which the drug formulation is applied should be immiscible with the formulation and thus constitute a perfect sink for the released drug.[149]

According to the Higuchi model, drug release from an insoluble matrix is directly proportional to the square root of time and is based on Fickian diffusion.

$$Q_t = k_H t^{1/2} \qquad \text{(Equation 5.6)}^{148}$$

Where,

$Q_t$ = amount of drug released in time t

$Q_0$ = initial amount of drug in formulation

k = release rate constant ($k_0$ – zero order, $k_1$ – first order, $k_H$ – Higuchi model)

In 1983 Fick's first law was used to describe the transfer of a diffusing substance through a particular material using Equation 5.7.

$$J = -D\frac{\delta C}{\delta x}$$ (Equation 5.7)[64]

Where,

J = rate of transfer per unit area of the surface (flux)

D = diffusion coefficient

C = concentration of the diffusing substance

x = the spatial co-ordinate measured normal to the section.

In other words, Fick's first law may be interpreted to mean that the rate of transfer of drug is related to the velocity of molecular movement and the concentration of the molecule in motion.[62] Fick's first law gives the flux in the steady state.

Fick's second law of diffusion describes the non-steady state flow. It states that the change in concentration with time in a particular region is proportional to the change in the concentration gradient at that point in the system and is mathematically expressed as:

$$\frac{\delta C}{\delta t} = D\frac{\delta^2 c}{\delta x^2}$$ (Equation 5.8)

At steady state rate of penetration is given by:

$$\frac{dQ}{dt} = \frac{a_v}{\gamma_s}\frac{DA}{L}$$ (Equation 5.9)[150]

Where,

dQ/dt = steady rate of penetration

$a_v$ = thermodynamic activity of the drug in the vehicle

$\gamma_s$ = effective activity coefficient of the drug in the skin barrier

A = area of application

L = diffusion path length

It is thus apparent that increasing the thermodynamic activity of the formulation by altering vehicle composition would be expected to enhance the rate of penetration.

Drug release data from the microdialysis of proprietary formulations were applied to the Higuchi equation and the average cumulative amount was plotted against the square root of time. Linear regression analysis was performed for each plot and the rate of release determined from the slope of the plots. Lag time was obtained by extrapolating the Higuchi plot to the time axis.[151] The lag time corresponds to the delay that needed for drug molecules to be released from the dosage form and diffuse across the synthetic membrane if the membrane is not rate limiting.[152]

### 5.3.4 Method of Data Analysis

The drug release profiles were analysed using the Higuchi model to obtain release rates. Differences in the release rates were evaluated using ANOVA followed by Bonferroni *post hoc* testing.

## 5.4 Results and Discussion

### 5.4.1 Microdialysis Conditions Selected for Experimental Work

#### 5.4.1.1 Flow Rate Selection

Since the volume of perfusate to be collected is dependent on the sample size requirements of the particular analytical method being used, the frequency of sampling is a primary consideration. For example, if an assay requires a sample size of 20 µl of analyte, the sampling frequency needs to be at 20 min intervals if a flow rate of 1 µl/min is used. To increase drug concentration in the dialysate, low flow rates can be employed but these subsequently lead to decreased temporal resolution,[132] since a flow rate of 0.5 µl/min will result in a temporal resolution of 40 minutes. Lower temporal resolutions may become more important when very low amounts of drug are present in the analyte as is the case with lipophilic drugs. Where very low drug concentrations are encountered, the use of ultra sensitive analytical methods becomes a pre-requisite.

Perfusion rates normally used in microdialysis are in the range 0.1–5.0 µl/min.[139,153] During this research, a flow rate of 3 µl/min was used which provided sufficient sample

volume and also permitted satisfactory recovery rates. The effect of flow rate on the rate of drug recovery is tabulated in Table 5.3.

**Table 5.3 Effect of flow rate on the rate of recovery**

| Flow rate (µl/min) | Rate (µg/cm$^2$/hr$^{½}$) | Correlation coefficient ($r^2$) |
|---|---|---|
| 3 | 0.3 | 0.9979 |
| 6 | 0.2 | 0.9690 |
| 10 | 0.1 | 0.9977 |

It has been shown that, using flow rates between 0.5 and 5 µl/min, no net loss of liquid across the dialysis membrane occurs.[153] Higher flow rates interfere with the diffusion process that controls microdialysis, i.e. a lower perfusate flow will allow more time for the drug to diffuse into the perfusate[132,140] even though the temporal resolution of the measurements will be reduced.[139]

Although equilibrium can be achieved at any particular perfusate flow rate, attainment of such equilibrium does not give the absolute concentration of the drug of interest in the peri-probe fluid or the formulation in which the probe is embedded. This is termed relative recovery and is always below 100%. Relative recovery describes the efficacy of microdialysis.[154]

### 5.4.1.2 Perfusate Selection

The perfusate was selected primarily on the basis of the solubility of MF in the binary composition of PG/water. Generally, the solution in which MF is optimally soluble is the most appropriate one to sure that sink conditions are maintained during the experiment. During this research, a 70/30 PG/water composition was chosen as the perfusate for the microdialysis experiments. The solubility of MF was found to be 170 µg/ml and MF was stable after one month of refrigeration in 70/30 PG/water at 4 ˚C (*vide infra* Section 6.4.2).

PG was selected as it can be considered for use with *in vivo* microdialysis in addition to its ability to dissolve corticosteroids.[155] It is non-toxic and is readily metabolized and excreted. When used topically, PG has minimum irritancy. A daily intake of about 25

mg/kg of body weight is acceptable according to the WHO standards. It is also used in concentrations of between 10–60% in parenterals as a cosolvent or as a solvent.[155] However, when being considered for *in vivo* use the tonicity of the binary solution is of paramount importance. The use of a hypertonic perfusate will cause a net flow of extracellular fluid (ECF) into the perfusate. This will result in an alteration of the ECF fraction thereby affecting the extraction efficiency. A shift in ECF volume affects the local analyte concentration. If a hypotonic perfusate is used, the perfusate will tend to move out through the microdialysis probe into the ECF thereby diluting the drug in the peri-probe area. Hence, the use of either hypotonic or hypertonic perfusate in *in vivo* experiments will affect the recovery of the drug being investigated.

#### 5.4.1.3 Selection of Connection Tubings

The retrodialysis method is also referred to as reverse (micro)dialysis.[125,127] The relative loss of drug from the perfusate in retrodialysis is equal to the relative recovery of that same drug in microdialysis. As such the calculated relative loss from retrodialysis can be termed the relative recovery. This relative recovery is calculated as shown in Equations 5.2 and 5.3.

**Table 5.4 Retrodialysis results from the use of different tubings in the microdialysis probe: Portex® and PEEK tubings**

| Time (minutes) | Portex® (% Recovery) | Standard deviation (%) | PEEK (% Recovery) | Standard deviation (%) |
|---|---|---|---|---|
| 30 | 17.1 | 7.8 | 41.2 | 6.4 |
| 60 | 3.5 | 0.8 | 20.1 | 7.5 |
| 90 | 2.7 | 1.7 | 9.5 | 3.8 |
| 120 | 4.7 | 4.3 | 8.7 | 2.1 |
| 150 | 2.9 | 1.4 | 9.6 | 4.4 |
| 180 | 2.8 | 0.6 | 8.4 | 4.1 |
| 210 | 3.7 | 1.2 | 6.3 | 3.8 |
| 240 | 3.2 | 0.5 | 5.9 | 2.5 |

Microdialysis probes in which PEEK tubing was incorporated showed a significantly higher percent recovery (8%) than Portex® (3%) at equilibrium ($p = 0.0017$, $\alpha = 0.05$). However, lower %RSD (below 2%) was observed with Portex® than with PEEK

(between 2 and 5%). In addition, equilibrium was reached much faster using the microdialysis probe connected with the Portex® tubing (60 minutes) compared to that with the PEEK tubing (90 minutes) as shown in Table 5.4. Microdialysis probes made using Portex® tubing only for both the inlet and outlet were chosen over those that incorporated PEEK as these rigid probes were difficult to handle whilst the Portex probes are quite flexible and relatively easy to manipulate.

The final conditions selected for the *in vitro* microdialysis experiments are summarized in Table 5.5.

**Table 5.5 Microdialysis conditions selected**

| | |
|---|---|
| Perfusate | 70/30 propylene glycol/water |
| Flow rate | 3 μL/min |
| Membrane | Gambro GFS Plus 12 |
| Molecular weight cutoff | 5 kDa |
| Length | 4 cm |
| Tubing membrane | Portex® tubing, 10 cm on either side of membrane |
| Temperature | Ambient |
| Stirring rate | Quiescent (No stirring) |

Gravimetric assessment of the mass of dialysate was made under the conditions depicted in Table 5.5 at the end of each 30 minute interval. It was observed that no drug solution was lost due to either convective fluid loss or evaporation.

## 5.4.2 Retrodialysis and Microdialysis

Microdialysis and retrodialysis of MF yielded the results depicted in Figure 5.3. In 4 hours the percent of recovered drug increased to about 3%. The increase in the recovery rate was small and was thus expected not to increase significantly from 3%. The % lost from retrodialysis of MF is about 97%. Combining the % lost in retrodialysis and the % recovered in microdialysis should give a total of 100% which was observed in this case.

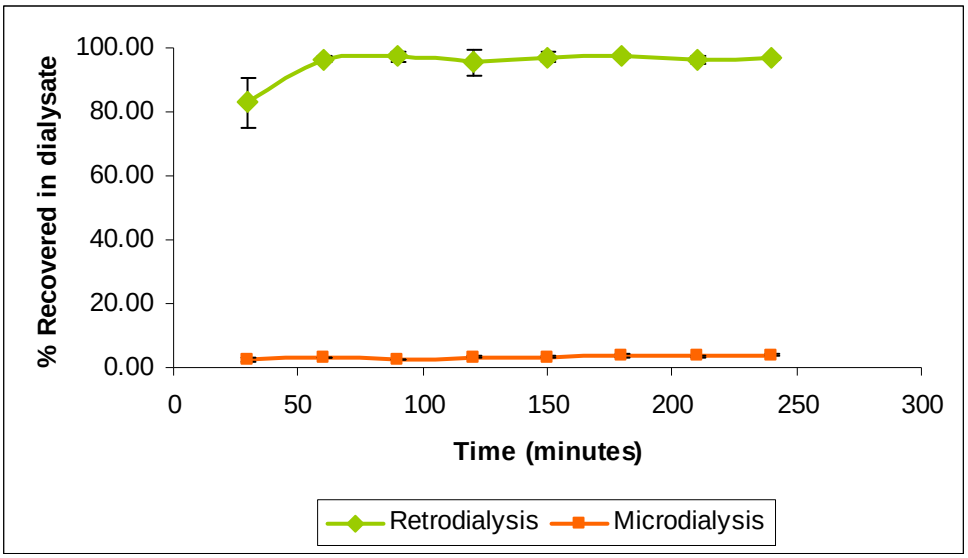



**Figure 5.3 Retrodialysis and microdialysis (n=4) - MF solution made up in 70/30 PG/water, dialysis using Portex® tubing**

### 5.4.3 Retrodialysis in Air in the Assessment of Adsorption

Retrodialysis in air was performed to investigate binding of MF to the membrane. The results showed that when the PG content was greater than 50%, MF did not bind to the probe; instead all the MF was recovered from the dialysate (Table 5.6).

**Table 5.6 Retrodialysis with Portex® tubing suspended in air**

| MF concentration (µg/ml) | Perfusate (PG/water) | % Recovered in dialysate |
|---|---|---|
| 0.5 | 70/30 | 100 |
| 0.5 | 50/50 | 100 |
| 0.5 | 30/70 | 0 |
| 0.5 | 20/80 | 0 |

At low PG and high water content it appeared that the water wetted the membrane such that MF had greater affinity for the membrane. In any system, the presence of an interface causes free energy increase. To reduce this increase in energy, the system spontaneously modifies the shape or the structure of the interface until a new equilibrium is reached. In the case of a solution the dissolved analyte moves towards the surface of the vessel or other rigid particles suspended in the solution (e.g. silica gel, zeolites or activated carbon) and becomes adsorbed.[156]

Non-specific binding to the membrane, tubing, glass syringe and plastic connectors may occur in the absence of organic solvent or protein.[105,130] Binding affinity depends on the partition of the compound to plastic or glass and into the solvent used as perfusate.[130]

Retrodialysis experiments were repeated with different peri-probe fluids (Table 5.7). The perfusate of 70/30 PG/water containing MF was used for all the experiments. Less than 5% of the drug was recovered in the dialysate in each case where buffer or water was used as the peri-probe whereas most of the MF was recovered using 70/30 PG/water. MF release is a function of its solubility in the perfusate.[150] The more soluble the drug in the perfusate the more drug is recovered in the dialysate. In this study, the osmotic differences between the perfusate and the peri-probe fluids were different. Consequently there was net movement of the peri-probe fluid (buffer and water) into the microdialysis probe. This resulted in the dilution of the 70/30 PG/water perfusate thus increasing the perfusate water content. The reduction of the PG content reduced the solubility of MF in the resulting PG/water mixture hence only about 5% was recovered in the dialysate.

**Table 5.7 Retrodialysis of MF in solution**

| MF concentration (µg/ml) | Peri-probe composition | % Recovered in dialysate |
|---|---|---|
| 54.1 | buffer | ~5 |
| 54.1 | water | ~5 |
| 54.1 | 70/30 PG/water | ~97 |

The volume of the peri-probe fluid used was about 40 ml so as to offer sink conditions. The drawback of such a set up was that the peri-probe fluid at the end of the experiment could not be tested for the presence of MF as it was too dilute. However, it can be inferred from the results from retrodialysis in air that the greater the water content of the perfusate, the more the MF binds to the microdialysis probe.

### 5.4.4 Assessment of the release of MF from Proprietary MF Formulations: Comparison of Results Using Portex and PEEK Tubing

Microdialysis drug release rates of MF from Elocon® ointment were lower than those from Elocon® cream (Table 5.8). This trend was obvious for both microdialysis probes, i.e. one incorporating Portex® tubing only and the other which incorporated PEEK tubing as well. Despite significant differences observed between the two microdialysis probes in retrodialysis, no significant differences were observed in the assessment of the Elocon® cream and ointment.

**Table 5.8 Cumulative amount of MF dialysed using PEEK and Portex® tubing**

| Formulation | Type of tubing used | Cumulative amount dialysed (µg) |
|---|---|---|
| Elocon® cream (S.A.) | PEEK | 1.8 ± 0.1923 |
| Elocon® cream (S.A.) | Portex® | 1.9 ± 0.4592 |
| Elocon® ointment (S.A.) | PEEK | 0.8 ± 0.2409 |
| Elocon® ointment (S.A.) | Portex® | 0.7 ± 0.2518 |

The maximum amount of drug dialysed from the cream was 1.9 µg in 720 µl at the end of 4 hours. The dialysed drug concentration of about 3 ng/ml is 50 000 times less than the solubility of MF in 70/30 PG/water which is 170 µg/ml. Sink conditions have been described as being 10 times the maximum achievable drug concentration during the course of an experiment.[157,158] This shows that adequate sink conditions are met.

MF is hydrophobic and thus is held more firmly by the ointment vehicle than by the cream base hence the release from the ointment base is slow. The greater solubility of MF in the ointment base may also account for the decrease in rate of drug release.[159] Cream bases which are generally oil in water (o/w) emulsions form a continuum with the water of the receptor phase[70,159] (70% PG: 30% water) hence the greater release. When the continuum is formed, the concentration of the drug dissolved in the water of the o/w emulsion is lowered as the drug redistributes in a larger volume of water. The equilibrium diffusion state between the concentration of drug in oil and that in water is disturbed, hence to re-establish this equilibrium more drug diffuses into the water component. The

water available in the matrix aids the dissolution of the drug as it moves towards the diffusion surface. The thermodynamic activity of the drug in the vehicle is the product of the concentration of drug and the activity coefficient of the drug in the vehicle. The concentration of the drug is the same in both formulations but they differ in the activity coefficient in the vehicle with the cream having a higher activity coefficient hence a higher thermodynamic activity.

In addition, the viscosity of a formulation has been implicated in the release rate of the drug. The higher the viscosity of a formulation the lower the rate of drug release.[160] The tortuousity of the ointment is far greater than that of the cream. Drug dissolution in the ointment is thus restricted to the minimum top layer that is just covering the microdialysis membrane in the amount of time that this experiment was done. It is possible that the oleaginous ointment vehicle plugs the pores of the membrane thus delaying drug penetration.

**Table 5.9 Release from Elocon® 0.1% (South Africa) cream and ointment (Portex® tubing)**

| Model | $r^2$ Cream | $r^2$ Ointment |
|---|---|---|
| Zero order | 0.9824 | 0.8917 |
| First order | 0.8802 | 0.7789 |
| Higuchi | 0.9899 | 0.9566 |

Correlation coefficient ($r^2$) values closest to unity are indicative of acceptable fit. The cream and the ointment both follow Higuchi kinetics hence the drug release from both formulations is a diffusion controlled process.

### 5.4.5 Bioequivalence Assessment of Proprietary MF Creams

The proprietary creams from the different countries show that there are differences between the creams. The Brazilian cream releases more drug in comparison with the other 2 creams, whilst the Canadian cream releases the least amount (Figure 5.4).

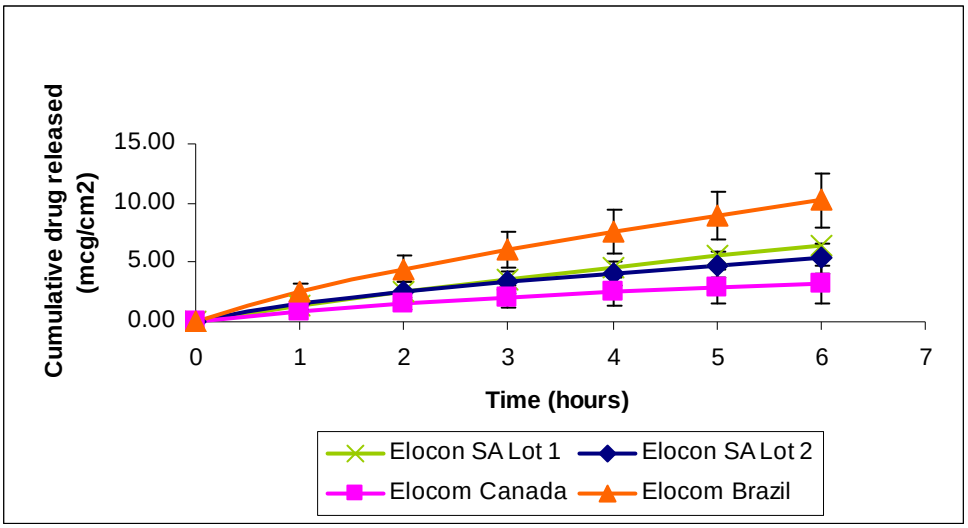



**Figure 5.4 Drug release profiles from proprietary 0.1% MF cream formulations**

Both lots of the South African cream showed release rates that were quite similar. Lot 1 had an apparent release constant of 3.6 μg/cm$^2$/hr$^{½}$ and Lot 2, 2.7 μg/cm$^2$/hr$^{½}$. Elocom 0.1% cream (Brazil) had the highest apparent release constant (ARC) which was found to be almost double that of Lot 2 of the South African cream and three times that of the Canadian cream. However, even though the ARC of the Brazilian cream was quite high, the lag time observed was four times greater than that of the Canadian formulation. The lag times of both Lot 1 and 2 of the South African cream are short compared to that of the Brazilian cream. A shorter lag time implies that the drug is more rapidly available for diffusion at the skin surface hence penetration to the site of action occurs faster and pharmacological action may be achieved earlier.

Some studies have been carried out to show the differences in ARCs between formulations of different bases e.g. lotions, gels and creams.[152] This study on the other hand, has shown that proprietary generic formulations may differ in the rate and extent to which the drug is delivered. The manufacturing processes between the different countries as well as formulation differences may thus contribute to the differences in ARCs found. Lot 1 and 2 of the MF creams, both made at the same manufacturing plant, Isando, South Africa, were quite comparable with respect to their ARCs and lag times in comparison with either the Brazilian or the Canadian creams.

**Table 5.10 Comparison of drug release kinetics from MF creams**

| Product | Apparent release constant ($\mu g/cm^2/hr^{½}$) | Lag time ($hr^{½}$) | Correlation coefficient ($r^2$) |
|---|---|---|---|
| Elocon® SA Lot 1 | 3.6 | 2.4 | 0.9940 |
| Elocon® SA Lot 2 | 2.7 | 1.3 | 0.9994 |
| Elocom™ Canada | 1.6 | 0.7 | 0.9983 |
| Elocom Brazil | 5.3 | 3.0 | 0.9974 |

The $r^2$ values obtained after linearizations using the Higuchi model show that drug release from the proprietary MF creams in the microdialysis experiments follow Higuchi kinetics ($r^2 > 0.99$).

Comparison of the Canadian and South African ointments also showed that they differed significantly as shown in Figure 5.5.

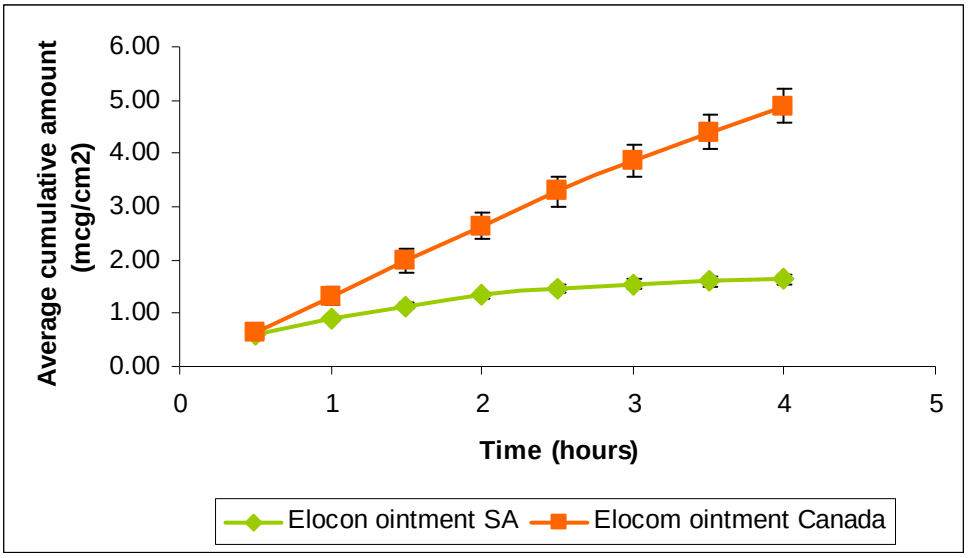



**Figure 5.5 Drug release profiles from proprietary 0.1% MF ointment formulations**

Whereas the Canadian cream released MF at a slower rate than the South African cream, the results from the study of drug release from the ointment formulations showed the opposite to be true. Elocom™ ointment (Canada) releases MF at a significantly faster rate that Elocon® ointment (South Africa). The rate of drug release from Elocon® ointment was about 1.0 $\mu g/cm^2/hr^{½}$ in the first 150 minutes. Above 150 minutes the rate dropped to

0.2 μg/cm$^2$/hr$^{½}$, closer to steady state. Consequently, the calculated lag time was negative. The overall release rate was calculated to be 0.8 μg/cm$^2$/hr$^{½}$. On the other hand, ARC from Elocom™ ointment was 3.4 μg/cm$^2$/hr$^{½}$.

**Table 5.11 Comparison of drug release kinetics from MF ointments**

| Product | Apparent release constant (μg/cm$^2$/hr$^{½}$) | Lag time (hr$^{½}$) | Correlation coefficient ($r^2$) |
|---|---|---|---|
| Elocon® SA | 0.8 | - | 0.9665 |
| Elocom™ Canada | 3.4 | 0.6 | 0.9914 |

The straight line plots of amount of drug released versus the square root of time for the products were obtained and the $r^2$ values are shown in Table 5.11. A negative lag time was obtained for Elocon® ointment (South Africa). Drug release profiles for formulations that have low release rates are almost parallel to the time axis hence the negative lag time. The ARC obtained for the Canadian ointment was 4 times that of the South African ointment. This may be due to the differences of the drug product formulations, probably different excipients both qualitatively and quantitatively and differences in the manufacturing process. The release data obtained by applying microdialysis to the different products were fairly approximated by Higuchi kinetics.

**Table 5.12 ANOVA results for pharmaceutical equivalence**

| Formulation | Mean difference | p-value | 95% CI for difference | | Significant/Not significant |
|---|---|---|---|---|---|
| | | | Lower limit | Upper limit | |
| Elocon® Lot 1 | 1.323 | p>0.05 | -0.901 | 3.548 | Not Significant |
| Elocom Brazil | -2.971 | p<0.001 | -5.195 | -0.746 | Significant |
| Elocom™ Canada | 0.7358 | p>0.05 | -1.489 | 2.960 | Not Significant |

Significant differences were observed between the reference product and Elocom cream (Brazil). No significant differences were observed between the reference product and the Canadian cream.

Used as described in this research *in vitro* microdialysis has the potential for use as a surrogate method of measuring release rates from formulations. Further modifications may be necessary before microdialysis probes can be used in quantitative and qualitative assessments.

## 5.5 Conclusions

The in-house fabricated linear microdialysis probes were seen to give reproducible and can be reliably used for these types of microdialysis experiments.

Recovery experiments are a pre-requisite for the application of microdialysis to assess drug binding to the various materials, membranes and tubing. Differences in the hardness of the tubings can lead to major differences in binding of the drug. Higher *in vitro* recovery was obtained when PEEK connection tubing was incorporated into the fabrication of the microdialysis probes in retrodialysis using 70/30 PG/water as a peri-probe fluid. When both probes were used for the assessment of the Elocon® formulation no significant differences were observed. In addition, Portex® probes had better reproducibility as evidenced by the smaller standard deviations, and they are flexible and easier to manipulate hence their selection over PEEK probes.

More MF was released from Elocon® 0.1% cream per unit time compared to Elocon® 0.1% ointment. These results indicate the utility of microdialysis for the determination of release rates of MF from topical formulations. Furthermore, release rates between different formulations can be readily assessed by this technique and therefore very good potential for use to assess the pharmaceutical availability of topical preparations.

Differences in formulation and manufacturing process(es) may be responsible for the differences in release and this has significant implications for the bioavailability of topical MF formulations (and other drugs formulated for topical use). Hence, such pharmaceutical availability information has good potential to predict possible differences that may occur with respect to bioavailability of active compounds used in topical

formulations. It can be used in preliminary investigations to assess the bioequivalence of different formulations during product development, as well as to assess differences between a test and reference product prior to undertaking a pivotal bioequivalence study. Further research and optimization of variables in *in vitro* microdialysis is needed. The results discussed show that the method has potential in the *in vitro* assessment of drug release from semi-solid formulations.

# CHAPTER 6

# *IN VITRO* RELEASE OF MF FROM TOPICAL PREPARATIONS USING FRANZ CELLS

## 6.1 Background

The establishment of quality, safety and efficacy is paramount for every new drug product prior to release onto the market. Controlled clinical studies are used for the assessment of safety and efficacy in the early phases of drug and drug product development. Safety and efficacy have to be demonstrated to ensure that product quality and performance are maintained in the presence or absence of change in later stages, after the drug has been approved. Controlled clinical studies are expensive and are also extremely time consuming. Whilst clinical studies are essential in the case of new drug entities and products, appropriate surrogate methods may be used to assess safety and efficacy of generic drug products, such as the currently accepted bioequivalence testing procedures.

For the API of a drug formulation to be absorbed it has to be released from its dosage form. Following drug release from a topical dosage form, the API comes into contact with the epidermis through which the drug must diffuse. Only after diffusion has occurred can drug absorption be possible. Consequently release rate determination is an important quality control parameter.[161]

*In vitro* release testing of semisolids has been accepted as a universal regulatory tool to monitor batch to batch uniformity in manufacturing.[162] This *in vitro* method tests product performance qualitatively and quantitatively. *In vitro* release testing is used for monitoring product reproducibility during component and compositional changes, manufacturing equipment and process changes, batch scale up or transfer to another manufacturing site,[163] and as such is a very valuable product development tool to assess pharmaceutical availability. *In vitro* release studies are carried out to quantify the rate of

drug release from the excipients/vehicle components of the dosage form. Information on the rate, degree and mechanism of penetration of drugs and other substances is required for the optimization of therapeutic activity of a drug product.

*In vitro* release is one of the standard methods used to characterize performance of finished topical formulations. The FDA Guidance for Industry, Non-sterile Semisolid Dosage Forms Scale-up and Post-approval Changes: Chemistry, Manufacturing, and Controls; *In vitro* Release Testing and *In vivo* Bioequivalence Documentation, released in May 1997[94], commonly known as SUPAC-SS, requires the use of validated methodology and provides a guide to the study design. The *in vitro* release study should use appropriate diffusion cells and membranes and the receptor medium used should be justified. Replicates of the drug release profile are required. Furthermore, an infinite dose should be applied and protected from vehicle evaporation. At least five sampling times over an appropriate period of time in which adequate drug release has occurred are essential. Quantification of the drug released has to be done using a sufficiently sensitive validated method of analysis such as HPLC.[94] Franz cells are widely used since they are relatively affordable, give reproducible results and are less time consuming[164] than most *in vivo* studies.

Drug release testing is considered as the single most useful *in vitro* method for assessment of batches,[165] lot-to-lot variation, stability, and for the development of new formulations as a preformulation tool.[94] *In vitro* drug release testing is also used to ensure continuing product quality after changes in the process of manufacturing, site of manufacture or after a scaling-up process.[94,147] Drug release *in vitro* is a property of the formulation and is thus used to assess pharmaceutical availability to provide information on the product sameness after minor changes.[166]

*In vitro* drug release testing of semi-solids has been performed on Franz cells,[164,165,167,168] flow through cells,[152,169] insertion cells,[170] plexiglass cells,[167] enhancer cells,[160] glass diffusion cells,[171,172] Perspex diffusion cells[151] and the modified version of Franz cells.[53,173,174] The choice of the particular cell is usually based on the solubility of the

drug and reproducibility. Satisfactory drug release needs to be demonstrated as a prerequisite for therapeutic efficacy.[167]

### 6.1.1 Diffusion Cells

The Franz static vertical cells as modified by Keshary and Chien[173] were used. These cells have been shown to be superior to the Franz diffusion cells in several ways. The star studded magnetic stirrer added to the receptor fluid rotates at 600 rpm and ensures complete mixing of receptor compartment contents.[71,173,175] The water jacket surrounding almost the whole cell results in better temperature control. The static diffusion cell is used for a drug whose ultimate concentration after permeation does not reach greater than 10% of the maximal solubility in the receptor fluid.[175]

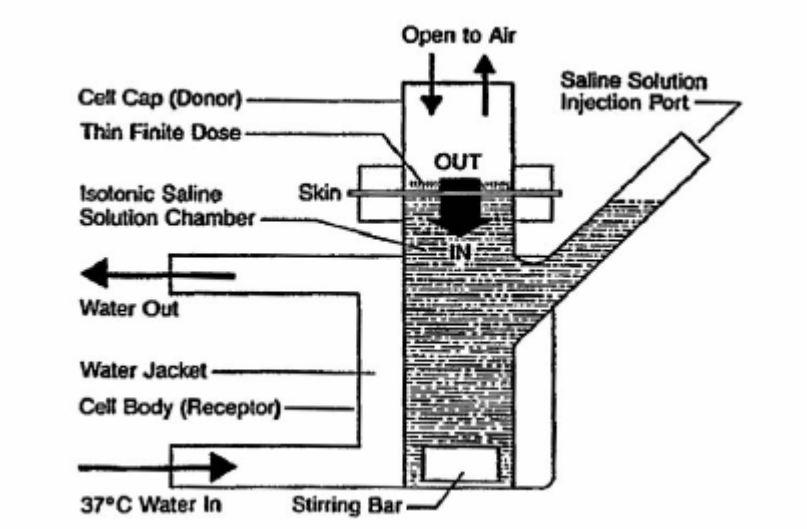



**Figure 6.1 Schematic diagram of a modified Franz cell[176]**

### 6.1.2 Temperature

Temperature affects drug release from a formulation. Temperature should thus be kept constant for the duration of the study. However, the temperature at which Franz cell diffusion studies are to be carried out is not specified in the SUPAC-SS guidance. Consequently a number of different temperatures have been used but most researchers maintain the receptor fluid at 32 ˚C.[15,71,177-179] with the justification that skin surface temperature is about 32 ˚C.[175,176] It was observed that when the receptor cell fluid was

heated using water in the water jacket at 37 ˚C, the surface temperature of the receptor fluid was maintained at 32 ˚C. Some portions of the cell are open to room temperature, such as the sampling port and the donor compartment hence some heat is lost.[144,175,180,181] High temperatures are associated with melting of semisolid formulation resulting in a reduction in viscosity of the vehicle in its molten state which increases diffusion of the drug from the vehicle.[169] In addition, kinetic energy imparted to the drug particles increases their thermodynamic activity thereby increasing drug release. Higher temperatures are not justifiable as these may result in degradation of the drug contained therein or the loss of volatile components of the vehicle.

### 6.1.3 Membranes

The ideal membrane for assessing percutaneous absorption *in vitro* is purported to be dermatomed human skin (thickness of 200 µm) excised from the anatomical site of topical application.[26] However, the availability of both fresh and human cadaver skin is limited due to ethical reasons and the danger posed as a result of the diseases skin can harbour including HIV.[165] In addition, the permeability of the anatomical site from which the skin is taken varies quite significantly from site to site.[71,165,182] Subsequent distribution, biotransformation and excretion processes occurring in skin are not separated from percutaneous permeation thus complicating the interpretation of results.[26]

Artificial skin that has been successfully used in skin grafting and the treatment of burns has been used as the membrane for *in vitro* assessment of topical preparations. Living skin equivalent models which consist of a homogenous layer e.g. reconstituted epidermis,[26] have also been used but these tissues were found to be fragile thus providing a less substantial barrier. Although these systems eliminate the disadvantages of the use of live tissue, where large numbers of experiments are to be carried out, artificial skin availability may be a problem that is limited by cost issues. There may also be an overestimation of flux across the artificial skin.[64]

Skin from guinea pigs, monkeys, pigs and rats have been suggested as suitable replacements for human skin.[64] Snake skin has been used due to its similarity to human *stratum corneum* in terms of structure, composition, lipid and water permeability.[15,26]

Synthetic membranes are easily and widely available. They allow for the measurement of percutaneous absorption without interference of distribution, biotransformation and excretion processes.[26] The objective of *in vitro* drug release is to develop a method that is simple, reliable and reproducible to assure batch to batch uniformity.[165] The manufacturing process of commercial synthetic membranes ensures a precise pore diameter and a consistent pore size hence batch-to-batch homogeneity and uniformity. As such the commercially available synthetic membranes provide reproducible results hence their use by many researchers in the assessment of lot-to-lot and batch-to-batch variations, and their choice for this project. The membrane chosen should be inert towards the formulation but be permeable to the drug hence should not be a rate limiting step in the drug release process.[152,169] The membrane should act as a physical support and protect the semisolid dosage form from surface erosion. The membrane should not allow back diffusion.[27,152,169] Mesh screens might seem to be the best to use instead of a membrane as mesh screens only offer a supporting platform for the semisolid without interfering with the diffusion process. The mesh method works well with water insoluble or water immiscible formulation bases. Water miscible and water-soluble formulations are sloughed off leaving the formulation exposed to the receptor from all sides. This introduces channels in the formulations thus increasing diffusion from the resulting larger surface area. Consequently, surface area cannot be quantitated and hence no basis of comparison can be established.[165]

Durapore® membrane (Millipore Co., Billerica, Massachusetts, USA) was used for the assessment of creams. Synthetic Durapore® is a hydrophilic polyvinylidene fluoride (PVDF) membrane with pore size of 0.45 µm and is relatively inert. The Durapore® membrane was trimmed to fit the aperture of the receptor cell through which diffusion took place.

### 6.1.4 Receptor Phase

A number of receptor fluids have been used: 50% (v/v) ethanol: water, varying concentrations of polyethylene glycol (PEG 20), or glycerol and serum albumin in saline solution for the assessment of *in vitro* diffusion studies of lipophilic molecules.[71] Due to the insoluble nature of MF it was necessary to add a suitable solvent to facilitate solubility so as to achieve necessary concentrations above the limit of analytical quantification. PG was selected as previously explained (Section 5.3.1.2).

## 6.2 Method and Procedures

### 6.2.1 Preparation of Buffer

Sodium dihydrogen phosphate (0.1 M) and citric acid (0.05 M) buffer were used to prepare Mcllvaine buffer at different pHs (pH from 2.5 to 8). Sodium hydrogen orthophosphate (14.2 g - NaHPO4) (Associated Chemical Enterprises (Pty) Ltd, Johannesburg, South Africa) and 10.5 g citric acid granules (Pal Chemicals, South Africa) were dissolved separately per litre of HPLC grade water. The solutions were separately sonicated for about 5 minutes (Model B-12 Ultrasonic bath, Branson Cleaning Equipment Co., Shelton, Connecticut, USA). The solutions were mixed and then adjusted to the required pH by varying the proportion of the respective buffer solution component. Mcllvaine's buffer was used as it spans a large pH range (2-8). A Crison pH meter (GLP 21, Crison Instruments, Barcelona, Spain) was used for all pH measurements. The buffers were filtered under reduced pressure through a 0.45μm Durapore® PVDF membrane.

### 6.2.2 Preparation of Binary Compositions of Propylene Glycol/Water

Appropriate volumes of propylene glycol (Merck, Wadeville, South Africa) and HPLC grade water were mixed and added to a volumetric flask to make the following PG/water compositions: 10, 30, 50, 70 and 80 and 100% PG. The binary mixtures were briefly mixed by shaking by hand then sonicated for 5 minutes to ensure uniform mixing.

### 6.2.3 Solubility Studies

The Shake – Flask method was used for the solubility studies.[150,183] Excess (about 12.5 ± 1.5 mg) MF was weighed into a 10 ml Kimax glass test tubes and either Mcllvaine buffer or a binary composition of PG/water added to result in an approximate concentration of 1.25 mg/ml. Equilibrium was reached by either shaking on the oscillating water bath, Julabo PC, (Labotec, Johannesburg, South Africa) at 32 ˚C, or shaking on a Junior Orbit Shaker at ambient temperature at 200 rpm for 24 hours in all cases. At the end of 24 hours sample aliquots were filtered through a syringe filter, hydrophilic PVDF of pore size 0.45 µm (Millipore Co., Billerica, Massachusetts, USA). Five hundred microlitres of the filtered sample were then added to a vial containing 500 µl of methanol[184] to ensure that the drug did not precipitate at room temperature after removal from the water bath. The samples were analysed using HPLC.

### 6.2.4 Sampling Times

Diffusion is a relatively slow process hence assessment was made over 48 hours. Samples were taken at 2, 6, 10, 24, 30 and 48 hours after initiating the experiment in order to generate adequate points for the drug diffusion profile. At each sampling time the receptor compartment was completely emptied and re-filled with fresh receptor fluid.[164] The samples were kept at 4˚C until analysis by HPLC.

### 6.2.5 Assessment of Proprietary MF Formulations

The assessment of the proprietary formulations of MF was carried out using the conditions summarized in Table 6.1 below.

**Table 6.1 Diffusion study conditions**

| | |
|---|---|
| Membrane | Durapore® (PVDF) 0.45 µm |
| Formulation amount | 700 mg |
| Receptor phase | 70/30 PG/water |
| Temperature | 32 ˚C |
| Sampling times | 2, 6, 10, 24, 30, 48 hours |

Small star studded teflon coated magnetic stirrers were placed at the bottom of the cell containing the receptor fluid and the trimmed membranes were placed horizontally over the aperture of the diffusion cells between the receptor phase compartment and the donor phase compartment. The diffusion cell cap was secured into position using Parafilm 'M'® (American National Can™, Chicago, USA) and clamped tightly. The formulation (700 mg) was applied by spreading it on the exposed membrane area with a glass rod which was subsequently weighed to account for any possible removal of the dosage form. The donor cell was sealed using parafilm, an impermeable film and foil to prevent accidental contamination and drying up of the cream.[72] The diffusion cells were then filled with receptor phase fluid comprising 70/30 PG/water from the spout making sure that no air bubbles were present at the interface of the liquid and the membrane. The receptor phase was continuously stirred using the small magnetic stirrers. At predetermined time intervals, the receptor compartment was completely emptied via the injection spout shown in Figure 6.1 refilled with fresh receptor fluid and the concentration of MF in the samples was assayed by HPLC.

## 6.3. Data Analysis

### 6.3.1 Higuchi Kinetics

The Higuchi theory is valid under the following conditions:

- the percentage of drug released is not more than 30% of the total drug content in the applied formulation
- only a single drug species is included in the vehicle
- the diffusion coefficient does not vary with respect to time or position within the vehicle layer
- only the drug species diffuses out of the vehicle and sink conditions are maintained in the receptor phase.[185]

If the release pattern obeys the Higuchi model it means that the process of diffusion controls drug release from the formulation. The Higuchi equation predicts that a linear

plot of amount versus square root of time is obtained for an infinite dose of drug in a formulation.[186]

The kinetics of *in vitro* release studies as explained by the Higuchi model have been previously discussed and are summarized in Section 5.3.3.3. This model describes drug release from one side of a semisolid layer in which the drug is completely dissolved.[185]

### 6.3.2 Assessment of Pharmaceutical Equivalence

Pharmaceutical equivalence was assessed as previously discussed in Section 5.3.

## 6.4 Results and Discussion

### 6.4.1 Solubility

#### 6.4.1.1 Solubility in Mcllvaine Buffer

There were no significant differences in solubility at different buffer pHs after 24 hours. The solubility of MF in Mcllvaine buffer was found to be low, 0.7 µg/ml. Since the acid dissociation constant, pKa, of MF is 13.08 ± 0.20,[10] it remains virtually completely un-ionized throughout the entire pH range used of 2–8, thereby confirming its insoluble aqueous properties and lack of influence by pH.

**Table 6.2 Solubility of MF in Mcllvaine buffer at 32˚C in a water bath**

| pH | Solubility (µg/ml) |
|---|---|
| 2.5 | 0.7176 |
| 3 | 0.7313 |
| 4 | 0.7309 |
| 5 | 0.7225 |
| 6 | 0.7340 |
| 7 | 0.7208 |
| 8 | 0.7307 |

It was therefore necessary to use an appropriate solubilizer in order to maintain a workable concentration of MF in solution. Propylene glycol was thus chosen as the appropriate solvent using mixtures of PG/water.

### 6.4.1.2 Solubility in Binary Compositions of PG/water

MF solubility in the binary compositions of PG/water was found to be higher than that in aqueous buffer solutions. Solubility of MF in binary compositions of PG and water increased with an increase in PG content. At 10% PG content and ambient temperature the solubility was about 1 µg/ml. It increased by almost 2 00 times to 2 mg/ml at 100% PG. At 70/30 PG/water and ambient temperature, the solubility was found to be about 170 µg/ml. At this composition, the solubility of MF at 32˚C in the water bath was about 163 µg/ml. Above 70% PG the solubility of MF in the water bath at 32 ˚C reached a maximum and deviated from the results obtained from the shaker as depicted in Figure 6.2 below.

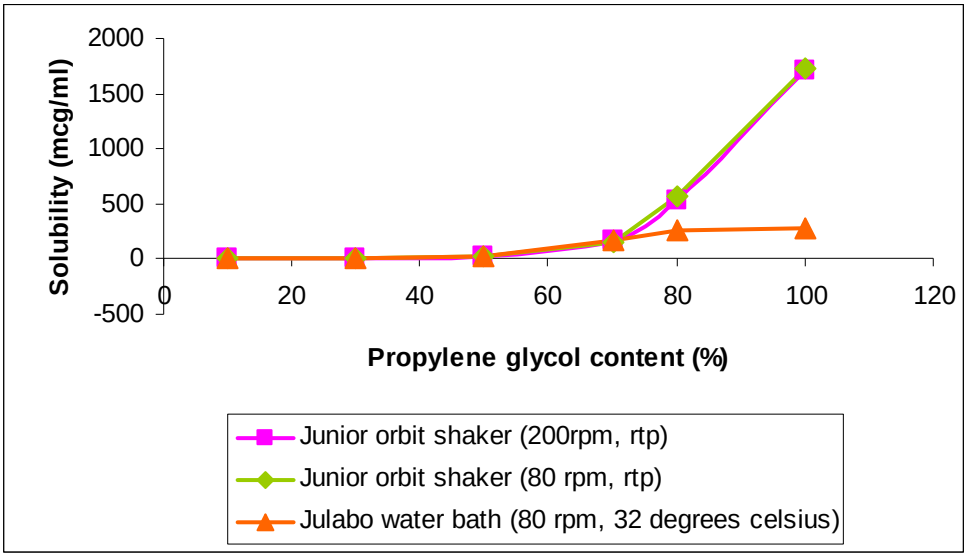



**Figure 6.2 Solubility of MF in binary compositions of PG/water under different conditions**

Results obtained show that the Junior Orbit Shaker mixed the drug and solvent much better than the water bath; higher solubility was observed at ambient temperature on a Junior Orbit Shaker than with the shaking water bath at higher temperature. Almost the same solubilities were observed in mixtures which had low content of PG, i.e. below

70%. This may be explained by the fact that below 70% PG the binary solutions are fluid enough to ensure adequate mixing with MF. At higher PG content, higher viscosity solutions need more force to be well-mixed. The Julabo shaking water bath which shakes the solutions with a relatively gentle side-to-side movement was not adequate, hence true solubility equilibrium was not reached.

Differences between the solubility after shaking for 24 hours at either 80 rpm or 200 rpm were indiscernible. The rotational shaking motion of the Junior Orbit Shaker was effective in ensuring complete mixing.

The solubility of MF in the binary compositions of PG/water tended to follow a logarithmic sequence. Semi-logarithmic relationships have been observed for other corticosteroids such as ethynodiol diacetate.[187] Betamethasone 17-valerate, hydrocortisone and hydrocortisone 17-butyrate give higher but comparable results as a function of solubility in binary compositions of PG.[188]

### 6.4.2 Stability of MF in 70/30 PG/water

MF was found to be sufficiently stable after one month storage in 70/30 PG/water in a refrigerator at 4˚C with the maximum percent of drug lost being less than 10% As shown in Figure 6.3. The pH measured at the end of the study was found to be 5.9. The slightly acidic pH may partly explain the stability of MF in PG/water.

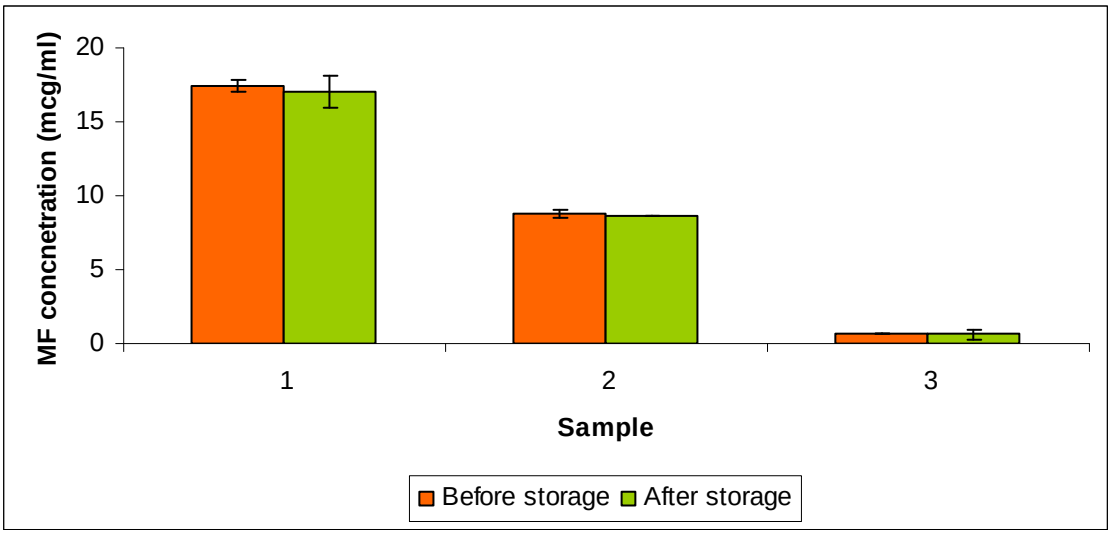



**Figure 6.3 MF stability after one month of storage in 70/30 PG/water at 4˚C**

The stability of MF in 70/30 PG/water allows this binary mixture to be used in the diffusion studies. The diffusion experiments were carried out over two days and the collected samples were refrigerated at 4 ˚C until the end of the experiment. The samples were then analysed on the third day. The demonstrated long stability of refrigerated MF ensures that no significant decomposition would have taken place between sampling, storing and analysing.

### 6.4.3 Receptor phase selection

PG (70%) mixed with water(30%) was chosen for use as the receptor phase in the Franz cell diffusion studies for comparative purposes with microdialysis of the same formulations (Chapter 5). The receptor fluid used should be highly fluid, i.e. non-viscous. At 70/30 PG/water the binary mixture is quite fluid and this is necessary to ensure that air within the membrane pores has been replaced completely by the liquid. The surface tension of the receptor fluid should be less than or equal to the critical surface tension for perfect wetting.[176] A combination of 70/30 PG/water is a good compromise between affording sink conditions in the diffusion cell receptor compartment and preserving the integrity of the HPLC column. The viscosity of PG may lead to increased operating column back-pressure hence incorporation of water to reduce the viscosity was useful.

### 6.4.4 Assessment of Proprietary Formulations

The *in vitro* release profiles of the different proprietary MF products measured through a synthetic membrane using the Franz diffusion cell are illustrated in Figure 6.4.

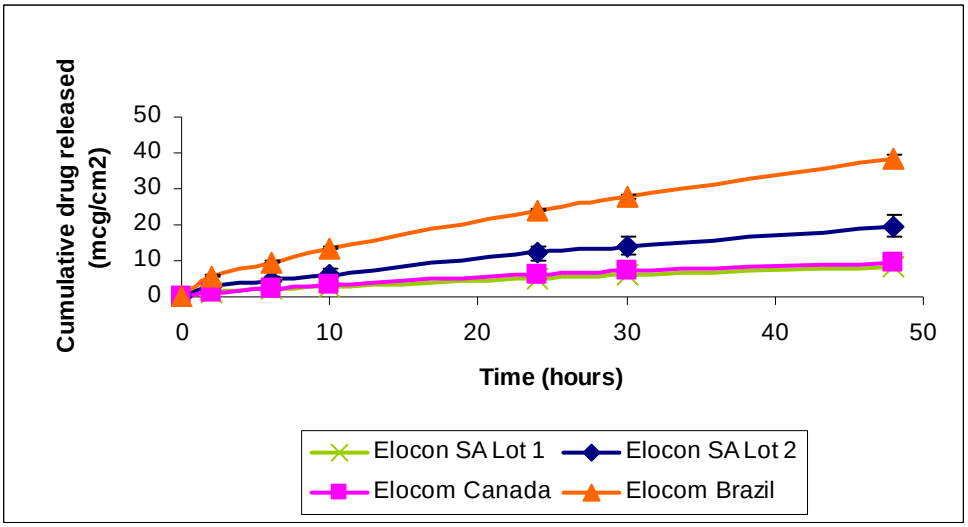



**Figure 6.4 Drug release profiles from 0.1% MF proprietary creams**

Drug release from Elocom cream (Brazil) was highest, followed by Lot 2 of the South African Elocon® cream. The release rates from Elocon® (South Africa) Lot 1 and Elocom™ (Canada) were virtually equal. Contrary to expectations, Elocon® cream (South Africa) Lot 1 showed a significantly lower release rate of MF compared to Lot 2 of the same manufacturer. The analysis of variance showed that there were significant differences among the formulations ($p=0.0116$, ANOVA). Further analyses using Bonferroni *post hoc* test showed that the significant differences were between the Canadian and Lot 1 of the South African creams when compared with Elocom cream (Brazil).

Changes in the manufacturing process, storage conditions or ambient conditions prevailing during processing time may cause differences in the release rates of the creams from the same supplier. This can possible be due to variable exposure conditions during transportation of the creams from the manufacturing plant to the community pharmacy. Transportation may cause settling of the cream due to vibration whereas variation in

temperature, especially excessive heat, may also cause the formulation to melt leading to separation of constituents and result in differences in the distribution of the API in the formulation.[15]

**Table 6.3 Comparison of drug release kinetics from MF creams**

| Formulation | Apparent release constant ($\mu g/cm^2/hr^{½}$) | Lag time ($hr^{½}$) | Correlation coefficient ($r^2$) |
|---|---|---|---|
| Elocon® Lot 1 | 1.4 | 0.9 | 0.9875 |
| Elocon® Lot 2 | 3.3 | - | 0.9936 |
| Elocom™ Canada | 1.5 | 0.8 | 0.9935 |
| Elocom Brazil | 5.9 | 0.7 | 0.9991 |

*In vitro* drug release from ointment formulations was negligible compared to that from creams. The cumulative amount of drug released from the Elocon® cream (South Africa) Lot 1 was greater than 200 $\mu g/cm^2$ whereas about 2 $\mu g/cm^2$ was released from the ointment (Elocon®, South Africa). This may be attributed to the different vehicles used in the respective formulations or other possible effects due to the interaction with vehicle components and the membranes.

MF is lipophilic hence is likely to be more soluble in the ointment as compared to the receptor phase leading to MF being retained in the ointment. When an ointment vehicle is used which is non-miscible with the receptor phase the synthetic membrane should be impregnated with lyophillic material such as isopropyl myristate.[161] For comparative purposes to the microdialysis set-up, impregnation of the membrane with solvent was omitted. Further analysis of the ointments was abandoned as the resulting low drug concentrations obtained could not be used to assess pharmaceutical availability.

The cumulative amount of drug released per unit area was linear and directly proportional to the square root of time. The slope, ARC (steady state flux) was calculated by linear regression. Drug release from all the cream formulations appeared to fit the Higuchi model ($r^2$>0.9800). Such correlation coefficients indicated that the release of MF from the cream formulations was probably diffusion controlled.

The drug incorporated into a formulation must leave the vehicle and reach the skin surface at an adequate rate and in sufficient amounts for therapeutic effectiveness.[76] This rate may be quantitatively assessed for generic formulations using pharmaceutical availability (*in vitro* release) testing. The assessment of the comparative ARCs, lag times and time taken to achieve the maximum rate can be compared to determine pharmaceutical equivalence. Statistical analysis involving the use of ANOVA was used to assess the pharmaceutical equivalence of each test product compared to the reference product, Elocon® Lot 2. The results of the ANOVA testing are tabulated in Table 6.4.

**Table 6.4 ANOVA results for pharmaceutical equivalence**

| Formulation | Mean difference | p-value | 95% CI for difference | | Significant/Not significant |
|---|---|---|---|---|---|
| | | | Lower limit | Upper limit | |
| Elocon® Lot 1 | -1.895 | p<0.001 | -2.510 | 1.280 | Significant |
| Elocom Brazil | -2.696 | p<0.001 | -3.311 | -2.081 | Significant |
| Elocom™ Canada | 1.748 | p<0.001 | 1.1320 | 2.363 | Significant |

Significant differences were observed between the reference product and Elocom™ cream (Canada). Bioequivalence assessment using the HSBA is the accepted method and hence provides the yard stick against which other methods are evaluated. The results of the Franz cell diffusion study showed that the difference between the Canadian cream and the reference products is very high ($p<0.001$). The Franz cell diffusion study appears to be quite discriminatory compared to *in vitro* microdialysis. All the test products showed significant differences with the reference product ($p<0.0001$, ANOVA). Significant differences were also observed between Lot 1 and Lot 2 of the Elocon® cream (South Africa) ($p<0.001$, Bonferroni *post hoc*).

Discrimination of a method depends upon the variables in the study. To validate *in vitro* methods against HSBA, a series of experiments have to be carried out. These should be in an attempt to produce the same rank order for the test products as the ones for the reference HSBA. As such more than three pharmacodynamic studies must be conducted

and the data ranked. The rank order resulting from the HSBA can then be compared to other *in vitro* methods with proper assessment.

## 6.5 Conclusions

MF was found to be readily soluble and stable in 70/30 (PG/water). As such the 70/30 PG/water was used as the receptor fluid.

The data obtained from the Franz diffusion study indicated that formulations of the same strength do not necessarily release the API at the same rate. Differences in the vehicle composition of the formulation either qualitatively or quantitatively may result in deviations in the drug release profiles of the formulations containing the same amount of the same drug.

The Franz cell diffusion study results were comparable to those obtained in microdialysis. In both methods, Elocom cream (Brazil) had the highest release rate followed by Elocon® cream Lot 2 which was also the reference product and Elocom™ cream (Canada) at the lower end. Drug release from Elocom™ cream (Canada) and Elocon® cream Lot 1 was almost the identical and these two formulations had the lowest drug release rates in the Franz cell diffusion study. Whereas Elocon® cream Lot 1 and 2 were significantly different from each other in the Franz cell diffusion study, these two lots did not show any differences in the microdialysis study. The Franz diffusion study method appears to be more discriminatory statistically compared to the microdialysis. To ascertain if these differences are real further studies are necessary. It is necessary to assess bioavailability of MF from Elocom cream (Brazil) and Elocon® cream Lot 1 compared to that from the reference Elocon® cream Lot 2. Based on the results from such a study, the statistical discrimination seen in the Franz cell diffusion study may be explained.

# CONCLUDING REMARKS

Patents on the early and most commonly used topical corticosteroids expired over 20 years ago resulting in a large number of generic formulations being released onto the South African market as well as all over the world. Comparative bioavailability for the purpose of establishing bioequivalence between the innovator or brand (reference) product and a generic (test) are necessary to establish that the safety and efficacy will be the same during clinical use.

A system was developed and subsequently patented for the handling and manipulation of a chromameter and which was found to be a more reliable and reproducible method of assessing skin blanching and skin colour, in general. Using this system, pressure applied to the application site during skin colour measurements was adequately controlled and consequently no significant differences were observed between operators using this patented system. Changes in skin colour due to circadian rhythm are difficult to monitor visually whereas use of the patented chromameter successfully assessed baseline skin colour changes in different races. This was important since changes in skin colour due to endogenous factors were more apparent in Caucasians compared to the other races.

At low dose durations the chromametric method is more discriminatory than visual assessment. Contrary to the expected, a* scale readings showed a stronger correlation with visual assessment compared to EDs. As a result the a* scale readings were used in the bioequivalence data analysis.

The results obtained from a pilot bioavailability study showed that the probability of a “responder” also being a “detector” was 50%. Based on those results 12 out 24 subjects were expected to be “evaluable” according to the FDA guidance. However, only seven were subsequently found to be evaluable following a pivotal study. A larger number of evaluable subjects would thus be necessary for adequate statistical power to establish bioequivalence. Although definitive conclusions on bioequivalence could not be drawn from the blanching study due to insufficient statistical power as a result of relatively low numbers of evaluable subjects, it can be inferred from the mean AUC ratio of the test to

the reference product (74%), that MF release from an MF cream marketed in Canada (Elocom™) 0.1% was lower than that from the South African product (Elocon® Lot 2).

The analysis of blanching profiles requires the use of methods that analyse the whole profile rather than multiple single point comparisons. It is proposed that future studies attempt to analyse blanching study profiles using mathematical functions. A typical parameter obtained from such mathematical functions is to determine the value of the slope after regression analysis. The derived parameters of the models are then compared with those from the reference product using ANOVA or the t-test. Mathematical functions that describe the whole profile are simpler to use for the evaluation of pharmaceutical equivalence. This makes it easier to compare pharmaceutical equivalence.

An HPLC method was successfully developed and validated. However, the limit of quantitation (LOQ) indicated that using HPLC would not provide the necessary sensitivity to measure MF in the dialysate following *in vivo* microdialysis.

*In vitro* microdialysis has good potential for use as a tool to monitor the release of MF (and other compounds) from topical dosage forms in order to assess pharmaceutical availability. Diffusion experiments using Franz cells provided data that was relatively in good accordance with the *in vitro* microdialysis data. Whereas the *in vitro* data obtained from microdialysis and Franz cells were compared with the *in vivo* data obtained from the HSBA, the low number of evaluable subjects from the pivotal HSBA study was insufficient to assess bioequivalence of the 2 creams tested. Hence, in order to utilise *in vitro* data as a surrogate measure for bioequivalence, many more subjects will need to be used in HSBA studies to validate both procedures and correlate them with *in vivo* data obtained from topical dosage forms. Such an IVIVC model will provide a valuable tool for the assessment of the bioequivalence of topical dosage forms that contain active compounds that do not blanch.

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