INVESTIGATIONS OF THE ASSESSMENT OF BIOEQUIVALENCE OF TOPICAL CLOTRIMAZOLE PRODUCTS USING A DERMATOPHARMACOKINETIC APPROACH

A thesis submitted to Rhodes University in fulfilment of the requirements for the Degree of

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by

Natalie Rae Parfitt
B.Pharm

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Faculty of Pharmacy
Rhodes University
Grahamstown
South Africa

ABSTRACT

The specialised nature of the *stratum corneum* makes it an efficient barrier to foreign substances, including drug molecules. Therefore, cutaneous drug absorption is a slow and complex process of which *stratum corneum* penetration is the rate limiting step. The rate and extent of *stratum corneum* penetration by a drug compound depends greatly on the presence of penetration enhancing/retarding excipients and therefore the clinical outcomes of a product rely greatly on the components and quality of the formulation. Hence, establishing bioequivalence between topical products is crucial to ensure that patients receiving multisource drug products are assured of the same efficacy and safety as the brand product.

Since locally acting topical formulations do not target the systemic circulation, conventional methods of assessing bioequivalence using plasma levels are not appropriate. Consequently, the current regulatory guidelines require comparative clinical trials to be carried out to show bioequivalence between topical products. As these studies are very expensive and time consuming, the development of a more direct and relatively rapid and inexpensive method for determining bioequivalence between topical products is required.

Clotrimazole is an anti-fungal agent where the target site of action is in the *stratum corneum*. In this work, tape stripping, which involves the sampling of *stratum corneum*, was investigated as a tool for the determination of bioequivalence between topical clotrimazole products. The tape stripping method involved the analysis of each tape strip individually and standardization of *stratum corneum* thickness between subjects was carried out using TEWL measurements. This approach provided detailed information regarding the amount of clotrimazole present in the *stratum corneum* as well as the extent of drug penetration.

Prior to the tape stripping studies an HPLC method was developed for the quantitative analysis of clotrimazole from the tape strip samples. This method was shown to be

accurate and reproducible across the required range. It was also shown to be selective for clotrimazole in the presence of possible interfering substances such as those present in the tape adhesive and also skin components.

The bioequivalence studies were conducted using a single “uptake” time point. In order to determine an appropriate dose duration for these studies a novel approach was employed, involving a preliminary dose duration study. For the bioequivalence investigations, Canesten® Topical cream was used as both test and reference products to determine if the method was capable of showing bioequivalence. Subsequently, Canesten® Topical cream was also compared to a 1 % gel formulation to determine if the method could detect formulation differences.

The conventional BE limits of 0.8 – 1.25 were used for the assessment of BE, however, the clinical relevance of using these limits for dermal studies is debatable since they are derived from oral pharmacokinetic studies. Therefore, the data from the tape stripping investigations were also assessed using more realistic limits of 0.75 – 1.33 and even 0.7 – 1.44.

In addition to the tape stripping studies a novel method of determining the amount of drug present in the *stratum corneum*, the “Residual Method”, was investigated. This method involved assaying the amount of clotrimazole found in the residual formulation after a specified dose duration had elapsed and subtracting that amount from the amount of clotrimazole initially applied.

The results of tape stripping investigations showed that, if the study is sufficiently powered, tape stripping may be used to determine bioequivalence according to the conventional limits, as well as possibly detect formulation differences between different clotrimazole products. Bioequivalence assessment using the widened intervals showed that fewer subjects were required to achieve a sufficient statistical power. The variability associated with this method was acceptable and tape stripping may therefore have the

potential to be used as a BE tool in a regulatory setting for clotrimazole or other antifungal topical formulations.

The “Residual Method” also showed promising results as a bioequivalence tool, but further investigation and extensive validation of this method is required before it can be suggested as a regulatory method.

The results of these studies have clearly indicated that tape stripping has the potential to be used as an alternative to comparative clinical trails for the assessment of bioequivalence between clotrimazole formulations and also to assess bioequivalence between other antifungal products.

This dissertation is dedicated to

my parents,
Royston Parfitt and Brigitte Parfitt.

Thank you for every opportunity you have given me.

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can be found on the accompanying compact disc

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
A_{max}	Maximum Amount
ANDA	Abbreviated New Drug Application
AUC	Area Under the curve
AUFS	Absorbance Units Full Scale
BA	Bioavailability
BE	Bioequivalence
$CI_{90\%}$	90% Confidence Interval
CLZ	Clotrimazole
C_{max}	Maximum Plasma Concentration
CRF	Case Report Form
CRS	Confocal Raman Spectroscopy
CV	Coefficient of Variance
CZE	Capillary Zone Electrophoresis
DMD	Dermal Microdialysis
DPK	Dermatopharmacokinetic
EPA	Environmental Protection Agency
FDA	Food and Drug Administration
GC	Gas Chromatography
HETP	Height Equivalent Theoretical Plate
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
HSBA	Human Skin Blanching Assay
ICH	International Conference of Harmonisation
IEC	International Electrotechnical Commission
ISO	International Organisation of Standardisation
IVIVC	*In vitro – in vivo* correlation
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantification
MCG	Membrane-Coating Granules
MECK	Micellar Electrokinetic Chromatography
MIC	Minimum Inhibitory Concentration
MS	Mass Spectroscopy
NDA	New Drug Application
NP	Normal Phase
PDA	Photodiode Array
RP	Reverse Phase
RSD	Relative Standard Deviation
R_t	Retention time
SC	*Stratum corneum*
SD	Standard deviation
SPE	Solid Phase Extraction
TEWL	Transepidermal Water Loss
TLC	Thin Layer Chromatography
TS	Tape Stripping
USP	United States
UV	Ultraviolet
UV/Vis	Ultraviolet-visible

CHAPTER 1

INTRODUCTION TO SKIN STRUCTURE, CLOTRIMAZOLE AND BIOAVAILABILITY / BIOEQUIVALENCE OF LOCALLY ACTING TOPICAL PRODUCTS

1.1 STRUCTURE AND FUNCTION OF THE STRATUM CORNEUM

1.1.1 The epidermis

The skin is the largest organ of the body, with many essential functions including thermoregulation, excretion and protection from mechanical, chemical, organismic and radiation damage. The skin is comprised of two layers: the dermis and the epidermis. The lower layer, the dermis, is made up of connective tissue, blood capillaries and lymphatic vessels. The epidermis is the avascular component which lies above the dermis and can be histologically defined as stratified squamous keratinizing epithelium tissue (1). Various types of cells are found in this layer including keratinocytes, melanocytes, Langerhans cells and Merkel cells. Keratinocytes are the most abundant. The keratinocytes are produced by the basal layer and move upward towards the surface of the skin to replace the outermost cells which are continually sloughed from the surface (1). As they migrate upwards they go through a radical differentiation process which has lead to histologists defining layers within the epidermis according to the cells' appearance (Figure 1.1).

The lowest layer of the epidermis, the *stratum basale*, acts as an attachment surface to anchor the epidermis to the dermis and also functions as the ectoderm for the keratinocytes. It is separated from the dermis by a thin membrane, the *basal lamina*, which acts as the area of contact between the epidermis and dermis.

As the keratinocytes progress towards the surface of the skin the cells start to differentiate, and appear to be studded with spiny projections. This has led to the next layer being known as the *stratum spinosum*. These spinous cells contain secretory granules known as membrane-coating granules (MCGs) which contain various lipids (1).

The layer above the *stratum spinosum* is the *stratum granulosum*. Its name originated because the cells contain many granules which become noticeable when the cells are stained with haematoxylin (1). These granules contain histadine-rich proteins that aid in the skin's inflammatory response (2). In this layer, the MCGs are positioned at the surface of the cell where their membranes fuse with the cell membrane and their lipid contents are discharged into the intercellular space.

Above the *stratum granulosum* the cells enter the *stratum lucidum*. In this layer the granular squamous epithelial cells lose their organelles and are transformed to cornified cells. The name of this layer is derived from the bright appearance of these cells when stained, which makes them easier to see than the older cornified cells (1). Here the cells are reinforced with kertatin filaments and the intercellular space is filled with a lipid matrix which imparts the water permeability barrier function.

The outermost layer is the *stratum corneum* (SC). This layer consists of older cornified cells. These cells are further strengthened by the proteins involucrin and keratolinin, which are added to the inner surface of cell membrane (1,3).

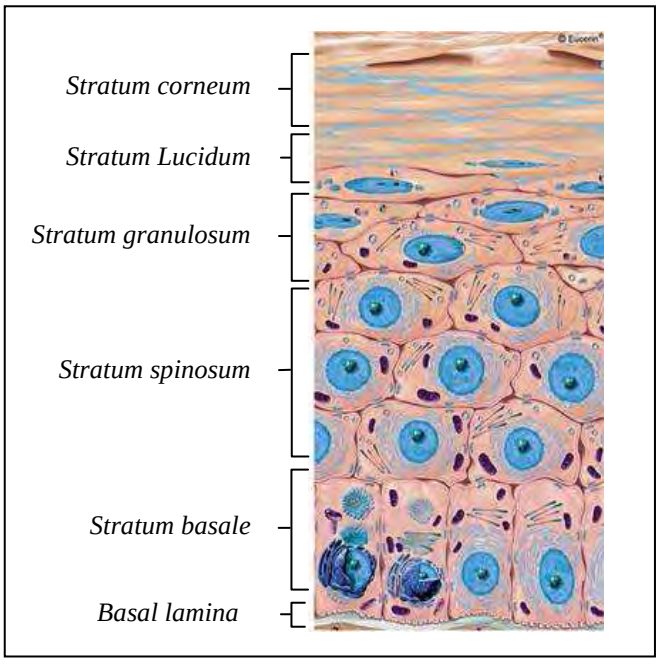


Figure 1.1: The histologically defined layers of the epidermis (4).

1.1.2 The *stratum corneum*

As the outermost layer of the epidermis, the SC is directly exposed to the external environment. It evolved to protect the sensitive and moist skin cells below it from abrasion and desiccation and plays a vital role in preventing the body from being damaged by the environment. The evolution of the SC is first seen in early reptiles and has been described as the "single most significant step in the evolution of tetrapod skin" (5).

The structure of the SC has been compared to "bricks and mortar" with the flattened keratinized cells as the "bricks" embedded in the lipid matrix which functions as the "mortar" (6). This effective design, depicted in Figure 1.2, provides the mechanism which allows the SC to function as an efficient water barrier.

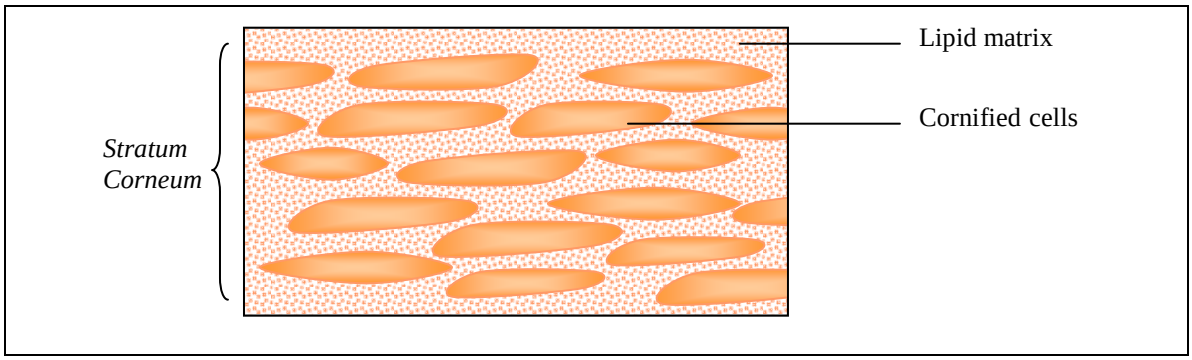


Figure 1.2: The "bricks and mortar" composition of the SC layer.

The physical attributes of the SC vary greatly depending on the anatomical site investigated (7-9), seasonal changes (10,11), and possibly gender (12). Differences in pH gradients within the SC do exist between Negro and Caucasian skin types, but, this affects only the outermost surface cells (13). One study investigating skin from the hip region has shown that the SC is comprised of 14-28 cell layers and ranges in thickness from 6.2 to 19.4 μm. In that study the density of the SC tissue was shown to vary greatly between individuals and was reported to range from 0.88 to 142 $g.cm^{-3}$ with a mean value of 1.20 (14). Most studies using SC density as a parameter assume the density of the SC to be 1 $g.cm^{-3}$ (15-19).

1.1.3 Fungal infections of the *stratum corneum*

The SC plays an important role in defending the body from pathogenic attack. It has a low water content and an acidic pH which makes for an unfavourable environment for the growth of pathogens (20). Commensal microflora and surface-deposited antimicrobial lipids are also present, which help defend against foreign organisms (20). However, some fungi have evolved to circumvent these measures. These organisms thrive in the SC environment as it has a rich supply of keratin that they use to produce chitin - an essential component in their cell wall structure (21). The group of dermatophytes responsible for cutaneous fungal infections comprises of the genera *Microsporum*, *Epidermophyton* and *Trichophyton.*

1.1.4 Topical drug delivery

When using pharmacotherapy to treat conditions of the SC, the use of the oral/systemic drug delivery route is not appropriate since the SC is an avascular tissue. Therefore, the use of topical preparations to treat SC conditions makes use of a more direct and efficient route. However, the SC provides such an efficient barrier to exogenous substances that drug penetration using this topical route is often very slow and only a very small percentage of the drug applied will penetrate the SC. Thus, when using the topical route for drug administration the penetration enhancing excipients and the quality of formulation play a pivotal role in the clinical outcomes of the therapy.

1.2. CLOTRIMAZOLE

1.2.1 History of clotrimazole

Prior to the 1950s the only treatments available for cutaneous mycoses were remedies such as Whitfield's ointment and Gentian Violet (22). These treatments were non-specific to the fungal pathogens and unpleasant for patients to use. In the early 1950s researchers started to look for more specific antifungal agents. This led to the discovery of the first

antimycotic azole, chlormidazole, in 1958 (23). Its action was specific to fungal cells, but it lacked potency (22). Fortunately the chemical arrangement of this compound allowed researchers to alter its structure with relative ease, thereby improving its activity. In 1969 the substantially more active compound Bay b 5097 was synthesised using chlormidazole as a lead compound (24). Bay b 5097 was registered as clotrimazole (CLZ) and marketed by Bayer (Pty) Ltd as the now well-established brand Canesten®. Today there are numerous generic CLZ products available world wide. Opportunistic superficial mycoses are often a complication for immuno-compromised patients and with the current prevalence of HIV/AIDS the use of topical antimycotic drugs such as CLZ still have an important role to play in modern medicine (25).

1.2.2 Physicochemical properties

1.2.2.1 Description

i) Organoleptic properties:

CLZ is a white or pale yellow, odourless, tasteless crystalline solid (26,27).

ii) Chemical formula and molecular mass:

CLZ has the molecular formula $C_{22}H_{17}ClN_2$ and a molecular mass of 344.84u (26,27).

iii) Chemical structure and name:

The chemical name for CLZ is 1-[(2-chlorophenyl)diphenylmethyl]-1*H*-imidazole(26). As can be seen in Figure 1.3, at the center of the CLZ molecule is an asymmetric carbon atom. The imidazole group joined to this carbon acts as the active pharmacophore. This, and the halogen-substituted benzene ring, are common features of all azole compounds (22).

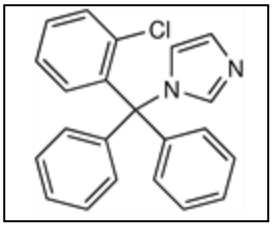


Figure 1.3: The chemical structure of CLZ (28).

1.2.2.2 Melting point

CLZ has a melting point of 141 °C to 145 °C (26).

1.2.2.3 Solubility

CLZ is a very lipophilic compound, making it practically insoluble in water. The table below (Table 1.1) shows the solubility of CLZ in some common solvents.

Common Solvent	Solubility at 25°C (mg/ml)
Benzene	>100
Chloroform	>100
Methanol	>100
Ethanol USP	95
Acetone	50
Propylene glycol	35
Diethyl ether	14
Mineral oil	0.8
Water	<0.01

Table 1.1: Solubility of CLZ in common solvents (27).

1.2.2.4 Dissociation constant

In an aqueous/ethanol solution (1:1) CLZ has a pKa = 4.7 (27).

1.2.2.5 Partition coefficient

The partition coefficient of CLZ, $[CLZ]_{oct}/[CLZ]_{water}$, has been reported as 704.5 (29).

1.2.2.6 Ultraviolet (UV) absorption spectrum

As can be seen from Figure 1.4, a methanolic CLZ solution has a high UV absorbance at low wavelengths (210 – 240 nm) and a λ_{max} at 260 nm.

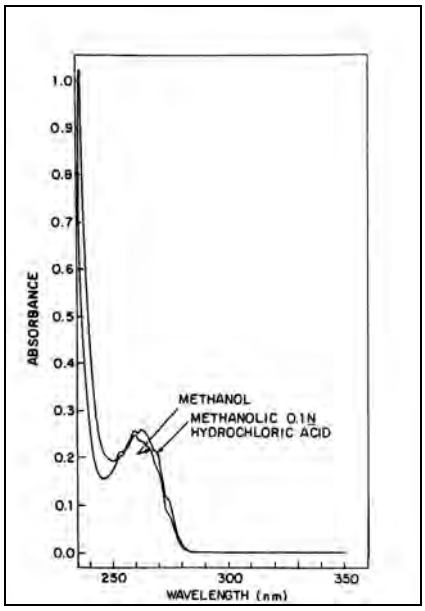


Figure 1.4: Ultraviolet spectra of a methanolic CLZ solutions (27).

1.2.2.7 Stability

When in solution, the stability of CLZ is dependent on the pH of the environment. As shown below (Figure 1.5), it rapidly hydrolyses in an acidic medium to form the degradation products diphenylmethanol and imidazole (27).

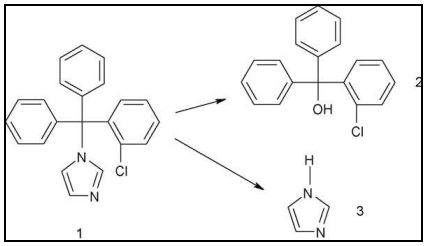


Figure 1.5: CLZ [1] and its degradation products diphenylmethanol [2] and imidazole [3] (30).

1.2.2.8 Storage

CLZ should be protected from light during storage (26).

1.2.3 Pharmacological properties

1.2.3.1 Classification

CLZ is classified as an antifungal agent and falls under the class of antifungal imidazole derivatives (31).

1.2.3.2 Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) is used to describe the potency of an anti-infective agent. It is defined as the minimum concentration of a drug required to inhibit organism growth by 100 %. In order to determine the MIC, an agar plate inoculated with a specific pathogen is spiked with a solution of CLZ. As can be seen in Table 1.2, for most harmful fungal pathogens the MIC for CLZ is less than 1 ug/ml (32).

Organism	MIC (ug/ml)	Organism	MIC (ug/ml)
E. floccosum	0.125–1	*T. balcaneum*	0.03–0.25
M. audouinii	0.06–0.25	*T. erinacei*	0.125–1
M. canis	0.01–4	*T. interdigitale*	0.06–1
M. ferrugineum	0.03–0.5	*T. mentagrophytes*	0.03–2
M. fulvum	0.125	*T. ajelloi*	1
M. gallinae	0.03	*T. rubrum*	0.03–2
M. gypseum	0.125–2	*T. simii*	0.25–0.5
M. praecox	0.06	*T. tonsurans*	0.03–1
M. racemosum	>16	*T. violaceum*	0.06–1

Table 1.2: MICs of CLZ required for various common pathogens (32).

1.2.3.3 Indications

Topical CLZ products are indicated for the treatment of cutaneous candidiasis (fungal infection caused by the *Candida* species, of which *Candida albicans* is the most common) and dermatophytoses (fungal infections of keratin in the skin and nails). It may also be useful in the treatment of tinea vesicolor (a common skin infection caused by the yeast *Malassezia furfur*) and *Corynebacterium minutissimum* (a bacterium which causes a superficial skin infection marked by reddish brown scaly patches known as erythrasma.)

These products are also indicated for skin diseases where there is a secondary infection caused by these pathogens. CLZ vaginal creams and pessaries are widely used to treat vulvovaginal candidiasis and CLZ lozenges are indicated for the treatment and prophylaxis of oropharyngeal candidiasis. A 1 % CLZ solution may be used for the treatment of fungal otitis externa (33).

1.2.3.4 Dosage

When using CLZ to treat cutaneous mycoses a 1% topical product should be applied to the skin 2 to 3 times a day for up to 4 weeks. When treating vulvovaginal candidiasis CLZ pessaries or vaginal creams are used with a dosing regimen of 100 mg daily for 6 days, 200 mg daily for 3 days or a single 500 mg dose. Vaginal creams are available in 1, 2 or 10 % strengths. When treating oropharyngeal candidiasis with CLZ lozenges a 10 mg lozenge should be dissolved in the mouth 5 times daily for up to 2 weeks. In immuno-compromised patients one lozenge sucked three times a day can be used as a prophylactic measure against oral candidiasis (33). CLZ is not a good candidate for oral use as it causes gastrointestinal side effects (34) and is a potent enzyme inducer (35).

1.2.3.5 Mode of action

Ergosterol is an important component of the fungal cell membrane and helps maintain the cells' integrity in a similar way to cholesterol found in animal cells(24,36). Ergosterol is synthesised in the fungal cells from lanosterol. The enzyme responsible for converting lanosterol to ergosterol is the CYP450 enzyme 14–α–demethylase. CLZ antagonises this enzyme thereby inhibiting ergosterol synthesis (36). The lack of ergosterol alters the structure of the fungal cell membrane causing an increase in cellular permeability. This causes the contents to leak out of the fungal cells thereby destroying them. The action of CLZ is specific to the particular pathogen and does not affect the integrity of the host cells (37).

1.2.3.6 Site of action

Cutaneous mycoses are caused by pathogenic dermatophytes invading the outermost layer of skin tissue, i.e. the SC (34). This layer of skin is made up of dead keratinized

cells on which the dermatophytes feed to obtain nitrogen for cell wall growth (21). The SC is therefore considered the site of action for antifungal compounds such as CLZ (38-40).

1.2.3.7 Adverse effects

Local reactions, including skin irritation and a burning sensation, may occur following topical treatment. Contact allergic dermatitis has been reported after topical use (33,41). It has been shown that topical exposure to CLZ can influence metabolising enzymes in the epidermis, however liver enzymes are not induced through topical application (42).

Nausea, vomiting, unpleasant mouth sensations and pruritus have been reported after the use of oral CLZ lozenges (33).

Intravaginal preparations of CLZ may damage latex contraceptives and additional contraceptive measures are therefore necessary during local application (33,41)

Gastrointestinal disturbance, dysuria and mental depression have been reported after the use of CLZ via the oral route. In cases of systemic absorption, lower abdominal cramps, increase in urinary frequency or skin rash may occur (33,41). Elevation of liver enzymes creating anti-androgenic effects has been reported after CLZ ingestion (35,37).

1.2.3.8 Contraindications

Topical 1 % CLZ creams are contraindicated for patients who are hypersensitive to CLZ and/or cetosteryl alcohol, the latter being an included excipient in the brand product, Canesten® (41).

1.2.4 Pharmacokinetics

1.2.4.1 Oral administration

CLZ is well absorbed when administered orally but plasma levels are not sustainable and decrease after two weeks of daily therapy (35). Single dose studies of 1.5 g and 3 g have

shown that CLZ has a C_{max} of approximately 2 hours, but there is great variability in peak concentrations, implying that absorption is erratic (43).

1.2.4.2 Topical administration

Some imidazoles, including CLZ, are reported to have an affinity for keratin (the main component of SC cells). It is suggested that this binding to keratin causes a long-term retention of the antifungal in the skin (44) While keratin binding is thought to decrease antifungal activity *in vitro* the bond appears to be weak and reversible, thereby not significantly hindering the performance of the drug (44).

1.2.4.3 Topical preparations

The available dosage forms of CLZ listed in the United States Pharmacopoeia (USP) are tabulated below:

Dosage form	**Condition it is used to treat**
Topical Solutions*	cutaneous mycoses
Topical Lotions*	
Topical Creams	
Vaginal Creams	vulvovaginal candidiasis
Vaginal Tablets	
Lozenges	oral candidiasis

*Dosage forms not available in South Africa(45)

Table 1.3: Available dosage forms of CLZ (46).

In South Africa available dosage forms of CLZ are topical and vaginal creams, pessaries and throat lozenges (45). The USP (46) and Martindale (33) also list topical solutions and lotions as possible dosage forms of CLZ but these are not marketed in South Africa. Gel and spray formulations are not mentioned as CLZ dosage forms in these references, however, these formulations are on the market in India (47,48).

CLZ creams are mostly manufactured as single-ingredient preparations, but combination formulations have also been developed. The USP lists one CLZ combination product, namely CLZ and betamethasone dipropionate cream (46). CLZ has also been used in combination with antibiotics to treat/prevent bacterial infection and various other

corticosteroids to reduce inflammation. Some examples include Neocip FC® (Cipla, India) which contains CLZ (1 %) and the fluoroquinolone antibacterial ciprofloxacin (0.5 %) as well as a corticosteroid, fluocinolone (0.025 %). Candiderma® (Glenmark, India) contains CLZ (1 %), beclometasone dipropionate (0.025 %) to reduce inflammation and the antibiotic neomycin sulphate (0.5 %). In South Africa only one combination CLZ product is available, namely Lotriderm® cream (Scherag, South Africa), which combines CLZ (1 %) with the corticosteroid betamethasone dipropionate (0.05 %). It is available in the USA as Lotrisone®.

1.3 BIOAVAILABILITY AND BIOEQUIVALENCE OF TOPICAL PRODUCTS

1.3.1 Background

Innovator drug companies developing a new drug have patent protection for the drug for a number of years, typically 15 - 20. Once the patent has expired, generic drug companies are permitted to make and market their own products as competitors to the innovator's product, usually at a substantially reduced cost. However, a prerequisite for registration with national drug registration bodies (and subsequent marketing) is that the rate and extent to which the drug is absorbed from the generic product is shown to be equivalent to that from the innovator product. This is an indirect way or surrogate measure of demonstrating that the generic product will be as safe and effective clinically as the innovator and is demonstrated by what are known as comparative bioavailability (BA) or bioequivalence (BE) studies.

1.3.2 Bioequivalence and locally acting topical products

Locally acting topical preparations pose a unique challenge to the FDA and other regulatory bodies when it comes to approving what is known as an Abbreviated New Drug Application (ANDA) in the USA or as a generic product in other countries. Unlike transdermal dosage forms, locally acting topical products are not intended to deliver the

active pharmaceutical ingredient to the systemic circulation and therefore do not reach detectable plasma concentrations (49). This means that the standard approaches used to determine BE for systemically active drugs are not applicable and alternative processes must be used.

1.3.3 Current legislation

Currently the FDA recommends the use of an appropriately designed comparative clinical trial to show BE between locally acting topical preparations even though this approach is described as "the least accurate, sensitive and reproducible" of all the measures used to determine BE (50). The only products exempt from this method are solutions for application to the skin (51) and corticosteroids intended for topical use, where the human skin blanching assay (HSBA), also known as the vasoconstrictor assay may be used in the latter instance (52).The European Agency for the Evaluation of Medicinal Products (EMEA) also requires pharmacodynamic or comparative clinical trials to be carried out in order to determine if two topical products are bioequivalent (53). It should be noted, however, that comparative clinical trials to determine BE are very costly and time consuming, and should only be used if it is not appropriate to measure the drug directly or measure a pharmacodynamic effect (54). Doubts as to the reliability of this method exist since a study conducted on 139 acyclovir products, from various European countries, using *in vitro* techniques, indicated differences between most products. In some instances, for example, the concentration of penetration-enhancing excipients were found to be different between formulations yet the products were available as generics (55). No indication was given as to how these products were approved in those countries.

1.3.4 *In vitro* techniques used to assess release from topical formulations

The *in vitro* assessment of semi-solid dosage forms gives an indication of the physical and chemical attributes of a formulation and is therefore a valuable tool in quality control. The use of *in vitro* techniques in maintaining the quality of a product has been shown to maintain the established clinical performance in the long term (56). In addition, *in vitro*

methods are also used to assess product stability and during component and compositional changes, manufacturing, instrumentation or other process changes as well as batch scale up or transfer to another manufacturing site (57).

In vitro release testing is used to quantify the rate of drug release from the excipients/vehicle components of the dosage form (58), and is one of the standard methods used to characterize the performance of a finished topical formulation. *In vitro* release testing is universally accepted as a regulatory tool to monitor batch to batch uniformity during the manufacture of semisolid dosage forms (59). Since drug release *in vitro* is a property of the formulation it is also used as a valuable product development tool to assess pharmaceutical availability (57) and to provide information on the product sameness after minor changes (60). It is also important to note that satisfactory drug release needs to be demonstrated as a prerequisite for therapeutic efficacy (61).

In vitro drug release testing of semi-solids has been performed using Franz cells (61-64), flow-through cells (64,65), insertion cells (66), plexiglass cells (61), enhancer cells(67),glass diffusion cells (68,69), perspex diffusion cells (70) and other modified versions of the Franz cell (71-73) . The choice of the particular cell is usually based on the solubility of the drug and the precision that can be achieve using the method.

In an industry setting, diffusion cell studies most often use synthetic membranes, but studies done to predict absorption/penetration of drug compounds may use human or animal skin as the membrane instead. If the intention of the study is to mimic human skin conditions the drawbacks associated with the use of these materials must be taken into account. Synthetic membranes do not realistically reflect the nature of the skin since they represent the skin as a homogenous layer with no enzymatic activity, microflora or hair follicles. The endogenous enzymes found in cadaver skin will not be active and its use as a membrane is likely to produce highly variable results as it is often excised from various anatomical sites and not pre-treated in a uniform manner (74). Attempts have been made to conduct *in vitro* studies where the viability and metabolic activity of excised human skin is maintained but this work is extremely challenging (75).The excised skins of

various animal species have been investigated as surrogates for human skin (76) but even though there may be some similarities between human and animal skin the differences that have evolved between the species are considerable (77). As commented by Barry "if at all possible, investigative problems should not be made more complex by selection of an animal tissue to represent human skin" (76).

In vitro release studies give an indication of the rate and extent of drug release but they cannot be used to predict *in vivo* penetration or clinical response unless a sound *in vitro-in vivo* correlation (IVIVC) has been shown. Attempts have been made to show IVIVC (60) but the high degree of variability surrounding these correlations has resulted in an unconvincing case for the use of this approach for the determination of BE and BA (56).

The FDA recognises the role of *in vitro* release testing in quality control and product development, however, the agency does not consider *in vitro* release an appropriate method for the determination of BA or BE (56). The use of *in vitro* methods to show BE is considered the least satisfactory approach (50) and therefore, except in some unusual cases (51), regulatory bodies will not accept *in vitro* data as proof of BE between products.

1.3.5 Methods used to determine bioavailability of topical products *in vivo*

Since determining BE between topical products, other than corticosteroids, poses unique challenges and is a largely unresolved issue in drug regulation, extensive research has been done in an attempt to develop innovative ways to investigate BA and BE of these dosage forms (74). In order for an *in vivo* method to be used for regulatory purposes it must provide quantitative data regarding the rate and extent of drug penetration into the skin, be shown to be relatively non-invasive and, most importantly, reproducible. The following sections discuss various methods used to determine bioavailability of topical products *in vivo* and a summary of the advantages and disadvantages of each is presented in Table 1.4.

1.3.5.1 Confocal Raman Spectroscopy (CRS)

This method is completely non-invasive and can be used to examine the chemical composition of the skin even several hundred micrometers below the surface (78). It is a vibrational spectroscopic method, but unlike infrared spectroscopy, which depends on light absorption, CRS is based on light scattering (78). CRS has been used to determine the hydration of the SC and assess the water concentration profile across the skin (78) as well as to investigate analytes, such as urea (79). However, in order to achieve significant results with CRS the compound under investigation must possess the necessary spectral features to make it distinguishable from skin components and it must be present in a sufficient concentration. Another major drawback of this technique is that it is not able to quantify the amount of drug present but determines only the relative concentrations (40). It will require substantial development in this field to achieve a technology that can be used to determine BA and BE of topical products.

1.3.5.2 Tape stripping (TS)

The TS technique is discussed more extensively in a later chapter. In brief, it involves the sequential removal of microscopic layers (0.5-1 μm) of the SC by placing a strip of adhesive tape onto the skin surface with uniform pressure, and then removing the tape strip (40). Drug uptake into the SC can be measured *in vivo* by harvesting SC previously exposed to a topical product. Adhesive tapes are used to strip the skin and the amount of drug present in the strip is subsequently quantified by a validated analytical method (18,19,38,39,80-82). The TS technique is recognized as simple and inexpensive, and has been described as minimally invasive (83) and relatively painless (40). However, this method still needs to be validated to show a correlation to clinical efficacy (40,84) and fine-tuned to produce results that are reproducible between laboratories (60,85).

1.3.5.3 Dermal Microdialysis (DMD)

Dermal Microdialysis (DMD) involves the insertion of a dialysis membrane under the skin within the dermis and the perfusion of a physiologically compatible solution through it in order to collect the active ingredient as it penetrates the skin from the applied formulation. One of the major advantages of this technique is that it allows for continuous

sampling and can be used to generate concentration-time profiles (74,86).The method has been described as “minimally invasive”(87), however there is some physical trauma involved as the skin needs to be punctured in order to insert the microdialysis probes. Another advantage is that with a large enough sample size and a well-designed trial, variability does not seem to pose too much of a problem (87,88). A major drawback of this method is that very high analytical sensitivity is needed to pick up the very low concentrations of drug obtained in the perfusate (86). In some instances, the use of technologies such as MS detection have been used to overcome analytical barriers (89) and DMD has been successfully used to determine BE of lidocaine (81) and ketoprofen (88) formulations. It is a promising technique for the determination of BE for drugs which have activity in or around the dermis but it is not applicable for drugs that have their site of action in the SC, such as antifungal drugs.

1.3.5.4 Blister

In the formation of a blister, the basal cells of the epidermis separate from the basal membrane and the cavity fills with a plasma-like fluid (90). This can be used as a compartment from which pharmacokinetic samples can be taken (40). This technique has been used to determine BA (91-93) but it is considered quite invasive (40) and it is not suitable for measuring hydrophobic compounds, as they are retained on the skin tissue (93). The technique has not been widely used and is generally considered too invasive and impractical to be used as for determining BE (40).

1.3.5.5 Biopsy

Skin biopsies are frequently used in general dermatological practise and because large amounts of information can be gathered from using this technique attempts have been made to determine the BA of topical formulations using this method (93,94). Shave biopsies remove a sample of skin down to the dermal layer and punch biopsies sample even deeper and include subcutaneous tissue. Since skin biopsies are generally performed under local anaesthesia they are deemed too invasive for serious consideration as a routine method for determining BE (40).

1.3.5.6 Novel approaches

In the search for novel techniques to determine BE between dermal formulations some researchers have abandoned the idea of assessing drug concentration and have instead focussed on comparing the clinical changes brought about by the test and reference products. Unlike conventional BE studies these studies are carried out in patient populations. Formulation comparisons based on clinical response criteria have been done in skin conditions such as dermal inflammation (95), psoriasis (96), atopic dermatitis and acne (74). The main drawback associated with this approach is the lack of sensitivity to distinguish between pharmaceutically equivalent products. However, advances in bioengineering methods have addressed this issue to some extent and further advances in technology may facilitate the popularisation of these alternative approaches (74).

Method	Advantages	Disadvantages
Confocal Raman Spectroscopy (CRS)	• Non-invasive	• The drug molecule must possess a specific structure to make it distinguishable from skin components. • The drug must be present in a sufficient concentration. • Provides mainly qualitative data
Tape stripping (TS)	• Simple • Relatively inexpensive • Minimally invasive • Relatively painless	• TS still needs to be validated to show a correlation to clinical efficacy. • Reproducibility between laboratories has been cause for concern.
Dermal Microdialysis (DMD)	• Allows concentration-time profiles to be generated	• Physical trauma involved as the skin needs to be punctured. • Very high analytical sensitivity required • Not applicable for drugs that have their site of action in the SC.
Blister	• Relatively large volumes of sample acquired	• The technique is quite invasive • Hydrophobic compounds cannot be measured • Impractical for routine use
Biopsy	• Provides large amounts of detailed information.	• Very invasive • Impractical for routine use
Novel approaches i.e. bioengineering methods	• Non-invasive • Minimal sample analysis required	• Indirect • Lack of sensitivity

Table 1.4: The advantages and disadvantages associated with various methods of determining BA of topical products.

1.4 CONCLUSIONS

The keratinocytes making up the SC undergo extensive specialisation as they migrate from the *basal lamina* to the skin surface. This makes the SC a complex layer that plays an important role in protecting the vulnerable skin cells below from mechanical and pathogenic damage. Fungal infections of this layer compromise its function and therefore agents are needed to treat infections with dermal mycoses.

CLZ is one of the older azole compounds still successfully used to treat cutaneous fungal infections. Its high lipophilicity does not make it a good candidate for oral administration but topical products are commonly used as first line treatment of cutaneous mycotic infections.

In order to make topical treatments like these affordable and accessible to the public the development of quality generic products is essential. However, determining BE between locally acting topical formulations poses unique challenges since traditional methods involving plasma or urine analysis cannot be used. Presently there are no official methods for determining BE of topical antifungal products other than lengthy and expensive clinical trials.

TS can be used to determine the BA of a drug within the SC and therefore is a promising technique in the search for methods to show BE between topical antimycotic products. It does not require the use of advanced technology like CRS and is much less invasive than acquiring biopsy or blister samples. Since TS involves sampling from the site of action, it is more applicable to the assessment of antifungal than DMD. In addition, there is the advantage of being able to use healthy human subjects rather than patients.

The following chapters discuss work done in an attempt to develop a tape stripping method that could be used to show BE between topical CLZ products.

CHAPTER 2
CLOTRIMAZOLE ANALYSIS

2.1 INTRODUCTION

Before investigations using the TS technique could be carried out on CLZ products, an analytical method for the quantitative analysis of CLZ was necessary. The method was required to be relatively rapid, so that a large number of samples could be analysed daily, and also be reliable to ensure that the results were acceptable. In addition, the formulations to be used in the TS studies needed to be assayed so that the amount of active ingredient applied to the skin could be accurately determined.

2.1.1 Published methods for the analysis of clotrimazole

In order to develop a suitable analytical method for the determination of CLZ, a literature search was conducted for previously published methods. Most of the methods described in the literature involve the determination of CLZ in semi-solid dosage forms such as creams or lotions. Most of these publications focus mainly on determining CLZ in the presence of its degradation products (30,97-102), concomitant active ingredients (97,101,103-105), preservatives (98,106) and other formulation excipients (46,99,100,107,108). Methods have also been published for the determination of CLZ in biological matrices such as serum (109,110). No methods are described for the analysis of CLZ in tape strip samples.

The techniques available for quantitative analysis of CLZ range from relatively unsophisticated titrimetric methods (102) to advanced systems such as gas chromatography coupled to mass spectroscopy (GC-MS) and liquid chromatography-tandem mass spectroscopy (LC-MS) (111). Spectophotometry (100,101) and spectrofluorimetry (100) have also been used successfully to determine CLZ in various dosage forms including vaginal tablets, topical powders, creams and solutions. In cream formulations, CLZ has been separated from its degradation products as well as from concomitant active ingredients, such as betamethasone and tinidazole, using TLC (97,104,108,112) and HPTLC (105). Capillary zone electrophoresis (CZE)

(109,113,114)and micellar electrokinetic chromatography (MEKC) (103,106)have also been used to quantify CLZ. MEKC methods have been used to determine CLZ in the presence of the preservatives methylparaben and propylparaben (106) as well as to assay CLZ in the presence of its degradation products and betamethasone (103). Electroanalytical methods such as polarography have also been developed for the analysis of CLZ (115). However, by far the most popular method used for the determination of CLZ is High Performance Liquid Chromatography (HPLC) coupled with ultraviolet (UV) detection (30,46,97-99,107,108,110,116,117). The table below (Table 2.1) summarises the HPLC conditions for the analysis of CLZ as published in the literature:

Stationary Phase	**Mobile Phase**			**wavelength**	**Reference**
	Organic component	**Aqueous Component**	**Ratio**		
30cm x 3.9mm (10 µm) C_{18} column	Methanol	Dibasic potassium phosphate	75 : 25	254nm	(46)
20cm x 4.6mm (5 µm) Lichrosorb RP-18	Methanol	0.02M orthophosphoric acid (pH 7.5)	70 : 30	215nm	(99)
15cm x 4.6mm Zorbax XDB-C8	Methanol	Phosphate buffer (pH2.5)	65 : 35	220nm	(97)
7.5cm x 4.6mm (3.5 µm) Zorbax SB-Phenyl	Acetonitrile	Water (pH 3.5)	65 : 35	210nm	(30)
25cm x 4.6mm (10 µm) U-Bondapak C_{18}	Acetonitrile	25 mM trishydroxymethyl aminomethane in phosphate buffer (pH 7)	55 : 45	260nm	(108)
12.5cm x 4mm (5 µm) Purospher RP-18e	Acetonitrile	Water	70 : 30	210nm	(98)
25cm x 4.6mm (5 µm) Hypersil C-18	Methanol	0.05M triethylamine phosphate buffer (pH 7)	85 : 15	270nm	(107)
5cm x 4.6 mm (5 µm) Supelcosil LC-8DB	Methanol	25 mM dibasic potassium phosphate (pH 6.3)	72.5 : 27.5	210nm	(110)
15cm x 4mm (5 µm) Nucleosil C_{18}	Methanol	Water	90 : 10	258nm	(116)
7.5cm x 4.6 mm (3 µm) Ultrasphere C_{18}	Methanol	10 mM sodium phosphate (pH 6.5)	41 : 59	237nm	(117)

Table 2.1: Summary of HPLC-UV methods found in the literature.

2.1.2. High Performance Liquid Chromatography (HPLC)

For compounds absorbing UV/Vis light, HPLC-UV provides a rapid practical method for quantitative analysis. It has advantages over other forms of separation such as GC because the sample is prepared as a solution and analysis is generally carried out at room

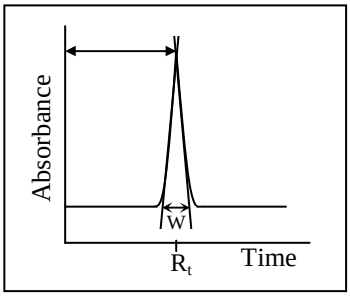
temperature. This allows non-volatile and/or thermolabile compounds to be easily analysed.

In order to achieve the best results when using HPLC-UV it is important that peaks are sharp and narrow, and that there is good resolution between peaks. The following sections discuss parameters used to assess the HPLC system, in particular the column, to monitor performance.

2.1.2.1 Column efficiency (N)

Column efficiency measures the performance not only of the column but of the entire HPLC system. It is also known as the theoretical plate count, a term derived from the distillation industry. Column efficiency refers to how well various compounds in a sample are separated from each other. The concept is based on the idea of theoretical "plates" or bands in the stationary column. In each band the sample compound must reach equilibrium between the solvent and the stationary material before transfer to the next section can occur. A large number of theoretical plates indicates good separation and overall system performance.

Various methods are available to calculate column efficiency. The tangent method (Equation 2.1) is a particularly useful equation because it is relatively simple and yet has the necessary discriminatory power.



$$N = 16 (R_t/W)^2$$

Equation 2.1: Where N = number of theoretical plates; Rt = retention time; W = width according to tangents

Column efficiency can also be described using the Height Equivalent to a Theoretical Plate (HETP) value, which takes the length of the column into account. Since column length is divided by N, the lower the HETP value the greater the performance of the system.

$$\mathbf{HETP = column\ length / N}$$

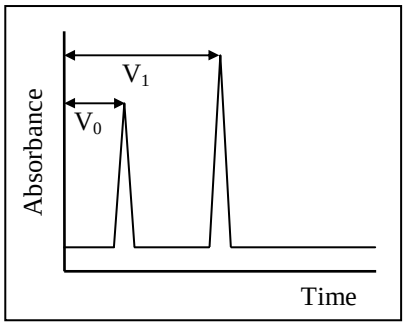
Equation 2.2: Where HETP = Height Equivalent to a Theoretical Plate;
N = number of theoretical plates

The column efficiency can be used to compare columns or track the performance of a column. Column efficiency should be assessed upon receipt of a new column or the set up of a new HPLC system and monitored over time. Factors that may reduce column efficiency include column aging, malfunctioning of the instrumentation (e.g. injector, flow rate) and changes in mobile phase viscosity.

2.1.2.2 Capacity factor (k')

The capacity factor (k') is a measure of how rapidly a compound is eluted from the column relative to an unretained probe molecule. In order for a mixture of compounds to be sufficiently separated by HPLC they must have different capacity factors so that they elute at different times. The structure and chemical nature of the compound is initially responsible for determining the capacity factor, but the k' value can be manipulated by changing the composition and flow rate of the mobile phase or the composition and surface area of the stationary phase. The capacity factor is significantly influenced by the column temperature. The rule of thumb is that an increase of 1 °C in column temperature will change the k' value by 1 % (118).

The capacity factor is a comparison between an unretained compound (uracil is frequently used in RP-HPLC) and the sample compound. As the column ages a change in the capacity factor may occur due to changes in the surface chemistry of the stationary phase. Therefore the k' value may also be used to track column aging and performance.



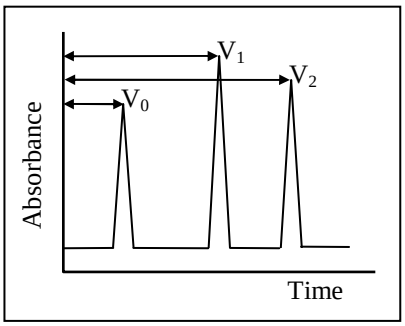
$$\mathbf{k' = V_1 - V_0 / V_0}$$

Equation 2.3: Where k' = capacity factor; V_0 = void volume;
V_1 = retention volume of sample compound

When running samples under isocratic conditions a k' value between 2 and 10 is considered optimal (118). Higher k' values will result in excessive run times and lower values are unlikely to give satisfactory peak resolution.

2.1.2.3 Selectivity (α)

The selectivity of a system is a measure of how well two peaks separate and is described by a ratio of the k' values of two peaks. An α value close to 1 indicates minimal separation and low selectivity, while values further from 1 show better selectivity. The selectivity can usually be altered by varying the polarity of the system by making changes to the mobile phase (e.g. pH or molarity of the aqueous phase and/or proportion of organic component) or changing the type of column. As with the capacity factor the column temperature and column aging can also influence the selectivity.



$$\mathbf{\alpha = k'_2 / k'_1 \text{ or}}$$
$$\mathbf{\alpha = (V_2 - V_0/V_0)/(V_1 - V_0/V_0)}$$

Equation 2.4: Where α = selectivity; k'_2 = capacity factor of peak 2; k'_1= capacity factor of peak 1

2.1.2.4 Resolution (R_s)

Resolution is a parameter describing the overall performance of the system. Only peaks that are sufficiently resolved from each other can be used for accurate analysis. Peak resolution depends not only on the selectivity of the system but also on the column efficiency. An R_s value greater than 1.5 indicates complete resolution, while a value less than 1.5 implies that the resolution is incomplete (119).

$$R_s = {}^1/_4(\alpha\text{-}1/\alpha)(\sqrt{N})(k'/1+k')$$

Equation 2.5: Where R_s = peak resolution; α = column selectivity; N = column efficiency; k'= capacity factor

2.2 HPLC METHOD DEVELOPMENT AND SYSTEM OPTIMISATION

The following sections describe the work involved in the development of an HPLC method for the determination of CLZ from tape strip samples. As a starting point, a published method for CLZ analysis using HPLC-UV was used to establish a set of HPLC conditions which have been termed the "original conditions" .These conditions were then optimised by investigating the effect of the organic solvent, the pH of the aqueous component, the column temperature, the injection volume and the wavelength used for analysis. The optimised method was then adjusted to ensure that the system was selective for samples of CLZ extracted from tape strips. Validation was subsequently done and is described in section 2.3.

2.2.1 Reagents and materials

The CLZ standard (Lot. 075K1032, 99% purity) was purchased from Sigma-Aldrich (Sigma-Aldrich®, Atlasville, South Africa) and was stored in a cool, dark cupboard. The HPLC grade organic solvents, acetonitrile (ACN) 200UV ROMIL - SpS™ Super Purity Solvent and methanol 200UV ROMIL - SpS™ Super Purity Solvent, were obtained from Romil Ltd. (Romil Ltd., Waterbeach, Cambridge, UK). The UnivAR® ammonium acetate

crystals (Saarchem, Krugersdorp, South Africa) were supplied by Merck chemicals (Pty) Ltd and stored in a tightly sealed container away from moisture.

The water used to prepare the mobile phase was purified by reverse osmosis and then filtered through a Milli-Q® system (Millipore, Bedford, MA, USA). The system consisted of a Milli-Q® Academic A10 with a Quantum™ EX Ultrapure Organex Cartridge equipped with a Q-Gard® 1 Prograd pre-treatment pack.

2.2.2 Instrumentation

Sample analysis was performed on an Alliance 2695 HPLC system equipped with a 2996 PDA detector, autosampler and column heater (Waters® Corporation, Milford, Massachusetts, USA). Instrument control, chromatogram recording and storage as well as peak integration was done using Empower Pro™ software (Waters® Corporation, Milford, Massachusetts, USA).

2.2.3 Original conditions

Conditions similar to those described by Adel-Moety *et al* (2003) (108) were used as the basis for method development. This work was selected because the described method was a simple isocratic system that made use of readily available materials. A summary of the original conditions used and the resulting chromatogram are shown below.

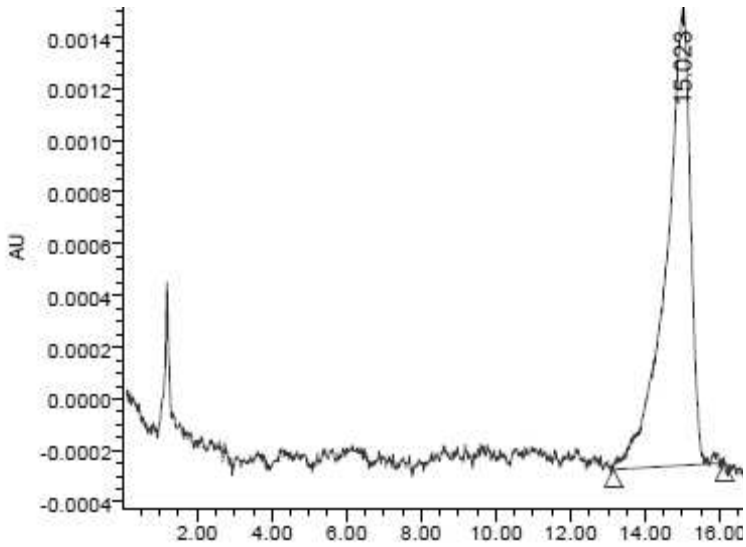
Original Conditions	
 Sample : CLZ 50 ug/ml in methanol	
Column type	Luna 5 µm (C_{18}) 100Å 150 x 4.60 mm (Phenomenex®)
Mobile Phase	
Organic component	Acetonitrile
Aqueous component	0.005M Ammonium Acetate (pH = 7.6)
Ratio organic:aqueous	55:45
Column Temperature	22 °C
Injection volume	10 µl
Flow rate	1 ml/min
Wavelength	260 nm

Table 2.2: Original HPLC conditions adapted from the method described by Abdel-Moety *et al* (2003)(108)

2.2.4 Method optimisation

Having a sensitive, accurate and reliable analytical method is imperative to successfully quantify a drug in a biological sample. Since the peak obtained using the original conditions was broad and had an unacceptably long retention time it was necessary to improve the method. The method optimisation process was focused on producing a chromatogram with a sharp, narrow and well resolved CLZ peak so that the necessary sensitivity for the analysis of the tape strip samples could be achieved.

2.2.4.1 Column selection

When HPLC is used to separate a mixture of compounds either normal phase (NP) or reverse phase (RP) chromatography may be appropriate. NP systems are made up of a hydrophilic stationary phase which is perfused by a non-polar mobile phase. Using this approach the hydrophobic components of the sample elute rapidly and close together while the more hydrophilic compounds are retained on the column longer due to their higher affinity for the stationary material. In most cases, higher retention times will result in better separation, hence NP chromatography is not considered ideal for the analysis of non-polar compounds.

RP-HPLC is a more popular method for the analysis of hydrophobic compounds since the polarities of the mobile and stationary phases are reversed and the non-polar compounds are retained on the column longer. Since CLZ has a largely non-polar structure, RP-HPLC is frequently used as an analytical tool (30,46,97-99,107,108,110,116,117). It was therefore appropriate to select a hydrophobic C_{18} column (Luna 5µm (C_{18}) 100Å 150 x 4.60 mm, Phenomenex®) as the stationary phase. In addition, a C_{18} guard column was used to protect the column from contamination due to possible undesirable components present in the tape strip extracts.

2.2.4.2 Mobile phase composition

In HPLC the polarity of the mobile phase is a key factor in determining the retention time of a compound. The mobile phase must be polar enough to allow the analyte to be retained on the stationary material yet hydrophobic enough to ensure that it elutes after a reasonable time interval. Using a combination of organic modifier and aqueous component allows the optimum polarity of the mobile phase to be achieved. Determining the appropriate proportion of each phase is critical. Too much organic solvent will result in the compound eluting too rapidly, leading to poor peak separation, whereas too low a proportion of organic phase will make the run time unacceptability long.

The effect of altering the ratio of organic phase to aqueous component in the mobile phase was investigated using a series of isocratic solutions ranging from 50:50 to 75:25.

ACN was used as the organic solvent and 0.005M ammonium acetate solution was used as the aqueous component.

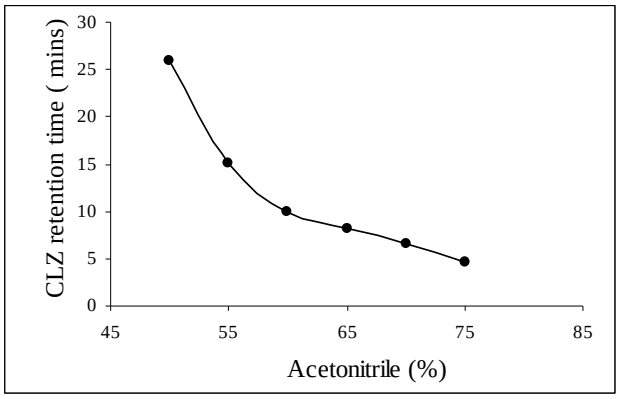


Figure 2.1: Effect of increasing the organic component of the mobile phase on the retention time of CLZ.

Figure 2.1 shows that as the percentage of ACN present in the mobile phase increased from 50-60 %, the retention time of CLZ decreased significantly. Increasing the percentage of ACN further from 60-75 % showed the same trend, but the decrease in retention times was not as great. A retention time of >10 mins is undesirable and therefore using < 60 % ACN was not appropriate. Since 75 % ACN gave the lowest retention time without significantly affecting peak shape, all further work was done using this ratio of organic to aqueous phase.

2.2.4.3 Effect of pH

The effect of mobile phase pH on the CLZ peak was investigated using 3 isocratic mobile phase solutions. Glacial acetic acid was used to adjust the pH of the 0.005 M ammonium acetate solution to pH 7.03 and 6.07 respectively and the unadjusted mobile phase, pH = 7.6, was also investigated. A methanolic CLZ solution (1 μg/ml) was injected (10 μl) into the system under each mobile phase condition and the retention time and peak width was recorded accordingly. The effect of increasing the mobile phase pH was not investigated, since CLZ is a fairly acidic compound (pKa = 4.7(27)) and therefore would experience a decrease in ionization in a basic mobile phase leading to an increase in lipophilicity and hence rapid elution.

pH of mobile phase	CLZ retention time	Peak width
pH 7.61	4.66 mins	0.32
pH 7.03	4.69 mins	0.35
pH 6.07	4.70 mins	0.40

Table 2.3 Summary of results obtained from altering the mobile phase pH

As can be seen in Table 2.3, decreasing the pH from 7.61 to 6.07 did not significantly affect the retention time for CLZ but it did affect the peak shape by broadening the peak width. It was therefore decided that the unadjusted mobile phase with a neutral pH would be used for future studies.

2.2.4.4 Column temperature

Altering the column temperature influences not only the retention time of a compound but also the peak shape. A narrow, sharp peak is more likely to be well resolved from interfering compounds and improves the accuracy of peak integration.

A 50 μg/ml solution was injected at 4 different temperatures ranging from 22 to 50 °C. The peak width and retention time for each temperature setting were recorded.

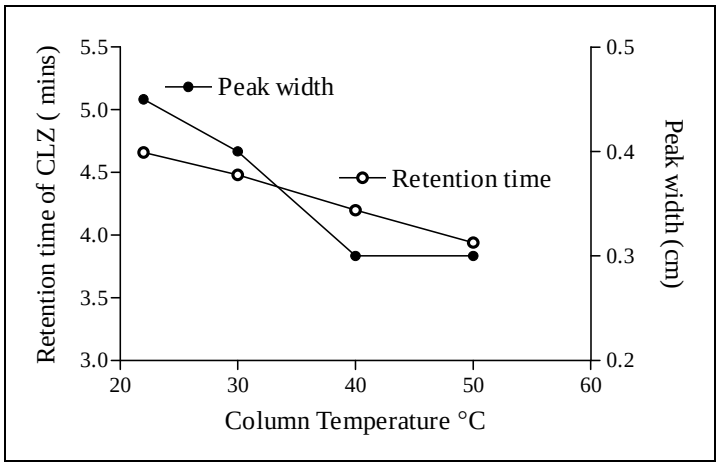


Figure 2.2: Effect of column temperature on the retention time and width of the CLZ peak.

As shown in Figure 2.2, as the column temperature was increased the retention time steadily decreased. An increase in temperature from 22 to 40 °C also caused the peak width to narrow, reaching a minimum of 0.3 cm at 40 °C. Increasing the temperature above 40 °C did not decrease the peak width further. Using a temperature above 40 °C

would not improve the peak shape or retention time significantly, hence 40°C was chosen as the column temperature at which all further studies were conducted.

2.2.4.5 Injection volume

Increasing the volume of sample injected into the HPLC system increases the amount of compound presented to the UV detector. This allows for greater absorbance values and therefore a more sensitive limit of quantification (LOQ) can be achieved. However, an increase in the injection volume also increases the load of compound on the column and this can lead to large, wide peaks unsuitable for quantitative analysis. In order to investigate the effect of increasing injection volume, a 1 µg/ml CLZ solution was injected 6 times using injection volumes ranging from 10 µl to 100 µl. The effect of injection volume on peak shape is shown in Table 2.4 below.

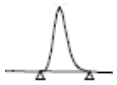
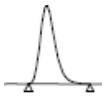
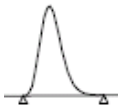
Injection Volume	Column Load	Peak Width	Peak Shape	
10 µl	0.001 µg	0.30 cm	Sharp	
20 µl	0.002 µg	0.40 cm	Sharp	
30 µl	0.003 µg	0.45 cm	Sharp	
60 µl	0.006 µg	0.48 cm	Rounded	
80 µl	0.008 µg	0.60 cm	Blunt	
100 µl	0.01 µg	0.70 cm	Peak splitting	

Table 2.4: Effect of increasing the injection volume.

Increasing the injection volume did not influence the retention time for CLZ, but the peak shape was dramatically affected. Injection volumes above 40 µl resulted in large blunted peaks unsuitable for accurate analysis. Injection volumes of 10 µl, 20 µl and 40 µl gave relatively sharp peaks, but as the injection volume increased, the peak width also increased. Since an injection volume of 10 µl resulted in the sharpest and narrowest peak, this volume continued to be used for all further analyses.

2.2.4.6 Wavelength selection

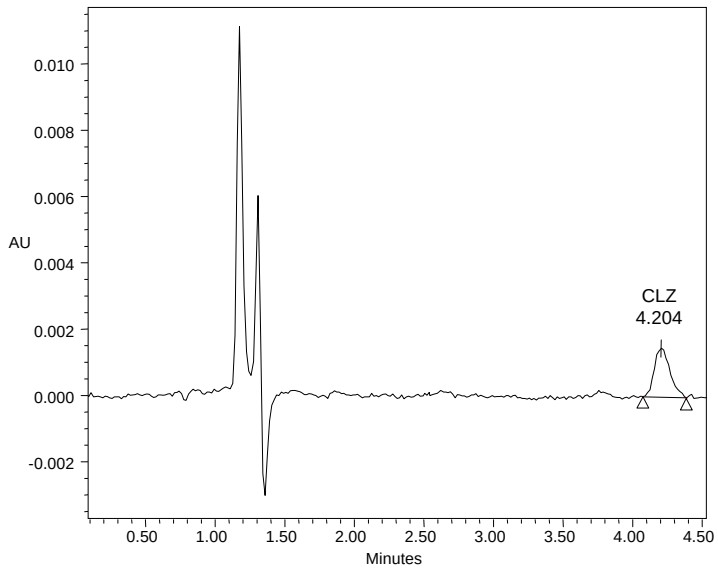
The choice of wavelength at which peak integration is done will influence the LOQ of the method. A wavelength providing the highest absorbance will allow samples of low concentration to be detected and therefore a lower LOQ can be achieved.

Previous HPLC-UV methods for the analysis of CLZ have used such wavelengths as 254 nm (46), 258 nm (116), 260 nm (108) and 270 nm (107), which are close to the λ maximum of 260 nm. However, it can be seen from the absorbance profile of CLZ (Figure 1.3) that much greater absorbance occurs at 210 nm. A disadvantage of using this wavelength is that many substances absorb UV light at this low end of the spectrum, thereby increasing the possibility of interference from other compounds present in the sample. However, the sensitivity gained by using this wavelength is significant and 210 nm was therefore chosen as the wavelength for all future sample analyses.

2.2.5 Optimised conditions

From this series of investigations a set of optimised conditions (summarized in Table 2.5) was obtained. Under these conditions CLZ had a run time of 4.2 mins and the peak was sharp and narrow.

Optimised Conditions



Sample : 0.1ug/ml CLZ in methanol

Column type	Luna 5µm (C_{18}) 100Å 150 x 4.60 mm (Phenomenex®)
Mobile Phase	
Organic component	Acetonitrile
Aqueous component	0.005M Ammonium Acetate (pH = 7.6)
Ratio organic:aqueous	***75:25***
Column Temperature	***40 °C***
Injection volume	10 µl
Flow rate	1 ml/min
Wavelength	***210 nm***

Table 2.5: Optimised HPLC conditions (bold italics indicate changes from the original conditions).

2.2.6 Method Selectivity

The optimised method produced satisfactory chromatography for samples of pure CLZ standard, however, the method needed to be able to analyse CLZ extracted from tape strip samples. When CLZ is extracted from tape strips it is likely that a variety of other substances such as tape adhesive or skin components may be present in the extracted sample. These substances could interfere with the CLZ peak and therefore it was necessary to select an adhesive tape for the TS studies such that components would not interfere with the CLZ peak. Similarly, components present in SC were also considered as possible sources of peak interference and the necessary measures taken to avoid interference with the assay.

2.2.6.1 Tape selection

In previous TS studies various types and sizes of tape strips have been used. Adhesive tapes, such as Scotch® Packaging Tape(120), Scotch® Book Tape (19) and Scotch Magic Tape(121) have been used to prepare tape strips in-house and commercially available adhesive discs, specifically designed for SC sampling e.g. D-squame®, have been used for TS studies (71,81,122).

When selecting a suitable tape strip material three factors were considered:

1. The presence of components that would interfere with the CLZ analysis.
2. The adhesive properties of the tape.
3. How easy it was to manipulate the tape. (Some adhesive tapes easily acquire a static charge which makes them difficult to work with.)

Four types of tape were investigated as possible candidates. These included 3M Transpore™ Surgical Tape (3M, Johannesburg, South Africa), Scotch® Magic™ Tape (3M, Johannesburg, South Africa), Sellotape® Original (Henkel Consumer Adhesives, Johanessburg, South Africa) and 3M Micropore™ Surgical Tape (3M, Johannesburg, South Africa). In each case a 2.4 x 2.4 cm square of tape was cut and placed in a 2ml centrifuge tube. Methanol (500µl) was added to the tubes and the samples were vortexed for 1 minute using an Eppendorf MixMate™ (model PCB-08, Eppendorf, Hamburg, Germany) at 2300 rpm and then centrifuged for 10 minutes (Eppendorf Centrifuge 5415, Hamburg, Germany) at 1300 rpm. The resulting extract was injected into the HPLC system.

The following table shows a summary of the properties of each adhesive tape as well as the corresponding chromatogram. The adhesiveness and ease of manipulation of each tape were subjectively determined by the investigator.

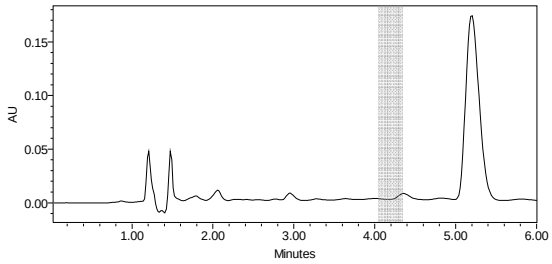
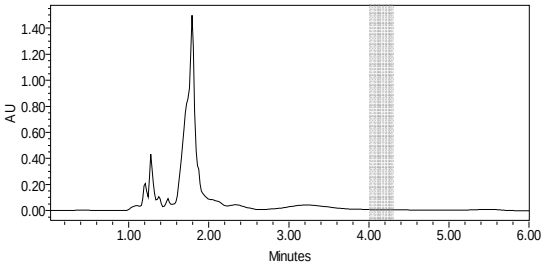
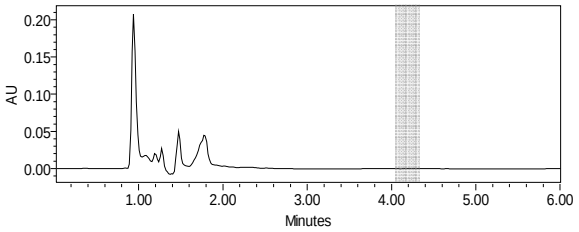
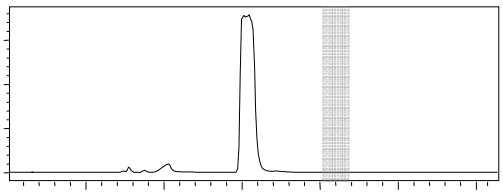
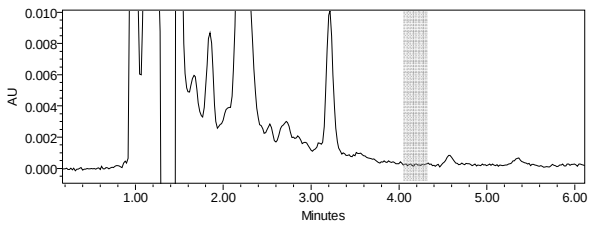
3M Transpore™ Surgical Tape	
Interference: likely Adhesiveness: very adhesive Ease of manipulation: easy	Sample: blank Transpore™™ tape strip
3M Micropore™ Surgical Tape	
Interference: unlikely Adhesiveness: poor Ease of manipulation: easy	Sample: blank Micropore tape strip
Sellotape® Original	
Interference: unlikely Adhesiveness: good Ease of manipulation: difficult	Sample: blank Sellotape® Original tape strip
Scotch® Magic™ Tape	
Interference: unlikely Adhesiveness: good Ease of manipulation: easy	

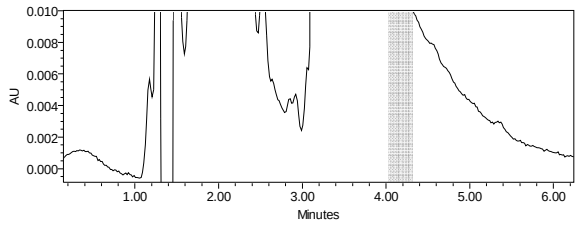
Table 2.6: The properties and chromatograms of various tapes
(The shaded area shows the position where CLZ elutes)

From Table 2.6 above it can be seen that Transpore™ and Micropore™ were unsuitable as tapes for use in the TS due to possible interference with the CLZ peak and poor adhesiveness respectively. Both Sellotape® and Scotch® Magic™ Tape were considered suitable as they did not show any interfering peaks close to the retention time of CLZ i.e. 4.2 mins.

In view of the fact that a relatively high sensitivity is required to detect CLZ extracted from the tapes, the AUFS setting needed to be in the range of approximately 0.01. It can be seen from Figure 2.3 that the profile obtained following the extraction of Scotch® Magic™ Tape resulted in a sloping baseline seen at the required AUFS of 0.01, making this tape unsuitable for the analysis of low CLZ concentrations. On the other hand, Sellotape had a relatively flat base line at the same AUFS, but a small peak eluting close to 4.2 mins was present. Nevertheless, Sellotape Original was considered to be the most promising candidate and was selected as the tape for further investigation. Clearly, some optimisation was necessary either with respect to the chromatography and/or extraction method to avoid interference by extraneous components.



Sample: blank Sellotape Original tape strip

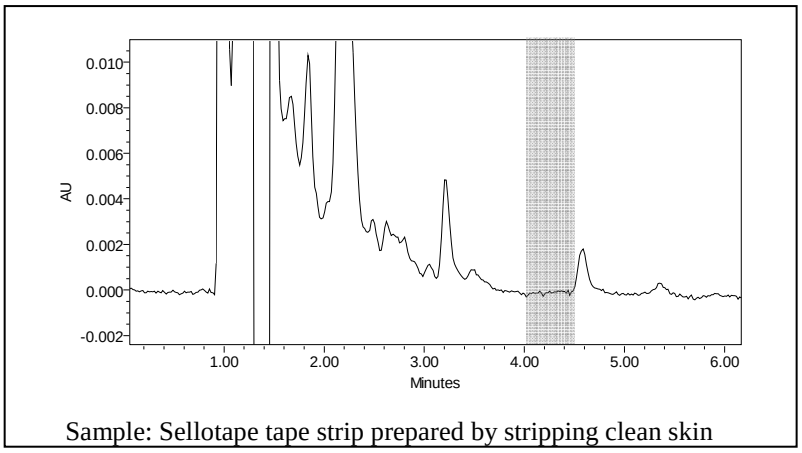


Sample: blank Scotch Magic Tape tape strip

Figure 2.3: Comparison of the baseline of Sellotape® Original and Scotch® Magic™ Tape.
(The shaded area shows the position where CLZ elutes)

2.2.6.2 Influence of *stratum corneum*

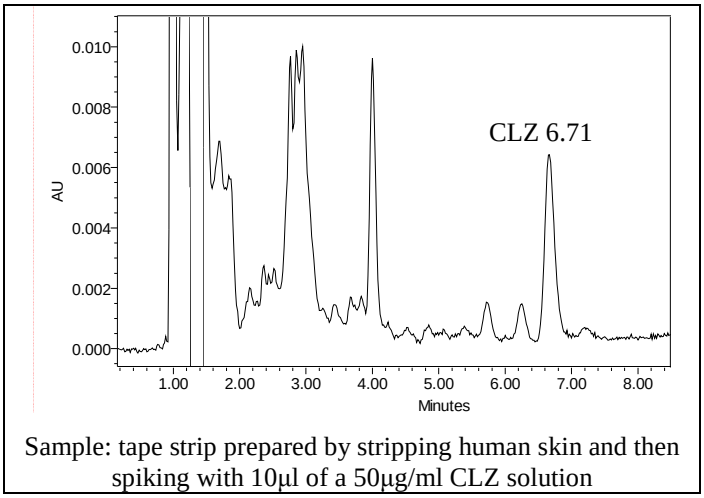
To determine whether the substances extracted from the adhered SC could interfere with the CLZ analysis, blank tape strips were prepared by stripping human skin. These tape strips underwent the same extraction process as described in 2.2.6.1 and the methanolic extract was then injected into the HPLC system.



Sample: Sellotape tape strip prepared by stripping clean skin

Figure 2.4: Chromatogram using the optimised HPLC conditions
(The shaded area shows the position where CLZ elutes)

Similar to the chromatogram for the blank tape, the chromatogram of this sample had a peak close to 4.2 minutes (Figure 2.4) that was likely to interfere with CLZ. Therefore the ratio of organic to aqueous solvent in the mobile phase was adjusted from 75:25 to 65:35 in an attempt to shift the interfering peak. The chromatograms below (Figure 2.5) show how this adjustment successfully created a system where the CLZ peak, now eluting later (6.71 mins) was free from interference.



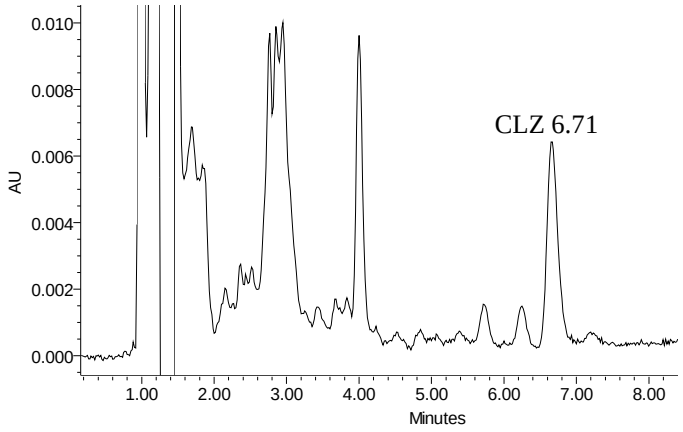
Sample: tape strip prepared by stripping human skin and then spiking with 10µl of a 50µg/ml CLZ solution

Figure 2.5: Chromatogram using the final HPLC conditions

2.2.7 Final conditions

The final HPLC conditions, adjusted to avoid interference from unwanted substances, are summarised below. From the chromatogram (Table 2.7) it can be seen that a desirable peak shape for CLZ was achieved and that it was well resolved from other components present in the tape and adhered SC. These features make it a suitable method for the analysis of CLZ extracted from Sellotape® Original tape strip samples.

Final Conditions



Sample: blank tape strip spiked with 10 µl, 50 µg/ml solution

Column type	Luna 5 µm (C_{18}) 100Å 150 x 4.60 mm (Phenomenex®)
Mobile Phase	
Organic component	Acetonitrile
Aqueous component	0.005M Ammonium Acetate (pH = 7.6)
Ratio organic:aqueous	***65:35***
Column Temperature	40 °C
Injection volume	10 µl
Flow rate	1 ml/min
Wavelength	210 nm

Table 2.7: Final HPLC conditions. (Italics indicate changes from the optimised conditions.)

2.2.8 Discussion

In order to optimise the original conditions for the HPLC analysis of CLZ, the effect of modifying various parameters was investigated. A summary of the stepwise approach that was used is represented by Figure 2.6. The use of a C_{18} column with a mobile phase

consisting of 65 % ACN and an aqueous component of neutral pH were shown to give an optimal run time and peak shape. Increasing the column temperature to 40°C was able to improve the peak shape further, however, increasing the injection volume widened the CLZ peak and therefore the injection volume of 10µl was not increased.

The use of a C_{18} column in this method of CLZ analysis was consistent with the published methods (46,98,99,107,108,116,117). However, even though most of the HPLC methods for CLZ analysis found in the literature made use of C_{18} columns, a variety of column types and specifications have been used for CLZ analysis. The types of C_{18} columns used include Lichrosorb®, U-Bondapak® and Purospher® columns. No methods using Luna columns were found. The lengths of the columns used in these methods varied from 5 to 25 cm and 2 studies (97,116) made use of 15 cm columns. The diameters of other columns used included 3.9 mm (46), 4.0 mm (98,116) and 4.6 mm (30,97,99,107,108,110,117). However, most methods made use of columns with a 4.6mm diameter. Various sizes of the packing material (e.g. 3.0 µm (117), 3.5 µm(30), 5.0 µm (98,99,107,110,116) and 10 µm (46,108)) have been used, but columns with packing material 5µm in diameter were the most common. Therefore, the Luna C_{18} column 150 x 4.6 mm, with 5 µm particle size, used for the HPLC analysis of CLZ, was similar to other columns used in published methods.

Previous studies have made use of either ACN or methanol as the organic component of the mobile phase and, in this method, a satisfactory retention time and peak shape for CLZ was obtained using ACN as the organic phase. Similar to other published HPLC methods for CLZ analysis that used plain water (30,98,116) or a salt solution (46,110,117), a 0.005M ammonium acetate solution was used as the aqueous component of the mobile phase. The pH of the aqueous phase was 7.6. This was consistent with the pHs used in similar studies (99,107,108).

The wavelength of 210 nm chosen for this method was in accordance with the wavelengths used in other studies (30,98,99,110) as maximum absorbance occurs at this wavelength and an injection volume of 10 µl was also consistent with previous studies.

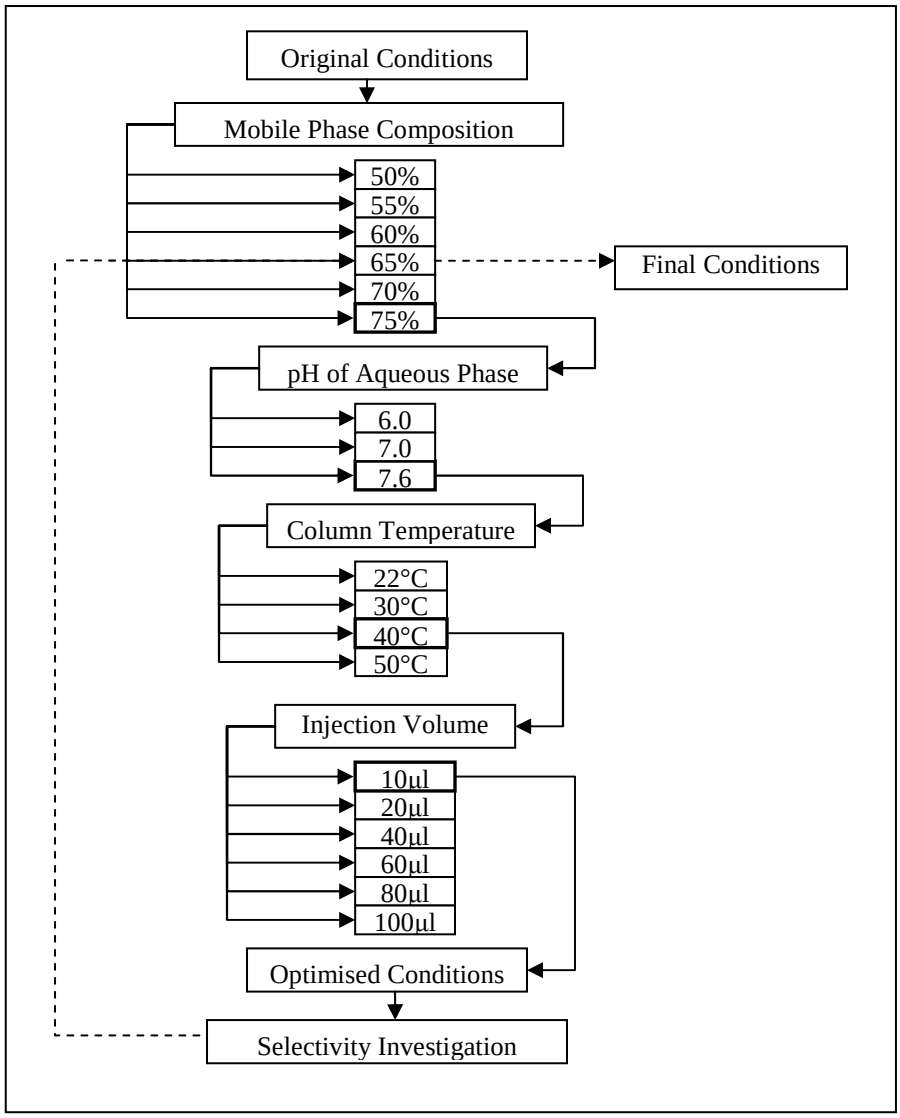


Figure 2.6: Flow diagram of method development process.

2.3 METHOD VALIDATION

2.3.1. Introduction

The parameters used to validate analytical methods have been defined by various regulatory bodies and interested parties including the FDA, the International Organisation for Standardization and International Electrotechnical Commission (ISO/IEC), the US Environmental Protection Agency (EPA) and the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). For the validation of this method, the ICH guidelines were followed

(123). In accordance with these guidelines, accuracy, precision, specificity and linearity were investigated across the required range. The limit of detection (LOD) and LOQ were also determined although the guidance document does not consider these parameters obligatory when validating assay procedures. The ICH guidance also does not require robustness studies to be done as part of routine validation, however, the publication does include guidelines for assessing this parameter and the robustness of the method was investigated accordingly.

2.3.2. Reagents and Materials

As described in section 2.2.1

2.3.3 Instrumentation

As described in section 2.2.2

2.3.4 Sample preparation

A stock solution of 2.5 mg/ml was prepared by dissolving 50 mg of CLZ in 20 ml methanol. This was used to prepare a series of dilutions ranging from 2.5 mg/ml to 10 μg/ml.

2.3.4.1 Calibration curve samples

In order to prepare a calibration curve for tape strip extracts, 10 blank tape strips (prepared by stripping human skin) were spiked with 10 μl of the relevant standard solution as follows: 2.5 mg/ml, 2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.1 mg/ml, 0.075 mg/ml, 0.05 mg/ml, 0.025 mg/ml and 0.01 mg/ml using a digital syringe (Hamilton 0.10ml Microliter™ Digital Syringe™, Nevada, USA). The spiked tape strips were allowed to dry before each strip was placed in a 2ml centrifuge tube and extracted as described in section 2.2.6.1.

2.3.4.2 High, medium and low concentration samples

The samples of high, medium and low concentrations used to determine accuracy, precision and robustness were made by spiking blank tape strips (prepared by stripping human skin) with 10µl of 1.5 mg/ml, 0.15 mg/ml or 0.015 mg/ml of CLZ stock solution respectively. The tapes were treated and extracted with 500 µl methanol as previously described (section 2.2.6.1). The resultant concentrations were therefore 30 µg/ml, 3 µg/ml and 0.3 µg/ml respectively.

2.3.5 Validation

2.3.5.1 Specificity

Specificity is defined by the ICH guidelines as the "ability [of a method] to assess unequivocally the analyte in the presence of components which may be expected to be present [in a typical sample]". Other guidances (e.g. FDA Guidance of Industry: Bioanalytical Method Validation(124)) prefer the use of the term "selectivity" since the word "specific" implies that a method will detect only the compound of interest, while "selective" infers that the analyte is detectable in the presence of other compounds. This term is more relevant since in most cases an HPLC-UV method will not be specific enough to detect only the compound of interest from a multi-component sample.

The manner in which selectivity is determined largely depends on the objective of the analytical procedure, however, the ICH guidance indicates three aspects that need to be considered when showing specificity. These are: identification of the peak, purity of the peak and ability to use the peak for assay purposes. In this study the following steps were taken to show the selectivity of the method for CLZ extracted from a layer of SC cells, previously exposed to CLZ 1% cream (Canesten® Topical cream) and harvested using a tape strip:

1. The peak was positively identified as CLZ by comparing the retention time of CLZ from a standard solution with that of an extracted sample.
2. The CLZ peak was shown to be pure and without interference from other compounds using a PDA detector coupled to the HPLC system.

3. The CLZ peak was shown to be sufficiently resolved from the other components and thus acceptable for use to analyse CLZ in TS study samples.

2.3.5.2 Limits of quantification (LOQ) and detection (LOD)

The LOD is the lowest concentration of analyte detectable by a system but not necessarily quantified. The LOQ is a similar but more meaningful parameter as it is the lowest concentration that can be quantified with accuracy and precision. Various methods are available to determine these parameters but analysis of the signal-to-noise ratio is one of the quickest and easiest methods to perform. The LOD and LOQ are defined as having a signal-to-noise ratio of 3:1 and 10:1 respectively.

In order to investigate these limits, the signal-to-noise ratios for a series of tape strips spiked with 10 µl of solutions ranging from 8 to 25 µg/ml were calculated. The LOD was found to be 0.05 µg/ml and the LOQ 0.2 µg/ml.

2.3.5.3 Range

Before accuracy, precision and linearity can be investigated an appropriate range of concentrations for which the method will be used needs to be established. The range of the method greatly depends on the intended application of the assay. In order to determine a suitable range the concentrations expected from actual samples must be kept in mind.

If low concentrations are expected in the sample, as with tape strip analysis, the LOQ is used as the lower limit of the range. To establish the upper limit the highest value that can reasonably expected must be determined.

In this method the LOQ was 0.2 µg/ml and the upper limit was chosen as 40µg/ml since this concentration was unlikely to be exceeded. Further tests regarding the accuracy, precision and robustness of the method were conducted using high, medium and low concentration samples (30 µg/ml, 3 µg/ml and 0.3 µg/ml respectively) representative of the range.

2.3.5.4 Linearity

For an analyte to be quantitatively determined using HPLC-UV, linearity needs to be established so that a calibration curve can be constructed. Ensuring linearity across the expected range is therefore one of the parameters included in the validation process.

Three calibration curves were constructed on each day of the validation study. All curves were shown to be linear, with R^2 values and slopes shown in Table 2.8.

	Day 1	**Day 2**	**Day 3**
R^2 value	0.9998 0.9999 0.9999	0.9987 0.9995 0.9996	0.9991 0.9986 0.9992
Equation	y = 59722x - 2986.5 y = 59391x - 675.31 y = 59512x - 2808.1	y = 58935x -1374.3 y = 59049x + 6937.7 y = 59304x + 8045.5	y = 61626x - 15917 y = 57719x + 16071 y = 62295x - 8859.3

Table 2.8: Results of the linearity investigations

2.3.5.5 Accuracy

The accuracy of a method indicates how close the values attained are to the "true" value when using the method. The "true" value is determined by using an accepted reference standard. One of the ways accuracy can be reported is by calculating percent recovery. This is done by adding a known amount of analyte to a sample and determining the percentage of the analyte recovered by the assay.

Accuracy was assessed using high, medium and low concentrations within the range, with 6 replicates of each concentration. This was done in accordance with the ICH guideline, which requires at least 3 different concentrations within the range to be investigated and at least 3 determinations to be made for each concentration. However, no acceptance limits for accuracy are specified in this document, therefore the FDA guidance for the validation of bioanalytical methods (124) was consulted. This guidance stipulates that the mean amount of analyte found should be within 15 % of the actual value present except at the LOQ where 20 % is acceptable. These limits are equivalent to a percent recovery of 85 to 115 % and 80 to 120 % at the LOQ, and were used as the criteria for accepting the accuracy of the method.

The percent recovery was found to be well within the stipulated limits for high, medium and low concentrations for both intra and inter-day analyses (Table 2.9).

		Amount CLZ expected (µg)	**Mean CLZ found (µg) (n=6)**	**Mean percent recovery (n=6) (90% Confidence Interval)**
High concentration	Day 1	15.084	15.576	103.26 % (101.3% to 105.2%)
	Day 2	15.119	15.743	104.13 % (103.83% to 104.43%)
	Day 3	14.980	14.707	98.17 % (95.65% to 100.70%)
	Inter-day accuracy			**101.85 % (99.60% to 104.11%)**
Medium concentration	Day 1	1.508	1.469	97.38 % (96.2% to 98.5%)
	Day 2	1.512	1.574	104.13 % (103.33% to 104.92%)
	Day 3	1.498	1.363	91.00 % (89.60% to 92.41%)
	Inter-day accuracy			**97.50 % (93.79% to101.21%)**
Low concentration	Day 1	0.151	0.140	93.13% (91.2% to 95.0%)
	Day 2	0.151	0.137	90.59% (88.47% to 92.71%)
	Day 3	0.150	0.168	112.14% (109.66% to 114.63%)
	Inter-day accuracy			**97.83% (90.97% to 104.68%)**

Table 2.9: Results of intra and inter-day accuracy investigations.

2.3.5.6 Precision

Precision can be defined as how closely a series of values, derived from the same sample, are to each other when using a prescribed method. Precision can therefore be expressed as variance, standard deviation or the coefficient of variance (CV) between multiple values obtained from assessing a single sample. Three levels of precision are defined in the ICH guidance (123).

1. Repeatability:

   Repeatability is defined as “precision under the same operating conditions over a short time interval”. As with accuracy the ICH requires a minimum of 9 samples (at least 3 different concentrations within the range with at least 3 determinations for each concentration) to show repeatability.
2. Intermediate precision:

   Intermediate precision takes into account within-laboratory variation by showing repeatability on different days, with different analysts, using different equipment etc.

3. Reproducibility:

   Reproducibility shows that precision exists between different laboratories. It is not usually necessary to show this level of precision unless collaborative work is being undertaken.

Once again the limits used to determine precision were based on the FDA guidelines for the validation of bioanalytical methods. Therefore %RSDs of less than 15% for samples of medium or high concentrations and 20% for the low concentration were considered acceptable. On these criteria the method was shown to be repeatable and intermediate precision was confirmed (Table 2.10)

		Mean amount of CLZ (n=6) (90% Confidence Interval)	**SD**	**%RSD**
High Concentration	Day 1	15.576 (15.287 to 15.864)	0.360	2.31%
	Day 2	15.692 (15.523 to 15.860)	0.211	1.34%
	Day 3	14.895 (14.844 to 14.945)	0.063	0.42%
	Inter-day precision	**15.342 (15.713 to 14.970)**	**0.569**	**3.71%**
Medium Concentration	Day 1	1.469 (1.452 to 1.486)	0.021	1.46%
	Day 2	1.586 (1.574 to 1.597)	0.014	0.91%
	Day 3	1.354 (1.348 to 1.360)	0.007	0.53%
	Inter-day precision	**1.469 (1.409 to 1.528)**	**0.091**	**6.19%**
Low Concentration	Day 1	0.140 (0.138 to 0.143)	0.004	2.53%
	Day 2	0.157 (0.153 to 0.162)	0.010	3.60%
	Day 3	0.030 (0.028 to 0.032)	0.003	9.57%
	Inter-day precision	**0.147 (0.137 to 0.157)**	**0.014**	**9.71%**

Table 2.10: Results of intra and inter-day precision investigations.

2.3.5.7 Robustness

Robustness describes how sensitive an analytical method is to small but deliberate perturbations. These changes are meant to emulate possible variations of the system parameters that can be expected during routine analysis. The ICH guidelines do not require robustness to be included as a validation test but it can be useful to investigate as it gives the analyst an idea of the capacity of the method to withstand small errors.

The robustness study was carried out to investigate how capable the analytical method was of withstanding small changes in composition, pH and molarity of the mobile phase, as well as column temperature and flow rate. Each change was investigated individually

and the adjustments to the mobile phase were made by preparing fresh isocratic solutions. The studies were carried out using 3 samples representative of the range. The results tabulated below (Table 2.11) show the peak areas and retention times (R_t) of the samples under the original conditions (in bold) and the results of altering the conditions. All the areas were found to be within 10 % of the area found under the original conditions.

		High Concentration		Medium concentration		Low concentration	
Parameter		**Area**	**Rt**	**Area**	**Rt**	**Area**	**Rt**
Mobile Phase							
Ratio Organic:aqueous phase	60:40	1715632	6.46	155923	6.47	15508	6.52
	65:35	1820657	5.80	172054	5.82	17762	5.83
	70:30	1869000	4.43	186331	4.44	18112	4.44
Molarity of ammonium acetate	0.0049M	1824710	5.81	172693	5.83	17471	5.84
	0.0050M	1820657	5.80	172054	5.82	17762	5.83
	0.0051M	1822018	5.34	176173	5.36	17736	5.35
pH of aqueous phase	7.84	1853735	5.03	175744	5.04	16557	5.04
	7.01	1820657	5.80	172054	5.82	17762	5.83
	6.06	1822766	6.64	174912	6.60	15783	6.65
Flow rate	1.1 ml/min	1821421	5.80	172425	5.82	17594	5.85
	1.0 ml/min	1820657	5.80	172054	5.82	17762	5.83
	0.9 ml/min	1858123	6.45	182656	6.46	18351	6.45
Column Temperature	35°C	1824285	6.02	172921	6.04	17526	6.07
	40°C	1820657	5.80	172054	5.82	17762	5.83
	45°C	1826136	5.59	171872	5.60	16590	5.60

Table 2.11: Results of the robustness study. (The bold font indicates the unadjusted conditions.)

The peak area of CLZ was largely unaffected by altering the system conditions but modifications to the mobile phase composition and pH were seen to influence the R_t considerably. A decrease in the flow rate of the mobile phase was also shown to increase the retention time of CLZ. In spite of changes to the HPLC conditions, which resulted mainly in changes to retention time, the analytical results were relatively unaffected and always found to be within 10% of the area obtained under the original conditions thereby confirming the robustness of the method.

2.3.6 Discussion

The validation studies described above show this method to be a reliable means of determining CLZ from tape strip samples. Since the method possesses the necessary

selectivity and linearity across the range, CLZ can be quantitatively determined in the presence of skin components, tape adhesives etc. Over the range of 0.2 μg/ml – 40 μg/ml the method was shown to be accurate and to display intermediate precision. Thus the method may be routinely employed in the analysis of CLZ from tape strip samples.

2.4 ASSAY OF CLOTRIMAZOLE IN SEMI-SOLID DOSAGE FORMS

2.4.1. Background

In order to confirm the label claim of the commercial CLZ products, samples were assayed for CLZ content. Numerous methods of assaying CLZ formulations, specifically creams, can be found in the literature (98,100,106-108,125). These methods often involve solid phase extraction (SPE) but since this is a rather complex procedure a more economic solvent extraction method, based on the work of Abdel-Moety *et al* (2003)(108), was used. Two products were assayed: Canesten® 1% Topical cream (Bayer, Germany) and Candid ® 1% gel (Glenmark, India).

2.4.2. Methods and materials

2.4.2.1 Reagents and materials

As described in section 2.2.1

2.4.2.2 Instrumentation

As described in section 2.2.2

2.4.2.3 Method

For each product, a sample (1g, equivalent to 10mg CLZ) was accurately weighed into a 100ml beaker. Five millilitres of econazole nitrate 1 mg/ml solution (made up in mobile phase) was added along with 40 ml of mobile phase. The beaker was heated over a water bath to 60°C for 15 mins with occasional shaking. The solution was then sonicated for 5 minutes before being briefly returned to the water bath. Once all the lipid components had melted, the beaker was placed in an ice bath and allowed to cool for 15 minutes. The

solidified lipophilic material was separated from the solvent by vacuum filtration. The filtered solution was transferred to a 50 ml volumetric flask and made up to volume with mobile phase. This solution was injected directly into the HPLC system. The same chromatographic conditions as described in 2.2.7 were used to analyse the assay samples.

2.4.2.4 Validation

A small validation study was conducted using Canesten® 1% Topical cream. Since the cream base (placebo) was not available, high, medium and low concentration samples could not be prepared by spiking a blank sample with a known concentration. Samples representing high, medium and low concentrations were therefore produced by weighing out 1.2 g, 1.0 g and 0.8 g of cream respectively.

2.4.3. Results and discussion

The validation study (Table 2.12) showed that the method gave repeatable results even when larger or smaller amounts of CLZ were present in the sample. Table 2.14 shows that the amount of CLZ recovered from a 1 g sample of Canesten was on average 10.16 mg, indicating that this batch contained 1.02 % m/m CLZ. This is well within the acceptable range of 90 – 110 % of the labelled amount as specified by the USP (46).

	Cream weighed (mg)	Amount CLZ expected (mg)	Amount CLZ found (mg)	Percent recovery
High	1.2011	12.01	12.05	100.3%
	1.2055	12.06	12.12	100.5%
	1.2065	12.07	12.16	100.8%
Medium	1.0020	10.02	10.21	101.9%
	1.0000	10.00	10.11	101.1%
	1.0070	10.07	10.17	101.0%
Low	0.8012	8.01	8.08	100.8%
	0.8036	8.04	8.06	100.3%
	0.8041	8.04	7.93	98.6%

Table 2.12: Results of the Canesten® Topical cream assay

Gel weighed (mg)	Amount CLZ expected (mg)	Amount CLZ found (mg)	Percent recovery
1.0241	10.24	11.51	112%
1.0031	10.03	9.57	95%
1.0026	10.03	10.22	102%

Table 2.13: Results of the Candid® gel assay

The percent recovery for the gel product was variable and gave inconclusive results. This indicates that the method, probably the extraction procedure, used to assay the cream formulation was not appropriate for the analysis of this gel product. However, for the purposes of this work Candid® gel represents a product not equivalent to Canesten® Topical cream and the results of the gel product, whilst variable, provide sufficient information regarding CLZ content.

2.5 DISCUSSION

In order to undertake a clinical study using the TS technique a rapid and reliable method for the analysis of tape strip samples was required. HPLC-UV has frequently been used to provide a rapid and practical method for the quantification of CLZ. However, a system specifically designed to analyse CLZ extracted from SC samples could not be found in the literature. Therefore, using a published method as a starting point, a systematic approach of system optimisation was undertaken. Through a series of investigations into the effects of various system parameters a selective and sensitive analytical method was developed with a run time of 7 mins.

The method was validated according to ICH guidelines and consequently assured that this method has the accuracy and precision to produce reliable results. This method can therefore be considered suitable for the analysis of tape strip samples taken during clinical TS studies for the determination of the BA/ BE of topical CLZ products.

These analytical conditions were also used in the assay of Canesten® Topical cream and Candid® gel. The results for the cream formulation were close to 100 % recovery and were shown to be repeatable and accurate. The gel product, however, gave relatively variable recoveries. This inconsistency was not expected to affect the outcome of the TS studies.

CHAPTER 3
DOSE DURATION STUDY (NP_TS 1)

3.1 BACKGROUND AND HISTORY OF TAPE STRIPPING

TS is often used in the field of dermatology to collect SC samples on adhesive tapes for microscopic examination in the diagnosis of certain skin conditions (126-129). However, by carrying out quantitative analysis of the tape strip samples, the applications of this simple sampling technique are greatly increased.

As described in section 1.3.5.2, the TS procedure involves the sequential removal of microscopic layers (0.5-1μm) of the SC which are subsequently assayed for compounds of interest. TS has been applied to determine endogenous SC components (130,131), investigate dermal exposure to toxic or irritant substances (132-134) and, most importantly, in the analysis of drug substances which penetrate the SC (19,38,39,82,122,135,136).It follows then, that TS has great potential for the *in vivo* assessment of topically applied dermatological formulations.

Because the SC acts as a barrier to cutaneous drug absorption, the rate and extent to which a drug penetrates this layer from a topical formulation is, in most cases, the rate limiting step in the absorption process (74). Therefore, the BA of locally acting topical products can be determined by quantitative analysis of drug found in the SC following product application. In the case of drugs having their site of action in the SC, this method has even more relevance (40,137). The method of assessing topically applied products by the determination of drug concentrations in the SC has been termed the dermatopharmacokinetic (DPK) approach (138). The term includes any method in which drug concentration in the skin is measured (139), however, since TS is presently the most widely used and acceptable method of SC sampling, the term has become synonymous with TS.

Since the TS method can be used to assess BA of topical products, it follows that it has been extensively investigated as a method for BE determination. In 1998 the FDA

published an industry guidance entitled “Topical Dermatological Drug Product NDAs and ANDAs-In Vivo Bioavailability, Bioequivalence, In Vitro Release and Associated Studies” (138) in which was described, a DPK approach, using TS, to determine BE between topical products.

This TS method involved a pilot study, in which the sampling scheme was optimized and the method validated, and a pivotal study, in which test and reference products were compared. The design of the pivotal study endeavoured to simulate the absorption and elimination phases seen in an oral pharmacokinetic study so that parameters analogous to those used in conventional BE studies could be determined. The absorption or “uptake” phase was determined by applying the formulation to 4 sampling sites and allowing each site to be in contact with the product for a different dose duration. The residual formulation was then removed with cotton swabs and the TS procedure commenced. The “clearance” phase was determined by applying the formulation to 4 different sampling sites and allowing each site to be in contact with the product for a dose duration determined in the pilot study and thought to represent steady state. The product was then synchronously removed from the sites and each site was tape stripped after a different time period had elapsed.

The use of 10 tape strips per site was suggested. The first 2 tape strips used on each site were discarded, as they were thought to contain residual formulation, and the remaining 8 strips were combined and underwent extraction and quantitative analysis. From the plot of drug amount in the skin vs. time , the AUC and maximum amount of drug found in the SC (A_{max}) was determined (Figure 3.1) and used as parameters to determine BE.

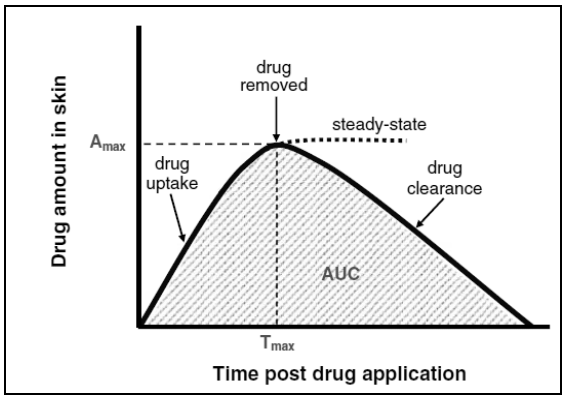


Figure 3.1: Schematic illustration of the profile achieved using the DPK method described in the FDA guidance (137).

The use of this protocol produced some promising results (122), however, in 2002 the FDA withdrew the guidance since major concerns were raised regarding the adequacy of the DPK method to assess topical products that did not target the SC and the reproducibility of the DPK method between laboratories (85). The latter concern was based on contradictory results generated by two reputable independent laboratories regarding the BE assessment of tretinion gel products (140-142). It was suggested that the differences may have been due to lateral spreading of the formulations in addition to differences in the actual stripping procedures (142). Thus it became apparent to the regulatory authorities that there was a need to re-evaluate TS methods.

Since the withdrawal of the guidance further concerns regarding the appropriateness of using parameters such as AUC and A_{max} have been raised, as these parameters have been derived from the principles of oral pharmacokinetics (137). After topical application of a drug, the concentration found at the site of action is determined primarily by SC penetration and processes such as partitioning, diffusion and keratin binding. In contrast, when using the oral route, the plasma concentration *vs*. time profile obtained is controlled by the processes of absorption, distribution, metabolism and elimination. This leads to the oral and topical routes having very different pharmacokinetic profiles. In addition, when a topical product is applied, normally only a very small amount of drug from the applied dose reaches the site of action (143) and drug levels are maintained until the residual formulation is removed (137). When using the oral route however (in the case of an

immediate release formulation) absorption occurs until the dosage form is depleted, after which elimination processes start to dominate.

The TS method described by the FDA was also associated with a high degree of variability. Even when results using the guidance protocol were satisfactory, large numbers of subjects were necessary to achieve a statistical power greater than 80% (122), indicating the inherent variability of this method (137). Some additional sources of the variability may be attributed to the inconsistency in the amount of SC adhering to each tape strip, the variability in the amount of drug present on the discarded tape strips and the variability in the effectiveness of the cleaning procedure (137).

Other weaknesses in the guidance included the trial-and-error approach taken to determine the time points at which TS should take place, the amount of time and labour required to carry out the procedure and the fact that the variability in SC characteristics, especially thickness of the SC, between individuals was not taken into account (40)

Since the withdrawal of the guidance in 2002, the TS approach has been re-evaluated and attempts have been made to improve the method and minimize the variability associated with it. The idea of using the AUC of an 8 point profile has largely been rejected as it is considered impractical (120), although the comparison of AUCs derived from 4 “uptake” points has been used to compare ketoprofen formulations (82). Comparing the total amount of drug found in the SC has been used as an alternative approach to the comparison of AUC. By comparing the total amount of drug present in the SC at 1 time point, differences have been shown between cream and ointment formulations (81). However, this is a relatively rugged approach and makes the assumption that the amount of SC harvested from each subject is the same. It also depends greatly on the chosen dose duration. An adaptation of this approach is the use of 2 time points, 1 representing the “uptake” phase and 1 representing the “clearance” phase (80,137) . This approach also uses the total amount of drug found in the SC as an assessment parameter, however, by including Transepidermal Water Loss (TEWL) assessments, individual SC thickness can be determined (15), and consistency in the amount of SC harvested from each subject can

be achieved. In addition to these methods, a novel approach has been described by Herkenne *et al* (19), in which a single dose duration is used. However, instead of totalling the amount of drug found in the SC, a profile is obtained which describes the amount of drug present at varying depths of the SC.

3.2 PURPOSE OF THE DOSE DURATION STUDY

For the TS work described in this thesis, the approach taken by Herkenne *et al* (19) was used, since this method provides the most detailed information regarding drug penetration and was thus considered to be the most applicable method for the determination of BE. From the drug amount/tape strip *vs.* skin depth plot generated using this method, various relevant transport parameters can be derived, among them AUC. The AUC parameter is influenced both by the amount of drug present in the SC and the extent to which it has penetrated the SC. Therefore, it is a good representation of drug penetration and was the parameter chosen for the BE assessment.

A major consideration when using a single dose duration for a TS study is the choice of dose duration. In other studies (19,80-82,120,144), the choice of dose duration has generally been unsubstantiated. Sampling when the concentration of drug in the SC is at steady state is likely to mask differences in formulations, thus it is important to have a validated method of ensuring that the chosen dose duration falls on a sensitive part of a dose – response relationship, such as a plot of the dose duration *vs.* drug penetration profile.

In order to determine a dose duration which will provide the necessary discriminatory power to identify significant differences or equality between products, the approach employed in the FDA HSBA guidance (52) (the pharmacodynamic method of determining BE between topical corticosteroids) was used. In the HSBA it is necessary to ensure that the pivotal study is carried out at the most sensitive part of the dose-response curve. Therefore, the guidance published by the FDA includes a pilot study which uses the E_{max} model to determine the dose duration where the maximum sensitivity can be

expected – i.e. the ED_{50}. Hence this approach was considered appropriate to determine the dose duration for TS studies.

3.3 STUDY DESIGN

The study involved a single phase sequential design conducted on 10 human subjects. As shown in Figure 3.2 below, 8 (2 x 2 cm) sampling sites on the volar aspect of the left forearm were demarcated using a template. One of the sites was assigned as a blank and the remaining 7 sites were used for product application. Approximately 15 mg (accurately dispensed from a calibrated dispenser) Canesten® Topical cream was applied to each application site at time zero. Each site was exposed to the cream for a different dose duration (0.25, 0.5, 1, 2, 4, 6 or 8 hours respectively) after which the residual formulation was removed and the site was tape stripped. The designation of the sites was randomised between subjects.

The blank site also underwent TS, but in addition, TEWL measurements were taken after each strip in order that the thickness of each subject's SC could be calculated. After TS, the tape strips used on the blank site were spiked with standard solutions to prepare a calibration curve (as described in 2.3.4.1) and the tape strips used on the other sites were analysed as described in section 2.2.6.1. From these data a profile of dose duration *vs.* AUC was plotted and the ED_{50} determined.

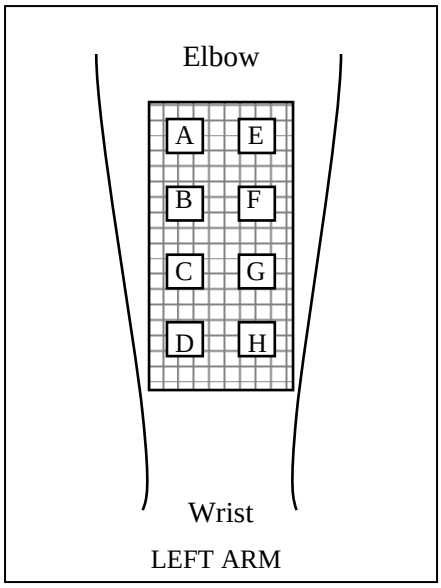


Figure 3.2: Illustration of the template used to demarcate sites A – H.

3.3.1 Materials

3.3.1.1 Study product

Canesten® Topical Cream (Bayer, Germany), Lot. BXPJCRT, containing 1% CLZ was investigated in this study.

3.3.1.2 Templates

Templates were prepared by reinforcing the non-adhesive side of Opsite™ Flexifix™ (Smith & Nephew Medical Ltd, London, England) with Scotch® Magic™ Tape (no.810, 3M, USA) and cutting out 2x2 cm squares to expose 8 sampling sites. The template was designed such that the Opsite™ was in contact with the skin. Opsite™ Flexifix™ was chosen since it is used as a medical tape and thus was unlikely to cause allergic or inflammatory reactions.

3.3.1.3 Tape strips

The tape strips were prepared the day before the study and stored in airtight containers. To make the tape strips, a small piece of Sellotape® Original was cut from the spool and one edge folded over to make a non-adhesive area that could be used as a handling tab.

The tab was made small enough to allow the exposed adhesive surface to be slightly greater than 2x2 cm. The tapes were individually weighed using a Precisa 180A balance (Precisa Balances Ltd., Geneva, Switzerland), sensitivity of 0.0001g, and placed on squares marked on the surface of each storage container, numbered from 1 to 15, to allow individual tapes to be tracked.

3.3.2 Study population

Subjects were recruited from the student body of Rhodes University by posting advertisements around the campus. Ten volunteers (1 male, 9 females) between the ages of 19 and 28 (mean 22 years) were enrolled in the study. Within this group, 7 subjects were Caucasians, 2 were of Indian or Malay descent and 1 participant was Negro. All subjects were verbally informed of the study procedure and also in writing. Written consent was obtained from all subjects before participating in the study. All enrolled subjects completed the study and were remunerated for the inconvenience of participating.

3.3.2.1 Inclusion and exclusion criteria

Inclusion criteria

1. Subjects had to be between the ages of 18 and 50.
2. Subjects had to be in general good health.
3. Subjects had to be available for the entire study period.

Exclusion criteria

1. Female subjects who were breast feeding.
2. Subjects who had known allergy/hypersensitivity to CLZ or any fungicides.
3. Subjects who had any history of drug or alcohol abuse.
4. Subjects who had any mental deficiency or handicap.
5. Subjects who had hairy ventral forearm surfaces and/or abrasions on the underside of their forearms.
6. Subjects who had engaged in sun-tanning within the last month.

7. Subjects who had participated in another dermal microdialysis or tape stripping study within 2 months of the study date.
8. Subjects who had used any topical fungicides within the last three months.
9. Subjects who suffered from any allergic conditions (hayfever, allergic rashes, asthma or childhood eczema).
10. Subjects who suffered from any skin disorder such as psoriasis, eczema or other relevant skin disorder.
11. Subjects who regularly took medicine or used any creams within the last week (contraceptive pills excluded).
12. Subjects with a history of any neurological, kidney or liver disorders.

3.3.2.2 Study restrictions

Subjects were asked to refrain from applying emollients, skin conditioning creams and/or tanning lotions to their forearms 24 hours before the study and from performing strenuous exercise for a period of 12 hours prior to the study. No prescription or over-the-counter medication was allowed to be taken one week before the study and with the exception of the study product no concomitant medication was permitted during the study. Subjects were prohibited from using alcohol 24 hours prior to the study and smokers were only included if they smoked less than 10 cigarettes per day.

If subjects deviated from the above restrictions it was recorded on the Case Report Form (CFR) and the decision as to whether the affected subject would be allowed to continue with the study was left to the investigator.

The subjects remained in the TS laboratory for the entire study period except when making use of the bathroom facilities. The motion of the subject's left arm was restricted by a specially designed constraint that prevented the subject from accidentally smearing the applied formulation. The subjects were allowed food and drink *ad libitum* and a bed was provided if the subjects wished to rest.

3.3.2.3 Criteria for removal from the study

Subjects could be withdrawn from the study at any time, as follows:

1. Voluntary withdrawal by the subject for any reason.
2. Illness or injury during the study if regarded as clinically significant by the principal investigator.
3. Any adverse event or signs of toxicity if regarded as clinically significant by the principal investigator.
4. Failure of the subject to comply with, or co-operate with, any study requirements or restrictions if such failure was regarded as clinically significant by the principal investigator.

3.3.2.4 Pre and post- study screening

A pre-study screening was conducted not more than 30 days prior to the start of the study. A post-study assessment of the tape stripped sites was conducted immediately after completion of the study. The following evaluations were included (Table 3.1):

Screening test	Pre-study	Post-study
Medical history[1]	X	X
Dermatological assessment[2]	X	X
Adhesive sensitivity[3]	X	-

Table 3.1: Pre and post study assessments.

[1]Demographic data (date of birth, age, sex, race), life style assessment (alcohol consumption, smoking habits) medical information (allergies, previous illnesses) and current state of health.
[2] General assessment of the volar aspect of the forearm for any dermatological abnormalities.
[3]Assessment of sensitivity to template material (done at least 2 days prior to the study by applying a small piece of Opsite® Flexifix™ tape to the skin for 4 hours and assessing any inflammatory response)

Appropriate follow-up action was taken for any study-related abnormalities that were observed post-study and followed up until the condition was resolved.

3.3.3 Ethical approval

The inclusion of human subjects in the studies followed the tenets of the Declaration of Helsinki promulgated in 1964 and its amendments (145).The study protocol (Appendix I) was approved by the Rhodes University Departmental Ethics Committee.

3.4 METHOD

3.4.1 Product application

Prior to product application, an Eppendorf® (0.5 ml) pipette was loaded with Canesten® Topical cream. An Eppendorf dispenser, dial set at 2, was used to deliver 2 doses of formulation, totalling 15.2 mg of cream (0.152 mg of CLZ) per site. This amount of formulation was in accordance with previous DPK studies (38,81,82). The precision of the dispenser was assessed prior to use. Each application was found to deliver 7.6 mg of the cream with a %RSD of 1.61%. A pre-weighed glass rod was used to spread the product within the area delineated by the template and was weighed following spreading in order that the amount of formulation applied to each site could be accurately determined.

3.4.2 Product removal

After the required dose duration had elapsed the residual formulation was removed using 2 cotton swabs per site. The swabs were used to wipe the area using small circular motions and care was taken to remove all the formulation visible to the eye. The swabs were placed in microcentrifuge tubes to which 1 ml methanol was added. The tubes were then sonicated for 20 mins before being vortexed and centrifuged as described for tape strip extractions in section 2.2.6.1. The resulting extract was analysed using the previously described HPLC conditions.

3.4.3 Tape stripping

To harvest the SC, each tape strip was placed on the demarcated site and uniform pressure was applied by rubbing the strip 10 times in both an upward and downward motion. The strip was removed with a single upward pull and immediately weighed to prevent SC desiccation. As shown in Figure 3.3, the direction of stripping was rotated (N,

W, S, E) to ensure uniform removal of SC (121). The tape strips were returned to the airtight containers and later extracted using the procedure described in 2.2.6.1.

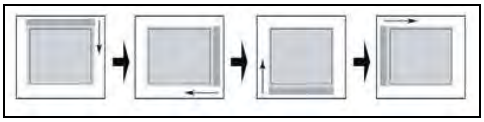


Figure 3.3: N, S, E, W directions used for tape application and stripping (121)

3.4.4 Tape strip weighing

One of the major factors interfering with the tape strip weighing was the static nature of the tape. Unlike some other types of tape, Sellotape® Original does not contain an anti-static agent and, therefore easily acquires a static charge, leading to unstable and inaccurate balance readings. In order to reduce the static charge on the tape strips a Zero-stat 3® anti-static device (Sigma-Aldrich, Atlasville, South Africa) was used and the tape strips were placed on tissue paper during storage to prevent static build up.

3.4.5 Sample analysis

Samples analysis was performed by HPLC using the conditions described in section 2.2.7

3.4.6 Transepidermal water loss (TEWL) measurements

TEWL measurements were taken at the blank site only, using a closed chamber Delifin VapoMeter® (Delfin Technologies Ltd., Finland) (Figure 3.4). A closed chamber system was selected as it is easier to use than an open chamber device and yields similar results (146,147). An initial reading was taken to determine the initial TEWL and then 15 tape strips were used to harvest the SC. After the removal of each tape strip, the VapoMeter® was placed vertically on the site without delay and a reading was taken. The tape strips were weighed immediately after stripping. Once the TEWL reading was completed, the next strip was promptly applied to the site to prevent water loss. The temperature of the room was maintained at 23°C throughout this procedure.

Figure 3.4: Delfin VapoMeter®(148)

3.5 DATA ANALYSIS

3.5.1 Determination of *Stratum corneum* depth

Since the thickness of the SC varies considerably between individuals it was necessary to determine the SC thickness for each subject so that the results could be normalized. In order to do this, the TEWL readings and TS data collected from the blank site were used.

The equation below is a linearized form of Fick's 1st Law and describes water loss from the surface of the skin (15).

$$\frac{1}{TEWL_x} = \frac{H}{K\Delta C \cdot D} - \frac{x}{K\Delta C \cdot D}$$ Equation 3.1(15)

In this equation, $TEWL_x$ represents the transepidermal water flux when x mm of SC has been removed by tape-stripping; K describes the SC-viable tissue partition coefficient of water; D is the average apparent diffusivity of water; ΔC is the water concentration difference across the membrane and H (μm) is the total thickness of the SC.

Therefore, since K, ΔC and D are constant, this relationship can be further simplified as $1/TEWL_x = – x + H$. Assuming that the SC adhering to each tape strip is uniform and has a density of 1 g/cm^3 (14), x can be calculated from the SC mass. Therefore, as shown in Figure 3.5, H can be determined by the x-intercept of the plot $1/TEWL_x$ *vs.* x.

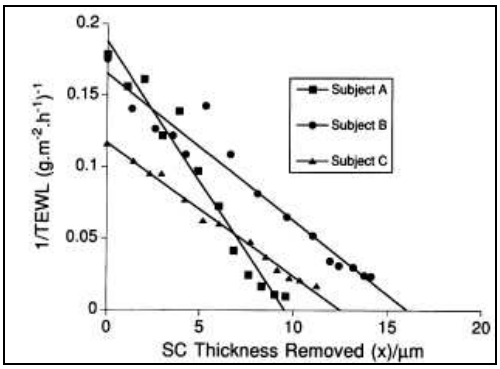


Figure 3.5: Plot showing how the relationship between TEWL and skin thickness removed by TS can be used to determine H (16).

3.5.2 Calculation of AUC

Before the dose duration profile could be constructed the extent of CLZ penetration occurring at each dose duration had to be determined. This was done by calculating the AUC (using the trapezoidal rule) of the curve obtained when the amount CLZ per tape strip was plotted against relative SC depth. The relative SC depth was calculated by dividing the thickness of SC removed by a particular tape strip (x) by H (19). The result was expressed as a percentage.

3.5.3 Dose duration profile

The dose duration profile was constructed using the mean of the AUC values obtained for each dose duration (n=10). An AUC value of 0 was assumed at time = 0 hours as no CLZ penetration could have occurred. This 8 point profile was fitted to the E_{max} model and the ED_{50} was calculated using GraphPad Prism® software Version 4 (GraphPad™ Software, San Diego, California, USA).

3.6 RESULTS AND DISCUSSION

3.6.1 Dose duration profile

The results showed rapid CLZ penetration within the first 2 hours, after which the rate of penetration appeared to level out, indicating that steady state had been achieved (Figure

3.6). This was contrary to other studies where other antifungals appeared to penetrate slowly (120).

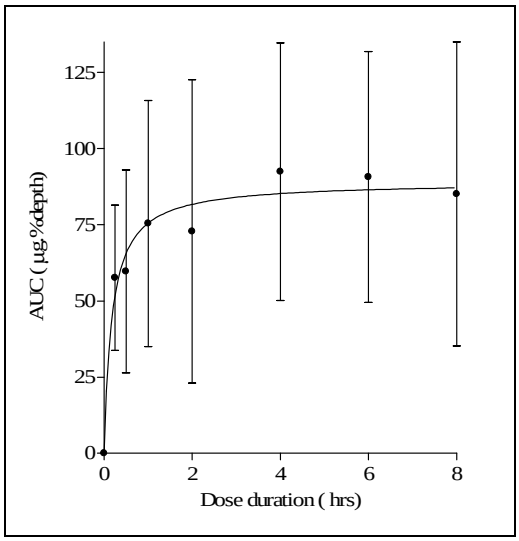


Figure 3.6: Dose duration profile for Canesten® Topical cream (n=10).

The data were fitted to the E_{max} model with $R^2 = 0.9648$, $E_{max} = 89.06$ and $ED_{50} = 0.801$ (10.8 mins). A dose duration on the steep part of the curve in accordance with the appropriate sensitivity was then selected. The ED_{50} is considered the time at which maximum sensitivity can be achieved. However, because it takes approximately 10 mins to carry out the TS procedure, a dose duration of 15 min was selected instead since a shorter dose duration shorter would have been impractical.

The results were associated with a relatively high degree of variability between individuals, as shown by the wide SD error bars shown above. This was expected as the nature of the SC is known to vary substantially between individuals and hence differences in AUC values between subjects were inevitable.

3.6.2 Determination of *stratum corneum* depth

The plots obtained from the blank sites (1/TEWL *vs*. cumulative skin depth) showed good linear correlations, with R^2 values ranging from 0.8995 to 0.9777 in accordance with previous reports (15). The SC thickness of the 10 subjects was found to be 19.5 ± 5.6 µm (mean ± SD) which was also within the expected range (15).

3.6.3 Total amount of *stratum corneum* removed

The use of 15 tape strips consistently removed an average of 83.37% ± 3.66 % (mean ± SD, n = 70) of the SC from the application sites. As can been seen by Figure 3.7, the amount removed was not influenced by the time the site was in contact with the formulation.

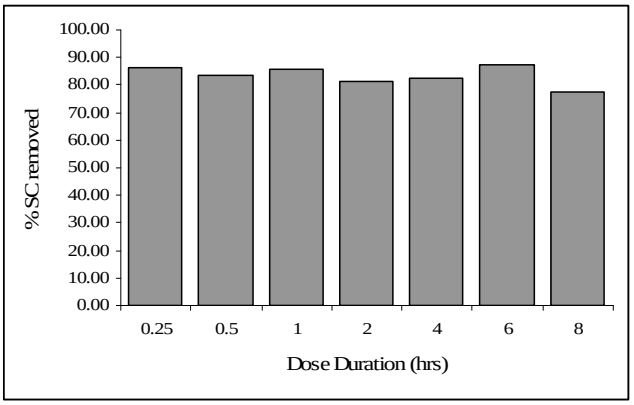


Figure 3.7: The percentage of SC removed for each dose duration (n=10).

3.6.4 Side-effects of tape stripping

The subjects expressed little or no discomfort during the TS procedure or while TEWL measurements were taken. The post-stripping condition of the sites ranged greatly between individuals, from mild erythema to raw inflamed skin. This was consistent with previous findings. However, in all cases a full recovery of the SC was seen within a few weeks (149).

3.6.5 Number of tape strips

Table 3.2 below reveals that the CLZ was not homogeneously distributed throughout the SC. Most of it was present in the first 60 % of the SC. The relative amount of CLZ found after the10th strip was negligible. This confirmed that the use of 15 tape strips was adequate.

Tape strip number	Percent SC depth	Amount CLZ (µg)	Relative amount CLZ (%)
2	8.03	6.31	37.9
3	15.29	3.00	18.1
4	21.79	1.80	10.8
5	28.71	1.19	7.2
6	34.78	0.94	5.6
7	40.71	0.75	4.5
8	46.16	0.56	3.4
9	51.84	0.46	2.8
10	57.91	0.37	2.2
11	63.07	0.30	1.8
12	68.37	0.29	1.7
13	73.23	0.21	1.3
14	83.37	0.22	1.4

Table 3.2: Amount of SC and CLZ removed per tape strip (average for all sites n=70).

3.6.6 Amount of formulation applied

Following application using a calibrated Eppendorf dispenser and spreading of the cream with a glass rod, an amount of 14.47 ± 0.40 mg (mean ± SD) was consistently applied to each site.

3.6.7 Amount of clotrimazole removed by cotton wool swabs

The mean amount of CLZ removed by the cotton swabs (n=10) is presented in Figure 3.8. As can be seen, the longer the dose duration, the less CLZ was removed. This was assumed to be due to CLZ penetration into the SC, however, lateral spreading of the formulation cannot be ruled out.

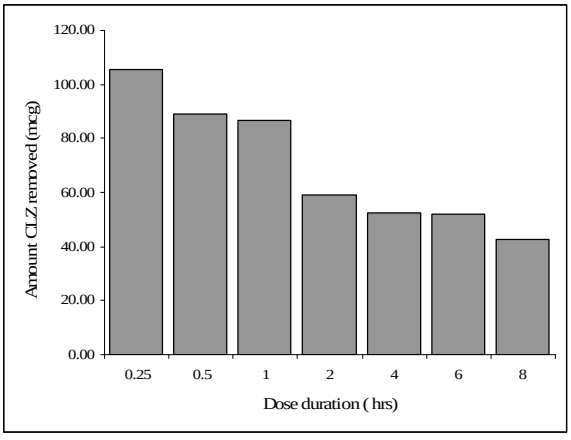


Figure 3.8: Mean amount of CLZ removed using cotton wool swabs (n = 10).

3.6.8 Amount of clotrimazole assumed to have penetrated the SC

In addition to considering the TS data obtained from this study, the total amount of CLZ that penetrated the SC was also investigated using a novel method. This novel approach, termed the "Residual Method", indirectly measures the amount of drug assumed to have penetrated the SC by calculating the difference between the amount of drug applied to the skin and the amount removed after a specific dose duration.

As can be seen below, when the amount of CLZ assumed to have penetrated the skin was plotted according to the dose duration, a plot showing the release of CLZ from the formulation was obtained. Unexpectedly, this plot also fitted the Emax model with R^2 = 0.9662 and ED50 = 0.734hrs ≈ 45mins.

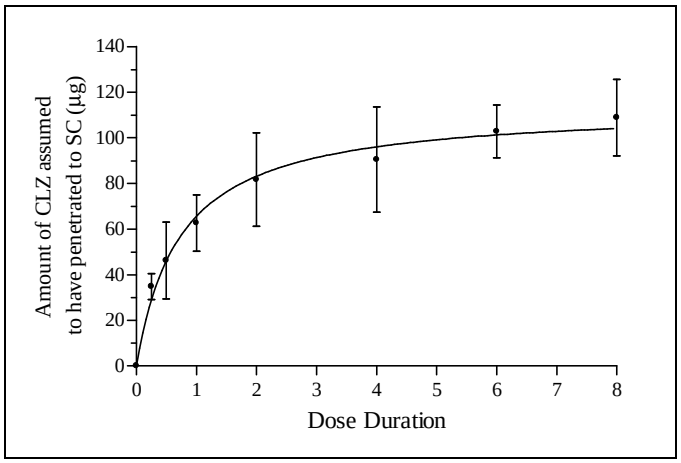


Figure 3.9: Dose duration profile for the "Residual Method" (n=10).

The simplicity and surprisingly low variability associated with this method was intriguing and further investigations using the Residual Method are described in the following chapter.

CHAPTER 4
DETERMINATION OF BIOEQUIVALENCE

4.1 INTRODUCTION

The first step in validating the TS method (i.e. Proof of concept) was to show that the method was able to detect BE between products when BE did in fact exist. In order to do this, BE studies were conducted using Canesten® Topical cream as both the "test" and the "reference" products.

The use of ln-transformed data for the BE assessment of oral products is appropriate since those data have been shown to follow a ln-normal distribution (150). There is some evidence from *in vitro* work to suggest that dermal absorption measurements also follow this log-normal distribution (151-153). However, for *in vivo* dermal pharmacodynamic studies (i.e. the HSBA) untransformed data are conventionally used (52), suggesting that untransformed data may be more appropriate for the assessment of topical products. The FDA draft guidance regarding the DPK method endorsed the use of transformed data (138), however, as this guidance has been withdrawn no guidelines for the analysis of TS data are presently available. Therefore, both ln-transformed and untransformed data were used for the BE assessments in these studies.

The conventional BE limits of 0.8 – 1.25 for the 90 % confidence interval of the ratio were used to define BE, even though the significance of using these relatively "tight" limits in a BE study involving topical formulations is debatable.

In addition to the TS studies, the "Residual Method" was also investigated to determine if there was any merit in pursuing this simplistic method as a BE tool . Since this method was simple and easy to carry out it was conducted in conjunction with the TS studies.

4.2. BIOEQUIVALENCE STUDY (NP_TS 2.1)

4.2.1 Study design

The study involved 10 human subjects. A template, similar to that described in 3.3.1.2, was used to delineate four application sites and one blank site on the left arm of each subject (Figure 4.1). Canesten® Topical cream (15.2 mg) was dispensed to each site as previously described (section 3.4.1), however, since the dose duration was the same for each site the application process was staggered for convenience. In order to collect data suitable for BE assessment, the doses applied to two of the four sites were randomly designated "test product" and doses applied to the other two sites were designated "reference" product. Each site was exposed to the formulation for 15 mins (as determined by the dose duration study) and the removal of the residual formulation and TS were carried out as described in 3.4.2 and 3.4.3.

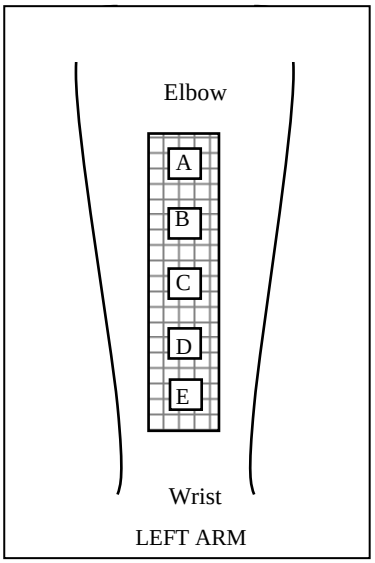


Figure 4.1: Template used for TS in study NP_TS 2.1

As in the dose duration study, the blank site was used to determine individual SC thickness. The AUC for each site was calculated from the profile of CLZ amount/tape strip *vs*. relative skin depth. The mean of the AUC values found for the "test" sites was used as AUC_{test} and similarly the mean of the "reference" AUC values was used as $AUC_{reference}$. A 90% confidence interval ($CI_{90\%}$) of the ratio $AUC_{test}/AUC_{reference}$ was

constructed and the conventional BE limits of 0.8 – 1.25 for the $CI_{90\%}$ of the ratio were used as the acceptance criteria to confirm BE.

In addition to the TS investigation, the right arm of each subject was used to investigate whether the Residual Method was able to determine BE between equivalent products. In order to do this, 4 sites were demarcated on the right arm (using the same template design as described above) and 15.2 mg of Canesten® Topical cream was applied to each site. As in the TS study, 2 of the sites were randomly designated "test" sites and the other 2 "reference" sites. After 45 mins (the ED_{50} determined from the dose duration study, section 3.6.6) the residual formulation was removed using 2 cotton swabs. The swabs were assayed as described in section 3.4.2. The amount of CLZ assumed to have penetrated the SC was then calculated by subtracting the amount of CLZ removed from the amount applied to each site. For each subject, the mean amount of CLZ assumed to have penetrated was determined for the "test" and 'reference" products (n=2). The data were then processed as in the TS study.

SECTION A

APPLICATION OF TAPE STRIPPING FOR BIOEQUIVALENCE ASSESSMENT

4.2.2. Methods and materials

Preparation of the templates and tape strips as well as the procedures for product application and removal, TS, sample analysis, TEWL measurements and SC depth and AUC determination were conducted as in study NP_TS 1, except that the templates demarcated 5 sites instead of 8 and a dose duration of 15 mins was used for all sites.

4.2.2.1 Study product

Canesten® Topical cream (Bayer, Germany), Lot. BXPJCRT, containing 1% CLZ was used as both the "test" and the "reference" products.

4.2.2.2 Study population

Ten human subjects were enrolled in the study (9 females, 1 male). They had a mean age of 22 yrs ± 1.8 (mean ± SD) and were of Caucasian (n=6), Negro (n=1), Indian (n=2) and Asian (n=1) ethnicities. All enrolled subjects completed the study in full compliance with the protocol.

The inclusion and exclusion criteria, study restrictions, criteria for removal from the study and pre/post study assessments were the same as described for study NP_TS1.

4.2.2.3 Ethical approval

The inclusion of human subjects in the studies followed the tenets of the Declaration of Helsinki promulgated in 1964 and its amendments (145) and the study protocol (Appendix III) was approved by the Rhodes University Departmental Ethics Committee.

4.2.3 Data analysis

An AUC_{test} and $AUC_{reference}$ value was determined for each subject by taking the mean (n=2) of the AUC values for each "product". As each subject received both the test and the reference product the study was considered to have a paired / crossover design.

As previously discussed, both ln-transformed and untransformed data were used for the assessment of BE. EquivTest 2.0 (Statistical solutions Ltd, Ireland) software was used to calculate the $CI_{90\%}$ for the $AUC_{test}/AUC_{reference}$ ratio for both sets of data.

For the untransformed data, the point estimate was calculated by dividing the mean AUC_{test} (n=10) value by the mean $AUC_{reference}$ (n=10) value and the $CI_{90\%}$ was determined using Locke's method (52). The CV% associated with the ratio was calculated using the following equation based on untransformed data:

$$CV\% = \sqrt{MSE} / \text{mean} * 100$$

Equation 4.1

In order to determine the number of subjects required for 80% statistical power the method described for "raw data from a cross over study design" by Chow and Wang (154) was used.

For the ln-transformed data, the Schuirmann two one-sided test (TOST) (155) was used to calculate the $CI_{90\%}$ and the point estimate. The following equation was used to determine the CV% associated with the ratio using ln- transformed data:

$$CV\% = \sqrt{e^{MSE} - 1} \; *100$$

Equation 4.2

The statistical power of the ln-transformed data was calculated using the MS Excel 2003 spreadsheet accompanying the 5th edition of *Pharmaceutical Statistics: Practical and Clinical Applications* (150).

4.2.4. Results and discussion

4.2.4.1 Bioequivalence assessment

Results for NP_TS 2.1 TS data		
	Untransformed data	Transformed data
n	10	10
$AUC_{test}/AUC_{reference}$	1.03	1.11
$CI_{90\%}$	0.93 - 1.18	0.94 -1.32
Bioequivalent? (0.8 – 1.25)	Yes	No
CV%	25.26%	21.02%
G	0.1964	n/a**
Power	n/d*	23.20%
n required for 80% power	47	40

Table 4.1 Summary of results for study NP_TS 2.1
*n/d: not determined **n/a: not applicable

As can be seen from the summary above (Table 4.1), the results of the BE assessment were similar using both transformed and untransformed data. For both sets of data the $AUC_{test}/AUC_{reference}$ ratio fell within the 0.8 – 1.25 limits, however, the $CI_{90\%}$ for the transformed data was outside the upper BE limit. The untransformed data showed bioequivalence between the products, however, the power of the study was too low to draw any definitive conclusions. According to the untransformed data an additional 37

subjects would be required to increase the power to 80% and according to the transformed data 30 additional subjects would be required. Increasing the number of subjects would narrow the $CI_{90\%}$ of the data around the point estimate and therefore, with the addition of more subjects, it is possible that the ln-transformed data would fall within the BE limits of 0.8 – 1.25.

4.2.4.2 Effect of expanding the bioequivalence limits

As there are presently no regulatory guidelines regarding the analysis of TS data, the BE limits used in this study (i.e. 0.8 -1.25) were based on those described in the guidance used for BE studies involving oral products (156). For oral products, the clinical relevance of these limits has been demonstrated over many years and hence the use of these limits has become internationally accepted. In the majority of cases, empirical clinical evidence has shown that oral products deemed be bioequivalent according to these limits have the same therapeutic outcomes as their innovator counterparts (157). However, the clinical relevance of applying these limits to BE studies involving topical products is questionable.

As the use of oral and topical dosage forms, as well as the pharmacokinetics associated with each are virtually incomparable, it is highly unlikely that the limits of 0.8 – 1.25 have any clinical significance for locally acting topical products. This was recognised in the draft DPK guidance issued by the FDA, where the limits for the BE parameter A_{max} were relaxed to 0.7 – 1.43 (138).

The data from this study were therefore reconsidered using the more realistic BE limits of 0.75 – 1.33 and even 0.7 – 1.43:

	Untransformed data	Transformed data
BE limits	Sample size required for 80 % power	Sample size required for 80 % power
0.8 – 1.25 Bioequivalent?	47 Yes	40 No
0.75 – 1.33 Bioequivalent?	30 Yes	18 Yes
0.7 – 1.43 Bioequivalent?	20 Yes	10 Yes

Table 4.2: Effect of widening the BE limits

As can be seen in Table 4.2, when using the conventional BE limits, the transformed data do not show bioequivalence even though the same product was used as both test and reference. However, by widening the limits both the transformed and untransformed data show BE.

When the limits of 0.8 – 1.25 are used in order to achieve a statistical power of 80%, a sample size of between 40 and 50 subjects would be required. However, widening the BE limits substantially reduces the number of subjects required for the study to have a power of 80%. This is particularly advantageous as the preparation and sample analysis involved in the TS method is relatively labour intensive, and reducing the number of subjects required without compromising the validity of the study would be beneficial for the routine use of this method.

4.2.4.3 Variability between application sites

Because all the sites used in this study were replicates of each other, the variability between all sites could be determined. The mean AUC value of all 40 sites (i.e. 4 sites x 10 subjects) was 61.49 with a %RSD of 73.35 %. However, due to the use of a paired study design and replicates of test and reference sites, the CV% associated with the $AUC_{test}/AUC_{reference}$ ratio was relatively low.

The variability between the sites within each individual was also investigated and the intra-individual %RSDs were found to range from 20 to 60%. As this was relatively high, consideration was given to investigating the reason(s) for this (*vide infra*).

4.2.5 Conclusions

Even though the ln-transformed and untransformed data gave similar results, only the untransformed data showed BE using the acceptance limits of 0.8 – 1.25. However, if the sample size was increased to achieve a power of 80%, it is likely that the $CI_{90\%}$ would

narrow around the point estimate and that $CI_{90\%}$ for both sets of data would probably fall within those BE limits.

As previously mentioned, however, the relevance of using 0.8 – 1.25 as the BE limits is in itself doubtful. By widening the BE limits to a more realistic range, both sets of data showed bioequivalence. In addition to this, widening the BE interval has the added advantage of requiring fewer subjects to achieve a statistical power of 80 %.

The use of replicate "test" and "reference" sites was advantageous in reducing the effect of the relatively high inter-site variability. However, even though the CV% associated with the $AUC_{test}/AUC_{reference}$ ratio was acceptable, the source of the inter-site variability required further investigation.

SECTION B

APPLICATION OF THE "RESIDUAL METHOD" FOR THE ASSESSMENT OF BIOEQUIVALENCE

4.2.6 Introduction

The Residual Method is based on the notion that the difference between the amount of drug applied to the skin and that removed after a specific dose duration can be assumed to be the total amount of drug which has penetrated the SC. While this approach is very simplistic it has the advantage of being completely non-invasive, easy to perform and requiring minimal equipment.

The dose duration study (section 3.6.6) showed that this method is associated with a surprisingly low degree of variability and, therefore, the following study was undertaken to investigate whether this method may be useful in BE assessment. As in the previously described TS study, this was done using Canesten® Topical cream as both test and reference products.

4.2.7 Methods and materials

The Residual Method only involves the formulation application and the removal of the residual formulation. These procedures were carried out as for the TS studies (i.e. as described in 3.4.1 and 3.4.2 respectively).

4.2.7.1 Study product

See section 4.2.2.1

4.2.7.2 Study population

See section 4.2.2.2

4.2.7.3 Ethical approval

See section 4.2.2.3

4.2.8 Data analysis

The amount of CLZ assumed to have penetrated the SC was calculated by subtracting the amount of CLZ removed by the cotton swabs from the amount originally applied. For each subject the average amount of CLZ assumed to have penetrated the SC for each product (n=2), i.e. $Amount_{test}$ and $Amount_{reference}$, was used to determine the ratio $Amount_{test}/Amount_{reference}$. As for the TS data both untransformed and ln-transformed data were used and the $CI_{90\%}$ of the ratio was constructed using both Locke's method and the TOST respectively. The relevant %CVs and power/sample size calculations were carried out as for the TS data.

4.2.9 Results and discussion

Results for NP_TS 2.1 residual data		
	Untransformed data	Transformed data
n	10	10
Amount CLZ_{test}/Amount $CLZ_{reference}$	1.01	0.96
$CI_{90\%}$	0.84 – 1.19	0.79 – 1.17
Bioequivalent? (0.8 – 1.25)	Yes	No
CV%	22.63 %	24.57 %
G	0.0180	n/a**
Power	n/d*	14.88 %
n required for 80% power	31	25

Table 4.3: Summary of the results for the residual method.
n/d*: not determined, n/a**: not applicable

In view of the fact that this method may be considered relatively rugged and possibly somewhat insensitive, it was encouraging that the point estimates, shown above (Table 4.3), were so close to unity. This indicates that it may be possible to determine BE between topical products by using this method instead of tedious tissue sampling procedures.

The variability associated with the method was also impressive as the CV% approximated that found for the TS study. As in the TS study, the $CI_{90\%}$ for the untransformed data showed BE while the ln-transformed results were just outside the 0.8 – 1.25 limits. As previously discussed, if the sample size were increased to achieve 80% power, it is likely that the $CL_{90\%}$ would narrow around the point estimate and both sets of data could possibly fall within the conventional BE limits.

Since use of the tighter limits seemed to provide an indication of BE, no further calculations were done to extend the limits.

4.2.10 Conclusions

Considering the simplicity and possibly lower degree of sensitivity assumed to be associated with this method, the results of this BE investigation were heartening. However, prior to using such a method on a routine basis further investigation and

rigorous validation is necessary. In particular, the method of product removal would need to be validated and standardised and the ability of the method to detect bio-inequivalence between products would need extensive investigation.

4.3 BIOEQUIVALENCE STUDY (NP_TS 2.2)

4.3.1 Template as a source of inter-site variability

The previous TS study showed that there was a high degree of variability between the application sites. While a high inter-individual variability between sites is generally quite usual and expected, the relatively high intra-individual variability was of concern. The following sections describe how the choice of template coupled with the lateral spreading of the formulation could have contributed to this variability.

4.3.1.1 Choice of template

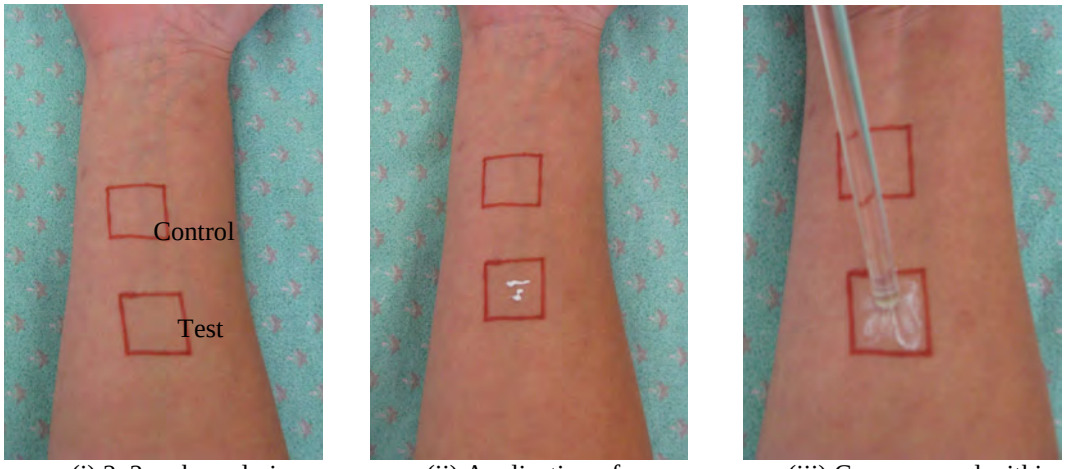
Various methods of site demarcation have been used in TS studies. The most common method is to demarcate the site using a felt-tipped pen and to apply the formulation within the delineated area (38,82,122). After product removal, the tape strips or adhesive discs are then placed directly over the area exposed to the formulation. While this is the simplest method, it has the disadvantage of requiring the tape to be placed very accurately on the sampling site and also the possibility of ink from the skin being transferred to the tape which may interfere with the analytical procedure. When using commercially available adhesive discs, this can be overcome by applying the formulation to an area slightly larger than the size of the disc (81). However, when tape strips are prepared in-house, the size of the tape strips is unlikely to be consistent for each strip and therefore this method is not appropriate. Another approach is to use an adhesive template (121) to delineate the TS area. This approach is adequate for formulations that have minimal lateral spreading, but for formulations with a high degree of lateral spreading the template is likely to absorb the formulation. This leads to non-absorbed drug adhering to the tape strips. In order to overcome the issue of template contamination, a demarcation method using two templates can be used. In this approach, the first template is used to demarcate

an area slightly larger than the TS site for formulation application and the second template is used to delineate the site for the TS (19,120).

Since the extent of lateral spreading exhibited by a formulation determines whether or not it is appropriate to use an adhesive template, the following investigation was carried out to determine if Canesten® Topical cream showed signs of lateral spreading upon application to the skin ,and as such contributed to the previously observed variability between sites.

4.3.1.2 Lateral spreading investigation

As illustrated in Figure 4.2, a 2x2 cm boundary was drawn on the ventral surface of a volunteer's forearm using a felt tipped pen and approximately 15 mg of cream was applied within this demarcated area.



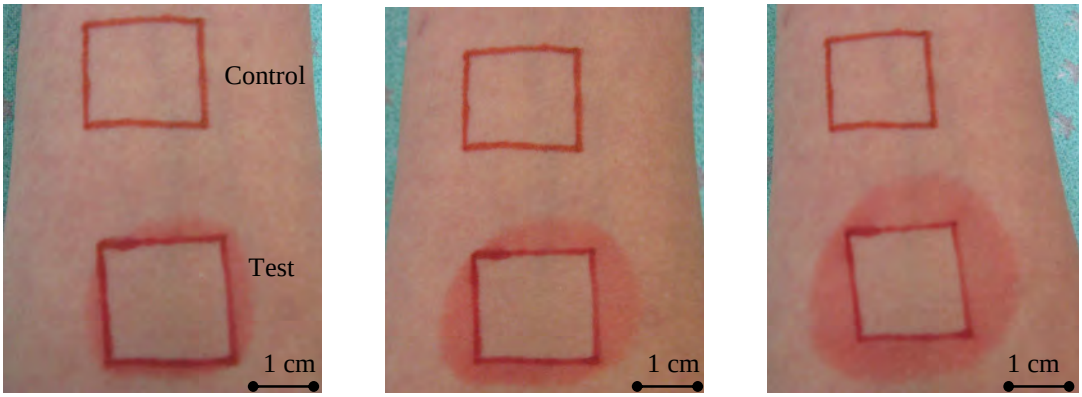
(i) 2x2cm boundaries | (ii) Application of Canesten® Topical cream | (iii) Cream spread within the boundaries

Figure 4.2: Lateral spreading investigation – application of formulation.

Because the ink of the pen was soluble in the cream, lateral spreading could be observed by the spread of colour around the site.

4.3.1.3 Results

As can be seen below (Figure 4.3), even after only 15 mins, lateral spreading of the formulation was clearly visible. After 30 and 60 mins respectively, significant spreading was seen.



(i) Lateral spreading after 15 mins (ii) Lateral spreading after 30 mins (iii) Lateral spreading after 60mins

Figure 4.3: Results of the lateral spreading investigation

4.3.1.4 Discussion

From this investigation it can be seen that Canesten® Topical cream exhibits lateral spreading. Therefore, it is likely that during the previous TS studies, the adhesive templates absorbed the formulation as it spread laterally across the SC. This was an undesirable situation since the template was likely to act as a reservoir and transfer unabsorbed CLZ to the tape strips during the TS process.

4.3.1.5 Conclusions

These results indicated the need to design a new template in order to prevent template contamination in the quest to reduce inter-site variability. In the following TS study, a new template design was introduced in an attempt to overcome the problems associated with the lateral spreading of the formulation. This new design used the approach of a double template, so that contamination of the template could be avoided.

4.3.2 Study design

This study involved the following amendments to the protocol described for study NP_TS 2.1:

1. A new template design was used to delineate the sampling sites.
2. Site E was used as the blank site in the case of all subjects.
3. A more sensitive balance was used for tape strip weighing.

These amendments were implemented in an attempt to improve the accuracy and precision of the TS method.

4.3.3 Materials

The preparation of the tape strips as well as the materials used in template preparation remained unchanged from study NP_TS 2.1 and, as in that study, Canesten® Topical cream was used as both the "test" and "reference" products.

4.3.3.1 Balance

The balance used in the previous studies was accurate to 0.1 mg, but since the mass of SC found per tape strip in the previous studies was in the range of 0.1 mg to 1.0 mg a more sensitive balance was required as it was considered undesirable to weigh a mass using the last decimal. Therefore, in the following TS studies a microbalance accurate to 0.001mg (MX5 microbalance, Mettler Toledo Ltd, Columbus, USA) was used to weigh the tape strips. Because the weighing pan was too small to hold the tape strip a platform was constructed of balsa wood to enable tape strips to be weighed. However, this balance was very sensitive to the static charge carried by the tape strips and it could take up to 30 minutes to weigh a set of 15 tape strips. This problem was largely overcome by using the Zero-stat 3® anti-static device.

4.3.3.2 Study population

This study involved the use of 13 subjects (9 females, 4 males) between the ages of 19 and 30 (mean ± SD was 25 ± 2.8). Four of the 13 subjects were Caucasian, 4 were Negro, 1 was of Asian ethnicity and 4 were of Indian or Malay descent.

The inclusion and exclusion criteria, study restrictions, criteria for removal from the study and pre/post study assessments were as described for study NP_TS 1.

4.3.3.3 Ethical approval

The inclusion of human subjects in the studies followed the tenets of the Declaration of Helsinki promulgated in 1964 and its amendments (145). All changes to the protocol of

study NP_TS 2.1 were approved by the Rhodes University Departmental Ethics Committee (Appendix V).

4.3.4 Method

All the procedures for product application and removal, TS, sample analysis, TEWL measurements, SC depth and AUC determinations and data analysis were conducted as in study NP_TS 1.

4.3.4.1 Site demarcation

Instead of using a single template to demarcate all the sites, the new template design marked each site individually and, most importantly, this new method involved the use of 2 templates per site to prevent contamination. As illustrated in the figure below, the first template was used to demarcate an area of 2.2 x 2.2 cm on the skin (Figure 4.4 (i)) and then a permanent marker was used to mark the corners, thereby outlining an area of 2 x 2 cm (Figure 4.4 (ii)). The formulation was then applied within the bounds of the marked areas and left in contact with the skin for 15 mins before removal (Figure 4.4 (iii)). Before TS commenced, a fresh 2 x 2 cm template was applied to accurately delineate the sampling area (Figure 4.4 (iv)).

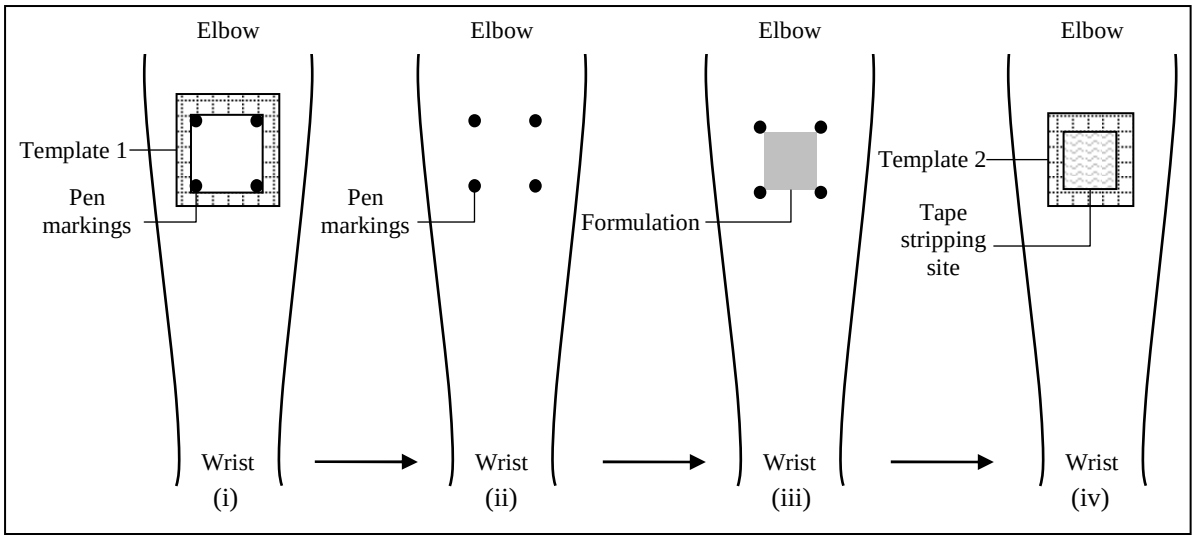


Figure 4.4: The double template design used in study NP_TS 2.2

4.3.5 Results and Discussion

4.3.5.1 Bioequivalence assessment

Results for NP_TS 2.1		
	Untransformed data	Transformed data
n	13	13
$AUC_{test}/AUC_{reference}$	0.94	0.97
$CI_{90\%}$	0.82 - 1.08	0.82 - 1.13
Bioequivalence? (0.8 - 1.25)	Yes	Yes
CV%	23.62%	23.40%
G	0.0399	n/a**
Power	n/d*	47.24%
n required for 80% power	19	21

Table 4.4: Summary of results for study NP_TS 2.2
n/d*: not determined, n/a**: not applicable

The results of this study (Table 4.3) suggest that regardless of whether transformed or untransformed data are used for analysis, if sufficiently powered, TS is capable of showing BE between CLZ products. According to the untransformed data, only 6 additional subjects (n=19) would be required to achieve a statistical power of 80% whereas using ln-transformed data, 8 additional subjects (n=21) would be required.

4.2.5.2 Effect of expanding the bioequivalence limits

Even though the results of this study fell within the limits of 0.8 to 1.25, the effect of widening the BE limits was investigated. Once again it can be seen (Table 4.4) how increasing the limits for the $CI_{90\%}$ of the ratio substantially reduces the sample size required. When the conventional limits are used, 19 or 21 subjects are required for 80% power. This is not unreasonable, but reducing the required sample size to 14 or less is still advantageous.

	Untransformed data	Transformed data
BE limits	Sample size required for 80 % power	Sample size required for 80 % power
0.8 – 1.25	19	21
0.75 – 1.33	14	13
0.7 – 1.43	<13	9

Table 4.5: Effect of widening the BE limits

4.3.5.3 Effect of the new template

The AUC values obtained using the new template design were much lower and less variable than those found in study NP_TS 2.1. As shown in Figure 4.5, in study NP_TS 2.1 the average AUC value (n=40) was 61.49 with a %RSD of 73.35%, while in study NP_TS 2.2 the average AUC value (n=52) found was only 24.62 and the %RSD was reduced to 49.83%.

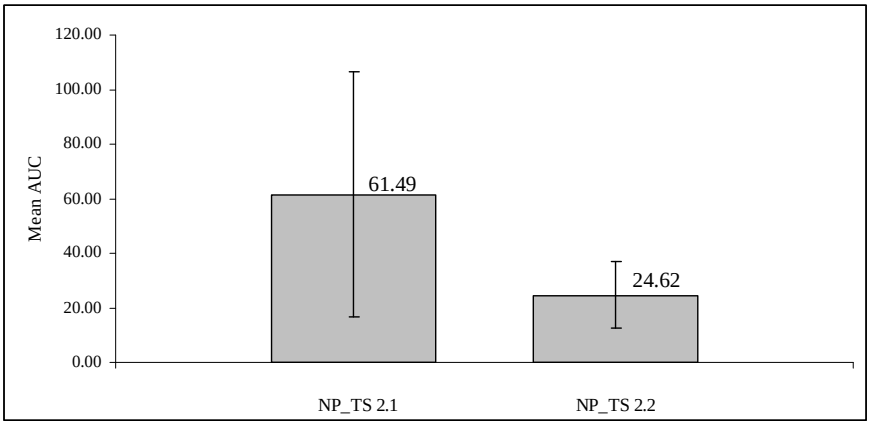


Figure 4.5: Comparison of mean AUC for studies NP_TS 2.1 and NP_TS 2.2

Since the dimensions of the sampling area were consistent with the previous study, the amount of SC removed per tape strip would not have been affected by the new template. Therefore, the decrease in the magnitude of the AUCs must be attributed to a decrease in the amount of CLZ removed by each tape strip, particularly the first few tape strips, where the majority of the CLZ is found. This supports the notion that the previous template design was causing the template to acts as a reservoir. Thus, the new template design can be considered successful in reducing template contamination.

4.3.6 Conclusions

The results of this study showed that TS can be used to determine BE, according to the conventional limits, if the study is sufficiently powered. In this study, the results attained using the transformed and untransformed data were virtually the same, showing that both methods of analysis may be considered acceptable for the BE assessment of TS data.

The effect of the new template was not evident in the variability associated with the $AUC_{test}/AUC_{reference}$ ratio, however, the inter-site variability as well as the magnitude of the AUC values was significantly decreased. As the new template design eliminated the effect of lateral spreading, these results support the hypothesis that the use of a single adhesive template for the Canesten® Topical cream studies resulted in artificially inflated AUC values due to contamination of the template, and subsequently the tape strips, with unabsorbed CLZ.

The investigations into the Residual Method provided some promising results regarding the ability of the method to determine BE between products.The fact that it is non-invasive and simple to perform makes it an attractive prospect for further research. However, until extensive investigation into the sensitivity of the method and rigorous validation studies are conducted this method is not suggested for routine use.

CHAPTER 5

PROOF OF CONCEPT TO ASSESS THE APPLICATION OF TAPE STRIPPING FOR THE ASSESSMENT OF BIOEQUIVALENCE AND BIO-INEQUIVALENCE

5.1 INTRODUCTION

The previous chapter investigated the ability of the TS method to determine BE when Canesten® Topical cream was used as both the test and the reference products. However, to fully assess TS as a BE tool, its ability to detect formulation differences also required investigation. If a method used for BE assessment is not able to detect bio-inequivalence, unsuitable products may be erroneously passed as bioequivalent leading to therapeutic failure or toxicity when used by patients. Hence it is important to confirm that any BE method used has the necessary discriminatory power both BE and bio-inequivalence between products where similarities and differences exist.

The following study was carried out to investigate whether the TS method was capable of determining BE between different products (i.e. cream *vs.* gel). This was done by comparing Canesten® Topical cream with Candid® gel. Although both these products contained 1% CLZ, because of the fundamental difference in formulation design, their rates and extent of dermal absorption were expected to be significantly different.

For the sake of completeness, this study also compared Canesten® Topical to itself by applying it as both "test" and "reference" products.

5.2 BIOEQUIVALENCE AND BIO-INEQUIVALENCE STUDY (NP_TS 3)

5.2.1 Study design

Since this was a "proof of concept" study, only 7 subjects were used to provide preliminary data. The study design was based on previous studies done in our laboratory (121) where only three sampling sites and one blank site were involved (Figure 5.1). The templates described in 4.3.3.1 were applied to the ventral surface of the left forearm to

demarcate the application sites. Canesten® Topical cream was applied to two of the three sampling sites. One of the doses of Canesten® Topical cream applied to these sites was designated the reference product (R) and the other as test product one (T_1). Candid® 1% CLZ gel was applied to the remaining sampling site and was designated test product two (T_2). The application of the products was randomised. Each site was exposed to the formulation for 15 mins, after which the residual formulation was removed and the site was tape stripped.

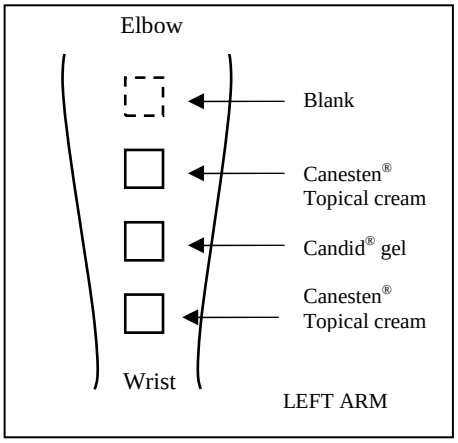


Figure 5.1: Example of a randomisation scheme used in study NP_TS 3

As in the previous studies the blank site was used to determine individual SC depth by means of TEWL measurements. The calculation of AUC was carried out as described in section 3.5.2.

5.2.2 Materials

5.2.2.1 Study products

Canesten® Topical cream (Bayer, Germany), Lot. BXPJCRT, containing 1% w/w CLZ was used as R and T_1. Candid® gel (Glenmark, India), Batch No. 34800141, containing 1% w/w CLZ was used as T_2.

5.2.2.2 Study population

Seven subjects were enrolled in this study (6 females and 1 male). Their average age was 28 ± 4.9 (mean ± SD) years. The ethnicities of the subjects included Negro (n=3), Asian

(n=1), Indian or Malay descent (n=2) and Caucasian (n=1). All the enrolled subjects completed the study.

The inclusion and exclusion criteria, study restrictions, criteria for removal from the study and pre/post study assessments were as described for study NP_TS1.

5.2.2.3 Ethical approval

The inclusion of human subjects in the studies followed the tenets of the Declaration of Helsinki promulgated in 1964 and its amendments (145) and the study protocol (Appendix VII) was approved by the Rhodes University Departmental Ethics Committee.

5.2.3 Methods

The procedures of product application, product removal, TS and skin depth and AUC determination were conducted as previously described.

5.2.3.1 Dispensing Candid® gel

The Eppendorf dispenser, set at 2 on the dial, was used to dispense two doses of 7.7 mg Candid® gel, i.e. a total of 15.4 mg (154 μg CLZ) per site. The precision of the Candid® gel delivery was assessed prior to use and each application was found to deliver 7.7 mg of the gel with a %RSD of 2.06%. As with the cream formulation, a clean pre-weighed glass rod was used to spread the product within the designated area and the rod was re-weighed following spreading.

5.2.4 Results and discussion

5.2.4.1 Bioequivalence assessment

Results NP_TS 3				
	T_1 / R		T_2 / R	
	Untransformed	Transformed	Untransformed	Transformed
n	7	7	7	7
$AUC_{test}/AUC_{reference}$	0.88	1.08	1.67	2.06
$CI_{90\%}$	0.51 - 1.73	0.58 -1.99	0.91 - 3.23	1.06- 3.99
Bioequivalence? (0.8 – 1.25)	No	No	No	No
CV%	36.64 %	27.67 %	27.74 %	24.61 %
G	0.2615	n/a**	0.1574	n/a**
Power	n/d*	<0	n/d*	0
n required for 80% power	29	44	n/a**	n/a**

Table 5.1: Summary of results for study NP_TS 3
n/d*: not determined, n/a**: not applicable

As can be seen above (Table 5.1), in the Canesten® *vs.* Canesten® investigation the T_1/R ratio was found to be close to 1 for both the transformed and the untransformed data. However, because a sample size of only 7 subjects was used, the variability associated with the study was relatively high and the $CI_{90\%}$ fell outside of the BE limits for both products. The small sample size also contributed to the study having a very low statistical power. In order to increase the statistical power of the study to 80%, approximately 30 – 45 subjects would be required. By increasing the number of subjects it is possible that the $CI_{90\%}$ would narrow around the T_1/R point estimate and confirmation of BE between the bioequivalent applications might be shown.

In contrast, the ratio found by comparing the inequivalent product with Canesten® (i.e. T_2/R) was approximately 2 for both sets of data. This indicated that the rate and extent of CLZ penetration from the gel was double that of the cream formulation. This clearly showed that these products were not bioequivalent. Because the point estimate was so much greater than 1, the probability of this study showing BE was completely unlikely regardless of the sample size used.

5.2.4.4 Influence of expanding the bioequivalence limits

As in the previous BE studies, the effect of widening the BE limits was investigated for the T_1/R study. Although the $CI_{90\%}$ for T_1/R did not fall within the BE limits of 0.75 – 1.33 or even between 0.7 – 1.43, the number of subjects required to confirm these results was calculated.

The table below (Table 5.2) shows how the number of subjects required to confirm the study results decreases as the BE limits are widened. As the point estimate and $CI_{90\%}$ for the T_2/R study was so far out of the BE limits, calculating the sample size required for various BE limits would have been meaningless.

	Untransformed data	Transformed data
BE limits	Sample size required for 80 % power	Sample size required for 80 % power
0.8 – 1.25	29	44
Bioequivalent?	No	No
0.75 – 1.33	22	23
Bioequivalent?	No	No
0.7 – 1.43	18	14
Bioequivalent?	No	No

Table 5.2: Effect of widening the BE limits on the power of the T_1/R study

5.3 CONCLUSION

Although, the small sample size resulted in the power of the studies being very low and the BE assessments being inconclusive, this small investigative study was able to demonstrate that the TS method is capable of detecting both differences and similarities between CLZ formulations, albeit not being able to confirm BE.

Considering the results from the previous Canesten® *vs.* Canesten® studies, it is possible that an increase in the number of subjects would provide more informative and realistic results. However, the data suggest that, regardless of the increase in subject numbers, the outcome of the Candid® gel *vs.* Canesten® Topical cream study would remain unchanged.

Whilst the data obtained from this "pilot" study were inconclusive because of the low number of subjects, the vast differences found between the cream and gel formulations

suggest the potential of the method to detect formulation differences if such differences truly exist.

CHAPTER 6
CONCLUDING REMARKS

The studies described in the previous chapters (NP_TS 2.1 and NP_TS 2.2) have shown that, if properly powered, it is possible for the TS method to determine BE between topical CLZ products. In addition, the small "proof of concept" study (NP_TS 3) indicated that this method may be capable of detecting formulation differences. These results are heartening because they suggest that TS has the potential to be used in a regulatory setting as a tool for the assessment of BE between topical CLZ products. As TS is a more direct method of assessing BE than comparative clinical trials, as well as being less costly and time consuming, it would be extremely advantageous to be able use this method instead. In addition, the relatively low degree of variability associated with this technique makes it a practical method for routine use.

Because the site of action for antifungals is the SC and the TS method takes samples directly from this tissue, this technique is particularly appropriate for the assessment of these formulations. Therefore, by using CLZ as a model compound, these studies have shown that the TS method has the potential to be used as an assessment tool not only for CLZ formulations, but also for other antifungal preparations.

The analytical method developed for the quantitative analysis of CLZ from the tape strip samples was shown to have the necessary accuracy and precision over the required range. Careful selection of the tape strip material ensured that the tape strips had the necessary adhesive properties to harvest SC, yet the adhesive compounds extracted from the tape strip during the preparation of the samples did not interfere with the CLZ analysis.

By assaying each tape strip individually and normalising the amount of SC removed, through use of TEWL measurements, detailed information regarding the amount and extent of CLZ penetration was obtained. This approach was different from some other TS studies where the total amount of drug present in the SC was used as the parameter for BE assessment and the distribution of the drug throughout the SC layer was not considered. However, it is recommended that data describing not only the total amount

but the distribution of the drug through the SC should be used when assessing topical formulations. From these TS studies it was clear that the CLZ was not uniformly distributed throughout the SC and it is possible that the distribution of drug throughout the SC will affect the efficacy of the product. Therefore, by considering the extent of drug penetration (AUC of the amount CLZ/tape strip vs. relative SC depth profile) as an indicator of bioavailability, this parameter is clearly appropriate to determine BE of such products.

In addition to these measures, the selection of an appropriate dose duration was based on a preliminary dose duration study. This was a novel approach since no method of determining an appropriate dose or dose duration for TS studies has been described. However, in this work a dose duration with the necessary discriminatory power was determined by applying the E_{max} model to the dose – response relationship found between the dose duration and AUC. Using this approach to determine the dose duration for the BE studies ensured that the TS method had the necessary sensitivity to discriminate between products and detect formulation differences.

A comparison of the results obtained using untransformed and ln-transformed data shows that both approaches are satisfactory for the analysis of TS data. However, the relevance of using ln-transformed data for dermal BE studies is as yet undetermined. It is possible that contrary to BE data of oral products, TS data are normally distributed and do not require logarithmic transformation.

One of the issues surrounding TS is the use of the conventional BE limits of 0.8 – 1.25. Since the relevance of using these limits in BE studies involving topical products is unsubstantiated, widening the limits may be appropriate. Because of the vast differences with regard to the method of dosing, the target site and the pharmacokinetics between oral and locally acting topical products it is unlikely that the 0.8 – 1.25 BE limits have the same clinical significance for topical products as they have for oral dosage forms. Moreover, as shown in the Canesten® Topical cream *vs.* Canesten® Topical cream studies, widening the BE limits decreases the number of subjects necessary to achieve

adequate statistical power. As this method of TS is a relatively labour intensive procedure reducing the number of subjects required for statistical power is particularly advantageous.

From this TS work the importance of the template design for TS studies was highlighted. The characteristics of the formulation, particularly the degree of lateral spreading, need to be considered when selecting a template design. If the template is contaminated with the formulation, unabsorbed drug will be present on the tape strips leading to artificially inflated AUC values.

In addition to the TS investigations the “Residual Method” was investigated as an entirely non-invasive way of determining the total amount of drug present in the SC. This method showed potential as a BE tool, but extensive development is still required. The main disadvantage of this method is that it does not measure the drug in the tissue directly and is possibly too insensitive to be used in a regulatory setting. However, the fact that no tissue sampling is required makes the prospect of further investigation attractive.

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APPENDIX I

Protocol NP_TS 1

1 BACKGROUND INFORMATION

1.1 Clotrimazole

1.1.1 Molecular structure

Clotrimazole is a widely used imidazole antifungal agent that was first synthesised in 1967 by Bayer [1, 2]. It has a molecular weight of 344.8g/mol and is found as a white crystalline powder [3]. The molecular structure of clotrimazole is represented below. The empirical formula for clotrimazole is $C_{22}H_{17}ClN_2$ [3].

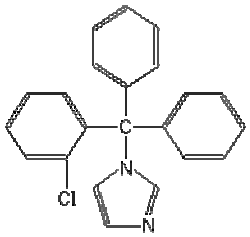


Fig. 1: Chemical structure of clotrimazole [4]

1.1.2 Mode of action and uses

Ergosterol is an important component of the fungal cell membrane and helps maintain the cells integrity in a similar way to the cholesterol found in animal cells [5]. Ergosterol is synthesised in the fungal cells from lanosterol. The enzyme responsible for converting lanosterol to ergosterol is the CYP450 enzyme 14–α–demethylase. Clotrimazole interacts with this enzyme thereby inhibiting ergosterol synthesis [2, 5]. The lack of ergosterol alters the fungal cell membrane causing an increase in cellular permeability. This causes the cell contents to leak out of the fungal cells thereby destroying them. The action of clotrimazole is specific to the pathogen and will not affect the host cells [6].

Clotrimazole is available in topical preparations (as a cream, lotion, spray, solution and powder), vaginal preparations (as a vaginal tablet and vaginal creams) and lozenges. The oral route has been used in the past but is largely superseded by other oral azoles [7]. The 1% topical cream that will be used in this study has the following indications: treatment of cutaneous candidiasis (yeast infection or thrush which is a fungal infection of any of the Candida species, of which Candida albicans is the most common) and dermatophytoses (fungal infections of keratin in the skin and nails). It may also be useful in the treatment of tinea vesicolor (a common skin infection

caused by the yeast Malassezia furfur which is normally found on the human skin and only becomes troublesome under certain conditions) and Corynebacterium minutissimum (a bacterium which causes a superficial skin infection marked by reddish brown scaly patches known as erythrasma.) [8].

1.1.3 Pharmacokinetics

A topical application of clotrimazole will penetrate the epidermis but there is little if any systemic absorption [7]. This is desirable since the stratum corneum is considered the site of action. (Dermatophytes that infect the skin are only able to survive in the dead keratinised cells of the stratum corneum). After vaginal use, however, between 3% and 10% of a dose has been reported in the systemic circulation [7]. Clotrimazole is metabolised by liver enzymes to form inactive compounds that are excreted in the faeces and urine [7].

1.1.4 Adverse effects

Local reactions including skin irritation and a burning sensation may occur following topical treatment. Contact allergic dermatitis has been reported. In cases of systemic absorption, lower abdominal cramps, increase in urinary frequency or skin rash may occur [7, 8]. Nausea, vomiting, unpleasant mouth sensations, and pruritus have been reported after the use of oral clotrimazole lozenges. Raised liver enzyme values have also occurred [7]. Intravaginal preparations of clotrimazole may damage latex contraceptives and additional contraceptive measures are therefore necessary during local application gastrointestinal disturbance, elevation of liver enzymes, dysuria, and mental depression have been reported after oral clotrimazole [7, 8].

1.1.5 Contraindications

Contraindications include a possible hypersensitivity to clotrimazole and / or cetostearyl alcohol [8].

A copy of the product information leaflet of the topical formulation to be used in this study is located in Appendix I.

1.2 Tape stripping technique

1.2.1 Overview of tape stripping

Tape stripping is a non-invasive technique employed to study the penetration, the distribution and the dermatopharmacokinetics of topically applied drugs and cosmetics products [9-12] within the stratum corneum. It is commonly used to disrupt the epidermal barrier to enhance the delivery of drugs *in vivo* [13] and to obtain information about stratum corneum function [11]. Tape stripping has been used extensively in dermatological and pharmaceutical fields to measure the stratum corneum mass and thickness, to collect stratum corneum lipids and protein samples, detect proteolytic activity associated with the stratum corneum, quantitatively estimate enzyme levels and activities in the stratum corneum and allow the detection of metal in the stratum corneum [11]. Although in use for over five decades there are no universally accepted protocols for tape stripping [14], however it has been identified to be of sufficient utility to have been proposed by the FDA as part of a standards method to evaluate the bioequivalence of topical dermatological dosage forms [15].

1.2.2 Principles of tape stripping

Tape stripping involves the sequential removal of microscopic layers (typically 0.5 - 1 μm) of the stratum corneum [10, 16] by placing a strip of adhesive tape onto the skin surface with uniform pressure, which is then removed [9,10]. Drug uptake into the stratum corneum *in vivo* can be measured by harvesting the stratum corneum previously exposed to a topical product with adhesive tapes, which are subsequently extracted and quantified for drug concentration by a validated analytical method [17]. The number of tape strips needed to remove the stratum corneum varies with age, gender, anatomical site, skin condition and possibly ethnicity [11]. Tape stripping is putatively simple, inexpensive and has been described as a minimally invasive technique [9].

1.2.3 Invasiveness of tape stripping

Although tape-stripping is considered to be essentially non-invasive, stripped sites in certain dark-skinned individuals may remain hyperpigmented for several months after healing. This effect will be communicated to the human volunteers before entering the study [10].

1.2.4 Measurement of Transepidermal Water Loss (TEWL)

TEWL has been shown to inversely correlate to stratum corneum depth [18]. Therefore, TEWL measurements in conjunction with the mass of stratum corneum harvested can be used to determine the stratum corneum thickness for each volunteer. This information will enable the data to be normalised. TEWL readings will be measured at the control site only. Readings will be taken after the removal of each tape strip. A Delfin® vapometer will be used to measure the TEWL. The instrument is placed on the surface of the skin, slight pressure is applied to ensure the chamber is sealed and a reading is obtained within 10 to 20 seconds.

2 STUDY PRODUCTS

2.1 Description

Product studied	
Commercial name	Canesten® topical cream
Generic name	Clotrimazole cream
Dosage form	Cream
Strength	1% m/m
Manufacturer	Bayer, Germany
MCC registration details	E/20.2.2/49
Expiry date	12/2009
Description	A soft, white cream
Dosage	± 5 mg per $1cm^2$ application site

2.2 Supply, storage and use

Sufficient Canesten® Topical Cream will be timeously supplied to the clinic by the principle investigator prior to the study. It should be used externally only. The product should be stored below 25° C and kept out of reach of children [8]. Dispensing and administration of test products will be recorded and administered only to subjects for the purpose of this study.

3 OBJECTIVES

The objective of this study is to measure dermal concentrations of clotrimazole using tape stripping to determine *in vivo* the extent of penetration from a topical cream formulation according to dose duration. Thereby, valuable information which will lay the foundation in an attempt to determine bioequivalence of clotrimazole topical formulations using this method will be obtained.

4 STUDY POPULATION AND MEDICAL ASSESSMENT

4.1 Number of subjects

Similar studies have made use of 6 - 18 human subjects for this type of study [13, 19-23] with equal numbers of male and female subjects. This study will make use of approximately 10 human subjects and will include male and female subjects. Eight application sites on the left arm of the subjects will be used for the tape stripping studies. Preliminary statistical analysis (Section 8) will be performed on the subjects completing the study. If needed, an add-on study of up to 10 subjects will be conducted to obtain a degree of variability of less than 30% if possible. If no further study is required, preliminary statistical analysis will be finalised and presented in the final report. If an add-on study is conducted, data for all evaluable subjects from both Group 1 and Group 2 will be used in the preparation of the final report. Homogeneity testing will be conducted between the two groups.

4.2 Inclusion/Exclusion criteria

Inclusion criteria

Only those subjects meeting the following criteria will be included in the study:

i. Subjects who are aged between 18 and 50.
ii. Subjects who are in general good health.
iii. Subjects who will be available for the entire study period.

Exclusion criteria

Subjects meeting the following criteria will be excluded from the study.

i. Female subjects who are breast feeding.
ii. Subjects who have a known allergy/hypersensitivity to clotrimazole or any fungicides.
iii. Subjects who have any history of drug or alcohol abuse.
iv. Subjects who have any mental deficiency or handicap.
v. Subjects who have hairy ventral forearm surfaces and/or abrasions on the underside of their forearms.

vi. Subjects who have engaged in any sun-tanning or taken any sunny vacations within the last month.
vii. Subjects who have participated in another dermal microdialysis or tape stripping study within 2 months of the study date.
viii. Subjects who have used any topical fungicides within the last three months.
ix. Subjects who suffer from any allergic conditions (hayfever, allergic rashes, asthma or childhood eczema).
x. Subjects who suffer from any skin disorder such as psoriasis, eczema or other relevant skin disorder.
xi. Subjects who take regular medicine or tablets or used any creams within the last week (contraceptive pills excluded).
xii. Subjects with a history of any neurological, kidney or liver disorders.

4.3 Subject restrictions

No prescription medication and OTC medication (e.g. cold preparations, vitamins, natural products used for therapeutic benefits, antacids, herbal or traditional remedies) will be allowed for at least one week prior to the study.

With the exception of study product no concomitant medication may be taken by subjects during the study.

No alcohol may be taken by subjects from 24 hours prior to product application and during the study.

No strenuous physical activity may be undertaken by subjects from 12 hours before product application and during the study.

Subjects must not smoke more than 10 cigarettes per day and will not be allowed to smoke during the study.

Subjects must refrain from applying emollients, skin conditioning creams and/or tanning lotions to their forearms for a period of 24 hours prior to the scheduled time of product application.

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

4.4 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 during the study if regarded as clinically significant by the principal investigator and the supervisor.

Any adverse event or signs of toxicity if regarded as clinically significant by the principal investigator and the supervisor.

Failure of the subject to comply with or be uncooperative towards any study requirements or restrictions if regarded as clinically significant by the principal investigator and the supervisor.

Subjects withdrawn or dropped out of the study will be fully documented and accounted for in the CRF.

5 STUDY PROCEDURE

5.1 Pre- and post-study medical screening

Pre-study screening will be conducted not more than 30 days prior to the start of the study. The pre-study evaluations will be conducted as listed in the table below.

Screening test	Pre-study	Post-study
Medical history	1	4
Dermatological assessment	2	5
Adhesive sensitivity	3	-

Medical history:

Demographic data (date of birth, age, sex, origin), skin (dermatological), allergies, alcohol consumption, smoking habits, dietary habits and sporting commitments.

Dermatological General assessment:

of the volar aspect of the forearm and any dermatological condition which may influence the barrier function of the skin and impact on the absorption of topically applied topical CLZ.

Adhesive sensitivity:

Assessment of subject’s sensitivity to adhesive on application site demarcation tape.

Medical history: Since start of study.
Dermatological: Examination of forearms and application sites.

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

5.2 Medical tape sensitivity screening

No less than 2 days prior to the start of the study, volunteers will undergo an assessment of their tolerance to the adhesive on medical tape used to demarcate application sites and for tape stripping procedures.
The following screening protocol will be utilised.
Adhesion of the Opsite® (Smith-Nephew, Hull, UK) medical tape will occur at 0800 hours.
The medical tape will be removed after four hours.
The forearm of the subject will be assessed for allergies to the adhesive medical tapes.
Subjects who exhibit allergies will not be eligible as candidates for the study.

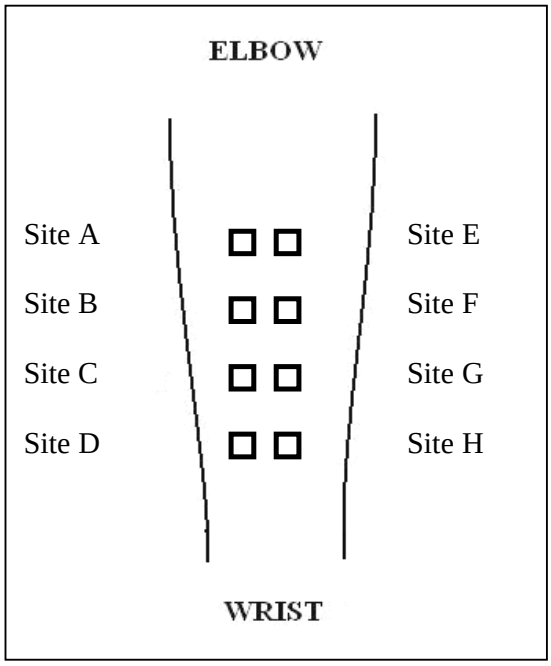
5.3 Check-in and confinement

Subjects will check-in at the clinic (Room T14) in the Faculty of Pharmacy building, Rhodes University, Grahamstown at 0900 hours or 1200 hours of the study day when they will undergo a brief medical examination, inclusion and exclusion criteria check and study restriction check. Subjects accepted into the study will then be prepared for product application. Subjects will remain in the study room for the entire duration of the study except to use the bathroom facilities.

5.4 Study design

The study will comprise of a single phase sequential design conducted on 10 subjects in the first instance, followed by another 10 subjects if necessary (see section 4.1). 1 subject will be studied on any study day and the time between the first and the tenth subject is expected to be approximately 2 weeks. A total of eight application sites will be employed on the right arm on each subject for the tape stripping study as shown in the diagram below. The dosage duration of the formulation will be

randomized between sites and individuals. The duration of the study for each subject will be approximately 8 hours.



The subjects will be requested to wash their forearm with soap during their usual ablutions (at least an hour before the study commences) and not apply any topical products to the arm. A template for use with the tape stripping study will be affixed on the left arm of the subject. The templates will be made from OpSite® (Smith-Nephew, Hull, UK) adhesive dressing reinforced with 3M Scotch® Magic tape (no.810) on the none-adhesive side. These will be used to demarcate 8 sites (2 cm x 2 cm) of the *stratum corneum*. Seven sites will be used for sampling and one as a control. The cream will be applied all sampling sites at time zero. The time of removal of the cream will take place at 0.25, 0.5, 1, 2, 4, 6 or 8 hours after application. Each site will be exposed to a different dose duration. The cream will be removed by wiping with a cotton swab. Adhesive tapes (Sellotape® Original) will be applied to each sampling sites with uniform pressure and then subsequently removed. 15 successive strips will be made from each site. No formulation will be applied to the control site but transepidermal water loss measurements will be recorded from the site in order to determine the thickness of the *stratum cornum*.

5.5 Pre-study day activities and procedures

This study is dependent on a number of preparations prior to the study. The following is a list of items that will be made available at least 24 hours prior to the study.

Calibration standards and mobile phase for clotrimazole analysis will be prepared.

The required number of tape strips will be cut for *stratum corneum* harvesting.

Templates will be prepared

The checklist will be performed (Appendix II).

The general setup of the clinic will be ensured.

5.6 Study day activities and procedures

- Adhesive tape strips (Sellotape® Original) for the tape striping study will be weighed out in the morning prior to start of study.
- The adhesive template for use with tape stripping will be affixed onto the left forearm to delineate the application sites on the *stratum corneum*.
- A foam guard will be strapped to the arm so that the elbow cannot be bent (this will help prevent the volunteer from smudging the cream
- The formulation (±20 mg) will be applied to the assigned application site.
- Excess formulation will be removed from the application sites after 0.25, 0.5, 1, 2, 4, 6 or 8 hours.
- Tape strips will be applied with uniform pressure to the site and then subsequently removed.
- 15 successive strips will be removed from each site.
- The removal of strips will be done in a clockwise manner (N, E, S, W) ensuring that equal amount of stratum corneum is removed in each of the 8 sites.
- Transepidermal water loss measurements will be measured from the control site after each tape strip.
- Each tape strip will be weighed immediately after tape stripping.
- The template will be removed from the subject.
- The forearm will be rinsed to remove any residual drug.

The actual time of study procedures and/or results/comments obtained during the study will be recorded on the '*Registration of Data during Tape Stripping Form*' (Appendix III).

5.8 Product application

Just prior to the product application, an Eppendorf® (0.5 ml) pipette will be filled with Canesten® 1% topical cream. The products will be dispensed twice (3 on the dial of the Eppendorf® dispenser) at each application site to allow ± 20 mg of the products (corresponding to 0.2mg of clotrimazole) to be applied at each site for tape stripping. The Eppendorf® dispenser will be used to ensure that an accurate amount of test product is dispensed to each application site for each subject. A pre-weighed glass rod will be used to spread the product and weighed following the spreading to determine the accurate amount of formulation applied to each site. Application of the product will be done by the principal investigator.

5.9 Posture and physical activity

Strenuous exercises will not be permitted as described in section 4.3.

5.10 Food and fluids

Subjects will not be restricted with respect to food and fluid intake.

5.11 Subject monitoring

The principal investigator will be present at all times during the study. Subjects will be asked open-ended questions about their health at the time of each assessment and any discomfort observed during the experience will be recorded in the CRF (Appendix VI).

6 SAMPLE ANALYSIS

Samples will be analysed for clotrimazole using a validated extraction procedure and HPLC analytical method. Analysis will be done within 48 hours after sample collection. Samples will be kept in the fridge at (4 – 8 °C) to prevent any degradation.

7 DATA ANALYSIS

Amount of clotrimazole per individual tape strip *vs*. fraction of skin thickness for each dose duration will be profiled. The AUC for each dose duration will be calculated from these profiles and then plotted against the dose duration time. The most discriminatory dose duration from the tape stripping study will be used in future studies. Deletion of any data from the analysis will be motivated. All individual

subject data will be documented and individual tape strip amount vs. curves will also be presented in linear/linear and log/linear scale.

7.1 Statistical analysis

Pharmacokinetic and statistical parameters will be determined by the principal investigator using GraphPad Prism version 4. The statistical analysis will estimate the variance associated with subject-to-subject variability. Summary statistics such as median, minimum and maximum will be given.

8 ETHICAL AND REGULATORY REQUIREMENTS

8.1 Ethical and institutional review

Approval by the Rhodes University Ethical Standards Committee (RUESC) (departmental) will be obtained before the study commences. The original signed copy of the ethical approval will be retained by the principal investigator.

The study will be conducted in accordance with the recommendations of the guidelines as set out in the Declaration of Helsinki (1964) and its amendments of Tokyo (1975), Venice (1983), Hong Kong (1989), Somerset West (1996) and Edinburgh (2000), according to ICH Good Clinical Practice (GCP) guidelines and in compliance with the Biopharmaceutics Research Group's (BRG) SOPs, RUESC requirements and guidelines on the conduct of clinical trials in South Africa.

8.2 Written informed consent

Preceding the study, the nature, purpose and risk of participating in the study will be explained to all volunteers. If volunteers desire, they will be given time to consider the information and any questions that they might have will be answered. They will also be informed that they may withdraw from the study at any time without penalty to themselves (other than a reduced remuneration) but that they will be encouraged to be committed to completing the study prior to their enrolment. They will sign a consent form in the presence of a witness. Subjects will receive written, detailed instructions concerning the study performance and restrictions.

8.3 Case report form

The Case Report Form (CRF) for this study will be designed and supplied by the principal investigator (Appendix VI). All case report forms will be quality assured and all major events such as final acceptance of a subject, adverse events and final release from the study will be signed by both the principal investigator and the study consultant.

8.4 Record retention

All source documents, study reports and other study documentation for which the principal investigator is responsible will be archived and retained by the Faculty of Pharmacy, Biopharmaceutics Research Group. Results will be published in the scientific journals and/or presented in a thesis submitted to Rhodes University in fulfilment of the requirement for the degree of Master of Science (Pharmacy) after the completion of the study.

8.5 Insurance

Subjects will be insured against any permanent adverse effect on their health which may arise in connection with the conduct of the study. A copy of the insurance certificate (Appendix VII) will be provided to RUESC (departmental) as part of the application to conduct this study. Adequate insurance cover in the event of negligence on the part of principal investigator and the supervisor will be ensured.

8.6 Termination of the study

The principal investigator reserves the right to terminate the study in the interests of subject welfare following consultation with the supervisor. The supervisor may terminate the study at any time for scientific or safety reasons. If the study is prematurely terminated or suspended for any reason the principal investigator will promptly explain to the subjects, take appropriate steps as deemed necessary under the circumstances to assure the subjects and where applicable follow up with therapy and inform the RUESC (departmental).

8.7 Adherence to protocol

Excluding an emergency situation in which proper treatment is required for the protection, safety and well-being of study subjects, the study will be conducted as

described in the approved protocol. Any deviation from the protocol will be recorded and explained.

Should amendments to the protocol be required, the amendments will be documented and signed by the principal investigator and the supervisor. If the protocol amendment(s) has an impact on the safety of subjects, such as a change in dosing regimen or additional formulations, the amendment will be submitted to the RUESC (departmental) for approval.

8.8 Blinding

Subjects will not be blinded and will be informed about the products for use at the application sites.

8.9 Adverse events/Adverse drug reactions

Subjects will be questioned on their health status at check-in, during the course of the study and before leaving the clinic at the end of the study. During the study, open-ended questions will be asked. If any adverse events are reported, the principal investigator will monitor the adverse event, initiate appropriate treatment if required and decide whether or not to withdraw the subject from the study. Signs and symptoms of any adverse events which occur during the study will be fully documented in the appropriate CRF (Appendix VI). If necessary adverse events will be referred to a suitably qualified medical practitioner for assessment and follow up.

Adverse events (which include illnesses, subjective and objective signs and symptoms that have appeared or worsened during the course of the study) will be assessed by the principal investigator and the supervisor during and after the study to determine whether or not they are related to the investigational test product (i.e. ADR), to the study procedure or other. The outcome of this assessment will be recorded in the appropriate CRF (Appendix VI).

AEs classified as severe or serious will be reported to the supervisor and Rhodes University Ethical Standards Committee (departmental) within 24 hours.

ADRs classified as serious and unexpected will be subject to expedited reporting as detailed in the ICH E2A and E2B guidelines on Clinical Safety Data Management and Data Elements for Transmission of Individual Case Report Forms respectively.

9 REPORTS

A full report on the study will be compiled by the principal investigator in the format requested by the supervisor and submitted to the supervisor. The analytical report will include results for all standard and quality control samples. A representative number of chromatograms or other raw data will be included covering the whole concentration range for all standards and quality control samples. The validation report will also be submitted.

ABBREVIATIONS

ADR	Adverse Dug Reaction
AE	Adverse Effect/Event
ANOVA	Analysis of Variance
AUC	Area Under the Curve
BRG	Biopharmaceutics Research Group
C_{max}	Maximum Concentration
CRF	Case Report Form
GCP	Good Clinical Practice
ICH	International Conference of Harmonisation
IRB	Internal Review Board
QA	Quality Assurance
RUESC	Rhodes University: Ethical Standards Committee
SOP	Standard Operating Procedure
t_{max}	Maximum Time
HPLC	High Pressure Liquid Chromatography

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APPENDIX II

Raw data NP_TS 1

Raw Data NP_TS 2 : AUCs found for the dose duration study

Subject	Dose duration		2	3	4	5	6	7	8	9	10	11	12	13	14	15	AUC
01	**0.25**	**SC**	7.85%	15.71%	19.63%	21.60%	29.45%	33.37%	35.34%	41.23%	45.15%	51.04%	53.01%	54.97%	60.86%	70.67%	**86.39**
		CLZ	7.54	3.45	1.75	1.34	1.41	0.66	0.73	0.52	0.61	0.39	0.30	0.28	0.23	0.20	
	0.5	**SC**	3.93%	11.78%	15.71%	19.63%	25.52%	29.45%	35.34%	43.19%	47.12%	54.97%	60.86%	70.67%	76.56%	80.49%	**62.17**
		CLZ	4.42	2.01	1.23	0.98	0.90	0.58	0.53	0.38	0.43	0.31	0.26	0.31	0.18	0.09	
	1	**SC**	11.78%	13.74%	15.71%	19.63%	27.48%	37.30%	41.23%	47.12%	51.04%	58.90%	68.71%	80.49%	90.31%	96.20%	**79.18**
		CLZ	7.80	3.97	1.83	2.24	1.51	1.18	0.69	0.75	0.60	0.33	0.46	0.24	0.28	0.16	
	2	**SC**	7.85%	11.78%	17.67%	25.52%	31.41%	33.37%	43.19%	51.04%	58.90%	66.75%	68.71%	76.56%	78.53%	86.38%	**114.65**
		CLZ	8.58	3.92	1.63	1.55	1.69	0.96	1.80	0.81	1.45	0.63	0.32	0.43	0.53	0.37	
	4	**SC**	5.89%	11.78%	15.71%	19.63%	27.48%	29.45%	35.34%	39.26%	41.23%	47.12%	51.04%	56.93%	68.71%	70.67%	**74.06**
		CLZ	5.15	2.82	2.18	1.19	1.32	1.50	0.60	0.33	0.29	0.66	0.34	0.27	0.64	0.30	
	6	**SC**	11.78%	17.67%	25.52%	33.37%	35.34%	39.26%	41.23%	43.19%	49.08%	58.90%	62.82%	66.75%	72.64%	82.45%	**102.08**
		CLZ	7.65	2.38	2.74	1.43	1.30	0.80	1.06	0.78	0.64	0.87	0.78	0.68	0.30	0.74	
	8	**SC**	3.93%	5.89%	9.82%	11.78%	13.74%	15.71%	17.67%	19.63%	21.60%	23.56%	25.52%	27.48%	29.45%	33.37%	**36.63**
		CLZ	4.76	2.37	2.14	1.42	0.87	0.79	1.02	0.45	0.45	0.67	1.04	0.83	0.47	1.27	
02	**0.25**	**SC**	3.28%	16.38%	22.93%	27.84%	32.75%	40.94%	52.40%	60.59%	70.42%	76.97%	78.61%	86.79%	88.43%	94.98%	**219.27**
		CLZ	16.17	5.48	2.64	1.09	1.05	0.77	0.84	0.61	0.60	0.32	0.31	0.28	0.23	0.25	
	0.5	**SC**	9.83%	14.74%	21.29%	24.56%	31.11%	36.03%	44.22%	47.49%	55.68%	65.51%	72.06%	78.61%	81.88%	85.16%	**77.03**
		CLZ	5.90	3.04	1.30	1.42	0.90	1.13	0.65	0.38	0.37	0.36	0.26	0.61	0.57	0.28	
	1	**SC**	6.55%	9.83%	13.10%	18.01%	22.93%	24.56%	26.20%	29.48%	36.03%	39.30%	42.58%	45.85%	50.77%	52.40%	**57.84**
		CLZ	4.22	2.92	1.93	1.01	0.88	1.23	1.03	0.76	1.56	1.13	0.62	0.38	0.66	0.24	
	2	**SC**	4.91%	13.10%	16.38%	21.29%	21.29%	26.20%	29.48%	37.67%	45.85%	47.49%	54.04%	63.87%	68.78%	75.33%	**117.80**
		CLZ	8.53	4.81	2.29	0.49	1.05	1.14	0.75	0.70	1.05	0.77	0.65	0.46	1.61	0.42	
	4	**SC**	6.55%	16.38%	22.93%	24.56%	32.75%	39.30%	40.94%	44.22%	47.49%	49.13%	52.40%	57.32%	60.59%	62.23%	**117.35**
		CLZ	6.22	4.02	3.55	1.81	1.23	1.03	0.89	0.88	0.76	0.60	1.01	0.70	0.58	0.33	
	6	**SC**	4.91%	6.55%	14.74%	22.93%	24.56%	32.75%	37.67%	39.30%	42.58%	42.58%	45.85%	47.49%	55.68%	60.59%	**88.47**
		CLZ	7.94	2.79	3.55	1.74	1.37	1.01	1.13	0.88	1.25	0.38	0.76	0.47	0.49	0.58	
	8	**SC**	8.19%	13.10%	18.01%	21.29%	21.29%	21.29%	22.93%	22.93%	27.84%	31.11%	36.03%	39.30%	42.58%	47.49%	**54.49**
		CLZ	7.25	2.06	2.74	0.98	0.83	0.62	0.49	0.65	0.47	0.46	0.89	0.34	0.30	0.58	
03	**0.25**	**SC**	4.04%	13.14%	32.36%	53.59%	68.75%	78.87%	81.90%	84.93%	87.97%	91.00%	94.03%	96.05%	98.08%	101.11%	**84.04**
		CLZ	2.52	1.07	1.98	0.48	0.44	0.16	0.13	0.11	0.11	0.14	0.14	0.07	0.00	0.00	
	0.5	**SC**	8.09%	27.30%	49.54%	65.72%	73.81%	77.85%	79.88%	82.91%	85.94%	87.97%	89.99%	91.00%	94.03%	97.07%	**64.46**
		CLZ	2.73	1.06	0.55	0.22	0.14	0.12	0.14	error	0.08	0.23	0.09	0.11	0.12	error	
	1	**SC**	4.04%	11.12%	21.23%	34.38%	45.50%	53.59%	60.67%	65.72%	69.77%	73.81%	76.84%	79.88%	83.92%	91.00%	**27.08**
		CLZ	2.09	0.72	0.48	0.25	0.15	0.12	0.10	0.07	0.08	0.11	error	0.07	0.08	0.05	
	2	**SC**	3.03%	10.11%	15.17%	17.19%	19.21%	21.23%	25.28%	32.36%	39.43%	44.49%	47.52%	50.55%	58.64%	66.73%	**33.51**
		CLZ	2.28	1.20	0.83	0.56	error	0.46	0.30	0.62	0.16	0.48	0.11	0.11	0.23	0.21	
	4	**SC**	2.02%	6.07%	12.13%	19.21%	26.29%	38.42%	50.55%	61.68%	71.79%	78.87%	83.92%	85.94%	87.97%	89.99%	**59.57**

		CLZ	5.08	1.15	0.85	0.70	0.77	0.70	0.62	0.39	0.32	0.23	0.15	0.08	0.32	0.13	
	6	**SC**	4.04%	11.12%	16.18%	20.22%	30.33%	52.58%	70.78%	75.83%	78.87%	81.90%	84.93%	87.97%	92.01%	98.08%	**87.36**
		CLZ	4.64	1.63	0.96	0.72	1.59	0.88	0.26	0.19	0.10	0.42	0.19	0.15	0.24	0.15	
	8	**SC**	1.01%	4.04%	7.08%	10.11%	25.28%	45.50%	53.59%	65.72%	77.85%	80.89%	83.92%	85.94%	87.97%	89.99%	**41.54**
		CLZ	2.40	1.30	0.62	0.73	0.71	0.50	0.14	0.18	0.13	0.09	0.10	0.18	0.08	0.14	
04	**0.25**	**SC**	10.82%	16.23%	20.28%	25.69%	35.16%	47.33%	50.03%	54.09%	60.85%	62.20%	63.56%	66.26%	67.61%	68.96%	**64.80**
		CLZ	8.04	3.07	1.66	0.86	0.65	0.57	0.23	0.15	0.17	0.08	0.16	0.05	0.06	0.10	
	0.5	**SC**	8.11%	13.52%	17.58%	22.99%	28.40%	29.75%	35.16%	36.51%	40.57%	44.62%	50.03%	51.38%	55.44%	62.20%	**64.32**
		CLZ	7.70	3.01	1.65	1.14	0.83	0.67	0.43	0.79	0.31	0.34	0.36	0.17	0.19	0.31	
	1	**SC**	12.17%	17.58%	20.28%	24.34%	28.40%	29.75%	31.10%	35.16%	36.51%	45.98%	48.68%	67.61%	70.32%	74.37%	**66.38**
		CLZ	8.58	3.16	1.90	2.08	1.37	0.95	0.57	0.42	0.19	0.24	0.19	0.21	0.12	0.28	
	2	**SC**	4.06%	8.11%	14.87%	22.99%	27.04%	28.40%	31.10%	32.45%	35.16%	39.21%	40.57%	45.98%	48.68%	58.15%	**71.63**
		CLZ	8.74	3.27	2.41	1.52	1.15	0.84	0.58	0.27	0.18	0.25	0.12	0.09	0.05	0.02	
	4	**SC**	8.11%	16.23%	20.28%	21.64%	27.04%	32.45%	33.81%	37.86%	43.27%	44.62%	45.98%	52.74%	58.15%	68.96%	**126.05**
		CLZ	10.75	5.29	3.23	1.98	1.94	1.70	0.86	0.92	0.61	0.60	0.30	0.41	0.36	0.45	
	6	**SC**	9.47%	16.23%	17.58%	29.75%	33.81%	35.16%	39.21%	41.92%	48.68%	54.09%	60.85%	66.26%	74.37%	85.19%	**124.47**
		CLZ	11.70	4.31	2.84	2.78	0.52	1.01	1.19	0.73	0.67	0.46	0.21	0.27	0.22	0.37	
	8	**SC**	6.76%	10.82%	12.17%	14.87%	18.93%	22.99%	25.69%	32.45%	37.86%	39.21%	45.98%	47.33%	51.38%	55.44%	**45.75**
		CLZ	6.81	3.05	2.35	0.99	0.59	0.92	0.46	0.51	0.38	0.29	0.39	0.05	0.11	0.08	
05	**0.25**	**SC**	4.96%	9.93%	14.89%	27.30%	29.78%	37.22%	47.15%	54.59%	64.52%	71.96%	81.89%	84.37%	91.81%	94.30%	**24.73**
		CLZ	5.75	2.55	2.03	0.89	0.82	0.37	0.20	0.20	0.09	0.08	0.06	0.00	0.00	0.00	
	0.5	**SC**	7.44%	12.41%	22.33%	37.22%	42.19%	44.67%	52.11%	54.59%	59.56%	69.48%	74.44%	81.89%	89.33%	94.30%	**39.20**
		CLZ	6.88	4.81	2.38	1.52	1.50	0.75	0.73	0.28	0.22	0.32	0.24	0.19	0.30	0.00	
	1	**SC**	12.41%	17.37%	32.26%	39.70%	47.15%	54.59%	57.07%	59.56%	64.52%	74.44%	79.41%	81.89%	86.85%	91.81%	**41.24**
		CLZ	8.97	5.56	3.19	1.72	1.06	0.46	0.32	0.97	0.33	0.16	0.13	0.00	0.00	0.00	
	2	**SC**	12.41%	22.33%	34.74%	44.67%	54.59%	62.04%	67.00%	71.96%	76.93%	89.33%	96.78%	109.19%	111.67%	114.15%	**39.74**
		CLZ	8.05	6.31	2.45	2.55	0.47	0.60	0.46	0.20	0.17	0.02	0.10	0.04	0.00	0.00	
	4	**SC**	14.89%	32.26%	39.70%	47.15%	54.59%	62.04%	71.96%	79.41%	86.85%	94.30%	101.74%	106.70%	111.67%	121.59%	**41.83**
		CLZ	9.99	5.50	3.34	3.04	1.55	1.11	0.80	0.53	0.25	0.30	0.47	0.04	0.01	0.04	
	6	**SC**	12.41%	22.33%	29.78%	42.19%	44.67%	52.11%	57.07%	59.56%	67.00%	69.48%	71.96%	76.93%	81.89%	89.33%	**33.08**
		CLZ	6.70	2.78	3.15	1.34	1.04	0.87	0.76	1.09	0.29	0.44	0.26	0.25	0.22	0.25	
	8	**SC**	12.41%	24.81%	27.30%	42.19%	49.63%	57.07%	59.56%	64.52%	69.48%	71.96%	79.41%	84.37%	91.81%	101.74%	**28.75**
		CLZ	6.29	3.69	1.68	1.71	0.60	0.79	0.26	0.29	0.21	0.02	0.23	0.09	0.12	0.11	
06	**0.25**	**SC**	5.77%	11.54%	17.30%	20.19%	25.96%	31.72%	34.61%	43.26%	54.80%	57.68%	60.56%	63.45%	66.33%	74.98%	**49.57**
		CLZ	5.02	2.20	1.37	0.64	0.83	0.61	0.28	0.13	0.25	0.13	0.09	0.04	0.06	0.11	
	0.5	**SC**	5.77%	8.65%	20.19%	23.07%	28.84%	31.72%	40.38%	49.03%	51.91%	57.68%	63.45%	72.10%	77.87%	83.64%	**47.11**
		CLZ	3.87	1.75	1.50	0.64	0.65	0.51	0.39	0.26	0.37	0.20	0.13	0.11	0.02	0.02	
	1	**SC**	5.77%	8.65%	11.54%	17.30%	25.96%	28.84%	31.72%	43.26%	57.68%	60.56%	72.10%	74.98%	80.75%	95.17%	**54.05**
		CLZ	3.31	1.62	0.98	0.76	0.94	0.68	0.52	0.30	0.25	0.16	1.30	0.10	0.43	0.33	
	2	**SC**	5.77%	14.42%	17.30%	25.96%	31.72%	34.61%	46.14%	51.91%	54.80%	57.68%	63.45%	69.22%	72.10%	74.98%	**112.77**
		CLZ	7.57	4.75	2.06	1.53	1.28	1.18	0.72	0.49	0.45	0.43	0.22	0.34	0.24	0.47	

	4	**SC**	11.54%	17.30%	31.72%	37.49%	43.26%	46.14%	46.14%	54.80%	72.10%	80.75%	86.52%	92.29%	95.17%	98.06%	**109.09**
		CLZ	7.38	3.64	1.60	1.80	0.65	0.45	0.83	0.74	0.28	0.25	0.27	0.06	0.12	0.13	
	6	**SC**	8.65%	17.30%	28.84%	37.49%	46.14%	51.91%	54.80%	60.56%	66.33%	74.98%	89.40%	100.94%	106.71%	115.36%	**139.59**
		CLZ	9.72	2.82	2.02	1.72	1.43	1.12	0.55	0.45	0.32	0.37	0.26	0.28	0.13	0.10	
	8	**SC**	8.65%	17.30%	25.96%	31.72%	37.49%	46.14%	49.03%	51.91%	54.80%	63.45%	72.10%	80.75%	83.64%	98.06%	**105.56**
		CLZ	5.88	3.13	2.31	1.63	1.00	0.80	0.51	0.23	0.26	0.47	0.12	0.68	0.17	0.16	
07	**0.25**	**SC**	4.68%	7.02%	9.37%	14.05%	23.41%	32.78%	42.15%	51.51%	58.54%	72.59%	84.29%	86.63%	88.98%	93.66%	**33.43**
		CLZ	4.72	1.14	0.75	0.56	0.74	0.46	0.37	0.27	0.10	0.00	0.08	0.00	0.00	0.00	
	0.5	**SC**	16.39%	30.44%	37.46%	51.51%	58.54%	67.90%	79.61%	88.98%	98.34%	100.68%	105.37%	110.05%	119.41%	126.44%	**88.40**
		CLZ	5.39	2.31	0.91	0.62	0.49	0.20	0.23	0.05	0.16	0.00	0.00	0.00	error	0.00	
	1	**SC**	14.05%	18.73%	21.07%	30.44%	32.78%	35.12%	37.46%	42.15%	44.49%	51.51%	58.54%	63.22%	65.56%	72.59%	**30.11**
		CLZ	5.64	2.37	0.78	0.53	0.20	0.06	0.06	0.02	0.00	0.00	0.00	0.00	0.00	0.00	
	2	**SC**	16.39%	32.78%	32.78%	35.12%	39.80%	49.17%	53.85%	56.20%	63.22%	72.59%	81.95%	84.29%	86.63%	93.66%	**105.80**
		CLZ	6.58	3.89	1.68	1.05	0.78	0.61	0.26	error	0.12	0.02	0.24	0.04	0.00	0.31	
	4	**SC**	7.02%	18.73%	25.76%	30.44%	39.80%	46.83%	56.20%	63.22%	74.93%	79.61%	91.32%	98.34%	105.37%	107.71%	**85.34**
		CLZ	4.26	3.44	0.97	1.16	0.41	0.35	0.37	0.07	0.24	0.08	0.04	0.21	0.00	0.05	
	6	**SC**	14.05%	21.07%	35.12%	46.83%	53.85%	58.54%	63.22%	70.24%	72.59%	74.93%	79.61%	84.29%	86.63%	93.66%	**134.42**
		CLZ	7.02	3.90	3.02	1.48	1.02	0.61	0.65	0.20	0.25	0.04	0.08	0.09	0.18	0.03	
	8	**SC**	11.71%	28.10%	37.46%	44.49%	46.83%	53.85%	58.54%	63.22%	70.24%	72.59%	74.93%	77.27%	84.29%	91.32%	**168.56**
		CLZ	8.49	4.38	3.02	0.83	0.84	0.44	0.21	0.56	0.23	0.14	0.02	0.03	0.21	0.04	
08	**0.25**	**SC**	6.00%	21.01%	27.02%	36.02%	45.03%	63.04%	69.04%	72.05%	90.06%	96.06%	99.06%	102.07%	105.07%	117.08%	**92.91**
		CLZ	4.93	3.10	1.15	0.81	0.31	0.23	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
	0.5	**SC**	6.00%	12.01%	18.01%	30.02%	33.02%	39.03%	42.03%	45.03%	48.03%	54.04%	57.04%	63.04%	69.04%	75.05%	**37.93**
		CLZ	4.54	1.80	0.93	0.48	0.18	0.15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
	1	**SC**	9.01%	18.01%	30.02%	42.03%	51.03%	60.04%	63.04%	75.05%	84.05%	90.06%	96.06%	99.06%	105.07%	111.07%	**72.08**
		CLZ	4.29	2.17	1.08	0.61	0.43	0.43	0.15	0.29	0.00	0.00	0.00	0.00	0.00	0.00	
	2	**SC**	9.01%	15.01%	24.02%	30.02%	42.03%	48.03%	51.03%	66.04%	72.05%	78.05%	81.05%	87.06%	90.06%	96.06%	**43.79**
		CLZ	4.86	1.99	0.79	0.36	0.36	0.16	0.10	0.01	0.03	0.00	0.00	0.00	0.00	0.00	
	4	**SC**	15.01%	33.02%	33.02%	39.03%	42.03%	45.03%	54.04%	63.04%	69.04%	75.05%	81.05%	84.05%	87.06%	90.06%	**80.91**
		CLZ	4.92	2.90	0.96	0.48	0.38	0.23	0.29	0.04	0.00	0.00	0.00	0.00	0.00	0.00	
	6	**SC**	15.01%	18.01%	24.02%	33.02%	42.03%	48.03%	57.04%	60.04%	69.04%	75.05%	81.05%	87.06%	90.06%	93.06%	**56.21**
		CLZ	4.45	2.26	1.08	1.39	0.78	0.86	0.38	0.27	0.12	0.24	0.00	0.04	0.02	0.00	
	8	**SC**	12.01%	18.01%	21.01%	30.02%	33.02%	39.03%	42.03%	51.03%	54.04%	60.04%	66.04%	72.05%	75.05%	84.05%	**44.23**
		CLZ	4.88	2.33	1.21	0.55	0.57	0.84	0.14	0.18	0.02	0.00	0.02	0.00	0.00	0.00	
09	**0.25**	**SC**	11.12%	16.68%	22.24%	33.37%	41.71%	52.83%	52.83%	58.39%	66.73%	69.51%	77.86%	83.42%	86.20%	88.98%	**76.93**
		CLZ	5.71	2.73	2.31	1.12	0.48	0.57	0.58	0.33	0.13	0.26	0.09	0.14	0.14	0.10	
	0.5	**SC**	11.12%	25.02%	27.81%	30.59%	36.15%	41.71%	50.05%	55.61%	66.73%	77.86%	88.98%	94.54%	97.32%	100.10%	**118.00**
		CLZ	8.40	3.20	1.15	1.00	0.78	1.09	0.35	0.73	0.24	0.07	0.11	0.14	0.08	0.03	
	1	**SC**	11.12%	22.24%	33.37%	44.49%	52.83%	63.95%	69.51%	77.86%	88.98%	97.32%	105.66%	108.44%	114.00%	122.34%	**159.32**
		CLZ	5.94	2.86	2.39	1.23	2.07	1.55	0.46	0.81	0.48	0.29	0.40	0.11	0.20	0.17	
	2	**SC**	8.34%	16.68%	33.37%	44.49%	52.83%	61.17%	75.07%	86.20%	88.98%	91.76%	97.32%	108.44%	116.78%	119.56%	**166.37**

		CLZ	6.98	3.62	2.34	1.38	1.05	1.54	0.51	0.79	0.67	0.30	0.20	0.27	0.13	0.07	
	4	**SC**	11.12%	19.46%	33.37%	38.93%	47.27%	52.83%	58.39%	66.73%	72.29%	75.07%	86.20%	91.76%	97.32%	102.88%	**148.31**
		CLZ	8.14	3.64	1.81	2.34	1.57	0.72	0.83	0.46	0.46	0.33	0.84	0.12	0.57	0.38	
	6	**SC**	5.56%	16.68%	27.81%	38.93%	47.27%	52.83%	55.61%	61.17%	69.51%	80.64%	94.54%	97.32%	100.10%	111.22%	**152.93**
		CLZ	7.08	3.86	2.04	1.83	1.26	0.89	0.40	0.37	0.47	0.35	0.32	0.26	0.15	error	
	8	**SC**	5.56%	16.68%	25.02%	33.37%	44.49%	47.27%	58.39%	66.73%	69.51%	75.07%	77.86%	83.42%	88.98%	102.88%	**119.22**
		CLZ	6.07	2.61	1.88	1.46	1.45	0.49	0.78	0.36	0.14	0.13	0.67	0.23	0.11	0.11	
10	**0.25**	**SC**	1.53%	7.63%	10.68%	13.74%	16.79%	21.37%	33.58%	41.21%	48.84%	51.90%	59.53%	62.58%	68.69%	70.21%	**61.1**
		CLZ	5.66	2.27	1.21	0.67	0.71	0.50	0.78	0.48	0.48	0.36	0.35	0.33	0.33	0.25	
	0.5	**SC**	1.53%	3.05%	7.63%	9.16%	13.74%	16.79%	19.84%	21.37%	22.90%	24.42%	27.47%	32.05%	33.58%	35.11%	**23.01**
		CLZ	3.93	1.29	1.00	0.57	0.88	0.47	0.49	0.47	0.38	0.40	0.35	0.35	0.33	0.37	
	1	**SC**	9.16%	13.74%	15.26%	21.37%	22.90%	29.00%	30.53%	33.58%	38.16%	41.21%	42.74%	45.79%	47.32%	53.42%	**47.95**
		CLZ	6.64	2.71	0.82	0.84	0.78	0.80	0.68	0.56	0.66	0.56	0.32	0.35	0.41	0.48	
	2	**SC**	1.53%	3.05%	7.63%	9.16%	12.21%	15.26%	21.37%	22.90%	24.42%	27.47%	29.00%	30.53%	33.58%	35.11%	**45.25**
		CLZ	3.16	3.62	1.80	1.03	1.51	1.48	1.29	0.66	0.61	0.71	0.59	0.45	0.47	0.75	
	4	**SC**	1.53%	4.58%	7.63%	9.16%	12.21%	15.26%	16.79%	21.37%	24.42%	27.47%	32.05%	35.11%	36.63%	41.21%	**34.94**
		CLZ	3.99	1.58	0.97	0.70	1.04	0.72	0.87	0.64	0.53	0.51	0.59	0.71	0.58	0.48	
	6	**SC**	4.58%	9.16%	15.26%	18.32%	21.37%	22.90%	29.00%	35.11%	39.69%	42.74%	47.32%	51.90%	58.00%	67.16%	**73.69**
		CLZ	4.46	3.10	2.25	1.29	1.14	1.47	1.00	0.64	0.52	0.64	0.53	0.41	0.44	0.54	
	8	**SC**	7.63%	15.26%	19.84%	21.37%	22.90%	25.95%	29.00%	32.05%	36.63%	41.21%	42.74%	45.79%	50.37%	56.48%	**83.49**
		CLZ	7.27	3.11	2.11	2.04	1.16	1.20	0.93	1.28	0.63	0.55	0.47	0.51	0.83	0.37	

Raw Data NP_TS 2 : Amount of CLZ assumed to be present in the skin

Subject	Dose Duration	Formulation applied	CLZ applied	CLZ removed	CLZ assumed to be in the skin
01	**0.25**	14.2	142	43.17	98.83
	0.5	13.7	137	22.92	114.08
	1	14.3	143	75.80	67.20
	2	14.4	144	44.56	99.44
	4	13.2	132	50.42	81.58
	6	14.9	149	92.95	56.05
	8	14.5	145	96.00	49.00
02	**0.25**	14.2	142	78.35	63.65
	0.5	13.9	139	88.20	50.80
	1	14.3	143	96.96	46.04
	2	14.2	142	72.74	69.26
	4	14.1	141	56.94	84.06
	6	14.6	146	55.74	90.26
	8	14.4	144	27.26	116.74
03	**0.25**	14.6	146	132.95	13.05
	0.5	14.1	141	95.66	45.34
	1	13.7	137	115.57	21.43
	2	13.4	134	83.15	50.85
	4	14.7	147	91.44	55.56
	6	14.7	147	71.34	75.66
	8	14.1	141	42.62	98.38
04	**0.25**	14.9	149	105.33	43.67
	0.5	14.6	146	90.88	55.12
	1	14.6	146	68.83	77.17
	2	14.8	148	54.12	93.88
	4	14.5	145	33.39	111.61
	6	15.1	151	29.82	121.18
	8	14.4	144	21.70	122.30
05	**0.25**	15.0	150	113.61	36.39
	0.5	14.3	143	74.40	68.60
	1	14.8	148	74.75	73.25
	2	14.6	146	72.06	73.94
	4	14.9	149	42.32	106.68
	6	14.9	149	45.26	103.74
	8	14.9	149	41.97	107.03
06	**0.25**	14.8	148	110.44	37.56
	0.5	14.5	145	110.69	34.31
	1	14.7	147	78.43	68.57
	2	14.8	148	52.53	95.47
	4	14.1	141	35.38	105.62
	6	14.4	144	41.41	102.59
	8	14.4	144	23.81	120.19
07	**0.25**	15.0	150	115.81	34.19
	0.5	14.9	149	109.86	39.14
	1	14.3	143	98.26	44.74
	2	13.8	138	72.76	65.24
	4	14.1	141	64.73	76.27
	6	14.7	147	45.18	101.82
	8	14.4	144	28.57	115.43
08	**0.25**	15.1	151	115.22	35.78
	0.5	14.3	143	119.80	23.20

	1	14.3	143	74.87	68.13
	2	15.0	150	95.68	54.32
	4	14.4	144	92.65	51.35
	6	14.5	145	61.33	83.67
	8	14.7	147	69.22	77.78
09	**0.25**	15.0	150	118.62	31.38
	0.5	14.0	140	75.26	64.74
	1	14.6	146	96.34	49.66
	2	15.0	150	42.17	107.83
	4	14.9	149	57.33	91.67
	6	14.2	142	31.95	110.05
	8	14.9	149	26.20	122.80
10	**0.25**	14.5	145	120.01	24.99
	0.5	14.4	144	105.26	38.74
	1	14.5	145	87.66	57.34
	2	14.0	140	error	error
	4	14.2	142	error	error
	6	14.1	141	44.11	96.89
	8	14.7	147	50.50	96.50

APPENDIX III

Protocol NP_TS 2.1

1 BACKGROUND INFORMATION

1.1 Clotrimazole

1.1.1 Molecular structure

Clotrimazole is a widely used imidazole antifungal agent that was first synthesised by Bayer in 1967 [1, 2]. It has a molecular weight of 344.8u and is found as a white crystalline powder [3]. The molecular structure of clotrimazole is shown in Figure 1 below and has the empirical formula, $C_{22}H_{17}ClN_2$ [3].

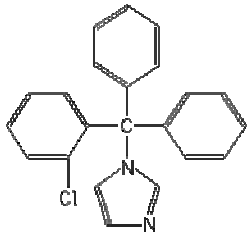


Fig. 1 Chemical structure of clotrimazole [4]

1.1.3 Mode of action and uses

Ergosterol is an important component of the fungal cell membrane and helps maintain the cells integrity in a similar way to cholesterol found in animal cells [5]. Ergosterol is synthesised in the fungal cells from lanosterol. The enzyme responsible for converting lanosterol to ergosterol is the CYP450 enzyme, 14–α–demethylase. Clotrimazole interacts with this enzyme thereby inhibiting ergosterol synthesis [2, 5]. The lack of ergosterol alters the fungal cell membrane causing an increase in cellular permeability. This causes the contents to leak out of the fungal cells thereby destroying them. The action of clotrimazole is specific to the particular pathogen and will not affect the host cells [6].

Clotrimazole is available in topical preparations (as a cream, lotion, spray, solution and powder), vaginal preparations (as a vaginal tablet and vaginal creams) and lozenges. Clotrimazole can also be administered orally but it has been largely superseded by other oral azoles [7]. The 1% topical cream that will be used in this study has the following indications: treatment of cutaneous candidiasis (yeast infection or thrush which is a fungal infection produced by the *Candida* species, of which *Candida albicans* is the most common) and dermatophytoses (fungal infections of keratin in the skin and nails). It may also be useful in the treatment of *tinea vesicolor* (a common skin infection caused by the yeast *Malassezia furfur* which is normally found on the human skin and only becomes troublesome under certain conditions) and *Corynebacterium minutissimum* (a bacterium which causes a superficial skin infection marked by reddish brown scaly patches known as erythrasma.) [8].

1.1.3 Pharmacokinetics

A topical application of clotrimazole will penetrate the epidermis but there is little if any systemic absorption [7]. This is desirable since the *stratum corneum* is considered to be the site of action (dermatophytes that infect the skin are only able to survive in the dead keratinised cells of the *stratum corneum*). After vaginal use, however, between 3% and 10% of a dose has been reported in the systemic circulation [7]. Clotrimazole is metabolised by liver enzymes to form inactive compounds that are excreted in the faeces and urine [7].

1.1.4 Adverse effects

Local reactions including skin irritation and a burning sensation may occur following topical treatment. Contact allergic dermatitis has been reported. In cases of systemic absorption, lower abdominal cramps, increase in urinary frequency or skin rash may occur [7, 8]. Nausea, vomiting, unpleasant mouth sensations, and pruritus have been reported after the use of oral clotrimazole lozenges. Raised liver enzyme values have also occurred [7]. Intravaginal preparations of clotrimazole may damage latex contraceptives and additional contraceptive measures are therefore necessary during local application. Gastrointestinal disturbance, elevation of liver enzymes, dysuria, and mental depression have been reported after oral clotrimazole [7, 8].

1.1.5 Contraindications

Hypersensitivity to clotrimazole [8].

Copies of the product information leaflets of the topical formulations to be used in this study are located in Appendix I.

1.2 The Residual Method

1.2.1 Overview of the residual method

The residual method for determining bioequivalence of a topical formulation has been derived from a previous study, namely NP_TS1. In that study, Canesten® 1% topical cream was applied to the skin and the tape stripping technique was used to remove the *stratum corneum* at different dose durations. Before the tape stripping commenced, residual formulation remaining on the site of application was removed using a cotton bud. When data from this study were analysed it was found that a strong correlation existed between dose duration and the amount of clotrimazole remaining in the skin i.e. the difference between the initial amount of clotrimazole applied and the amount removed using the cotton bud. When the mean amount of clotrimazole remaining in the skin (n=7) was plotted against dose duration, the plot fitted an Emax model [9] with R^2 = 0.9662 and with an ED50 = 0.734 hrs ≈ 45mins. From the dose duration study, the optimal dose duration (i.e. the ED50) to detect potential differences in release between formulations can be chosen.

1.2.2 Principles of the residual method

The residual method involves the application of a formulation to a demarcated site on the volar aspect of the forearm and the subsequent removal of the product at a specific time interval. The formulation is accurately applied using a calibrated dispenser and spread using a glass rod. The rod is weighed before and after spreading to account for any loss of formulation due to spreading. As the formulation is in contact with the skin,

clotrimazole is released from the formulation and penetrates the skin resulting in a decreased amount of clotrimazole left on the surface. The residual formulation is removed using two cotton swabs and the content of clotrimazole is analysed using a validated HPLC technique. It is assumed that the difference between the amount of clotrimazole applied and clotrimazole removed is the amount of clotrimazole that has entered the skin. This gives an indication of the release of clotrimazole from the formulation in an *in vivo* situation.

1.2.3 Invasiveness of the residual method

This method is pain- free and non-invasive and no biological samples are taken.

1.3 The Tape Stripping (TS) Method

1.3.1 Overview of tape stripping

Tape stripping is a non-invasive technique employed to study the penetration, the distribution and the dermatopharmacokinetics of topically applied drugs and cosmetics products [10-13] within the *stratum corneum*. It is commonly used to disrupt the epidermal barrier to enhance the delivery of drugs *in vivo* [14] and to obtain information about *stratum corneum* function [12]. Tape stripping has been used extensively in dermatological and pharmaceutical research to measure the *stratum corneum* mass and thickness, to collect *stratum corneum* lipids and protein samples, detect proteolytic activity associated with the *stratum corneum*, quantitatively estimate enzyme levels and activities in the *stratum corneum* and allow the detection of metal in the *stratum corneum* [12]. Although in use for over five decades, there are no universally accepted protocols for tape stripping [15]. However, TS has previously been identified to be of sufficient utility to have been proposed by the FDA as a method to evaluate the bioequivalence of topical dermatological dosage forms [16] and a preliminary guidance, which was subsequently withdrawn due to issues of variability, was issued by the US FDA.

1.3.2 Principles of tape stripping

Tape stripping involves the sequential removal of microscopic layers (typically 0.5 - 1 μm) of the *stratum corneum* [11, 17] by placing a strip of adhesive tape onto the skin surface with uniform pressure, which is subsequently removed [10,11]. Drug uptake into the *stratum corneum in vivo* can be measured by harvesting the *stratum corneum* previously exposed to a topical product and removed with adhesive tapes. The latter are extracted and quantified for drug concentration by a validated analytical method [18]. The number of tape strips needed to remove the *stratum corneum* varies with age, gender, anatomical site, skin condition and possibly ethnicity [12]. Tape stripping is putatively simple, inexpensive and has been described as a minimally invasive technique [10].

1.3.3 Invasiveness of tape stripping

Although tape-stripping is considered to be essentially non-invasive, stripped sites in certain dark-skinned individuals may remain hyperpigmented for several months after healing [11]. This effect will be communicated to the human volunteers prior to entering the study.

1.3.4 Measurement of Transepidermal Water Loss (TEWL)

TEWL has been shown to inversely correlate with *stratum corneum* depth [19]. Therefore, TEWL measurements in conjunction with the mass of *stratum corneum* harvested can be used to determine the *stratum corneum* thickness for each volunteer. This information will enable the tape stripping data to be normalised. TEWL readings will be measured at the control site only. Readings will be taken after the removal of each tape strip. A Delfin® vapometer will be used to measure the TEWL. The instrument is placed on the surface of the skin, slight pressure is applied to ensure the chamber is sealed and a reading is obtained within 10 to 20 seconds.

2 STUDY PRODUCTS

2.1 Description

	Innovator product	**Test product**
Commercial name	Canesten® topical cream	Canesten® topical cream
Generic name	Clotrimazole cream	Clotrimazole cream
Dosage form	Cream	Cream
Strength	1% m/m	1% m/m
Manufacturer	Bayer, Germany	Bayer, Germany
MCC registration details	E/20.2.2/49	E/20.2.2/49
Expiry date	12/2009	12/2009
Description	A soft, white cream	A soft, white cream
Dosage	± 4 mg per $1cm^2$ application site	± 4 mg per $1cm^2$ application site

2.2 Supply, storage and use

Sufficient Canesten® Topical Cream for use in the study will be timeously supplied and logged into a record book by the principle investigator.It is for external use only. The product will be stored below 25° C and kept out of reach of children [8]. Dispensing and administration of the products will be recorded and administered only to volunteers participating in this study.

3 OBJECTIVES

The objective of this study is two fold i.e. to assess whether the novel residual method and also the TS method can be used to determine bioequivalence between topical products. For both methods, the innovator product will be compared to a product known to be bioequivalent i.e. Test and Reference products will be identical.

4 STUDY POPULATION AND MEDICAL ASSESSMENT

4.1 Number of volunteers

Previous studies involved between 6 - 18 human volunteers to study the skin penetration of some topical products [10-15]. This study will make use of 10 healthy human volunteers and will include both male and female volunteers. Four application sites plus one blank site will be used on the left arm and four application sites will also be used on the right arm. Data will be collected from the sites on the left arm using the TS method and from the right arm using the residual method. Statistical analysis (Section 8) will be performed on all volunteers completing the study. If bioequivalence cannot be demonstrated because of a result of a larger than expected random variation or a relative difference, an add-on volunteer study using up to 10 additional volunteers will be conducted. If no further study is required, preliminary statistical analysis will be finalised and presented in the final report. If an add-on study is conducted, data for all evaluable volunteers from both Group 1 and Group 2 will be combined and used in the preparation of the final report.

4.2 Inclusion/Exclusion criteria

Inclusion criteria

Only those volunteers meeting the following criteria will be included in the study:

iv. Volunteers who between the ages of 18 and 50.

v. Volunteers who are in general good health.

vi. Volunteers who will be available for the entire study period.

Exclusion criteria

Volunteers meeting the following criteria will be excluded from the study.

xiii. Female volunteers who are pregnant, possibly pregnant or breast feeding.

xiv. Volunteers who have a known allergy/hypersensitivity to clotrimazole or any fungicides.

xv. Volunteers who have any history of drug or alcohol abuse.

xvi. Volunteers who have any mental deficiency or handicap.

xvii. Volunteers who have hairy ventral forearm surfaces and/or abrasions, scares, marks etc on the underside of their forearms.

xviii. Volunteers who regularly use cosmetic procedures on their forearms (e.g. shaving or waxing of arms, intense exfoliation, intense moisturisation, sunbed, spa or beauty treatments)

xix. Volunteers who have engaged in any sun-tanning or taken any sunny vacations within the last month.

xx. Volunteers who have participated in another dermal study within 2 months of the study date.

xxi. Volunteers who have used any topical fungicides within the last three months.

xxii. Volunteers who suffer from any allergic conditions (hayfever, allergic rashes, asthma or childhood eczema).

xxiii. Volunteers who suffer from any skin disorder such as psoriasis, eczema or other relevant skin disorder.

xxiv. Volunteers who take regular medicine or tablets or who have used any medicated creams within one week (contraceptive pills excluded) prior to the study commencement.

4.3 Volunteer restrictions

- No prescription medication and OTC medication (particularly any medicated creams, e.g. corticosteroids, high dose vitamin A, Roaccutane® or its generics) will be allowed for at least one week prior to the study.
- With the exception of study product no concomitant medication may be taken by volunteers during the study.
- No strenuous physical activity may be undertaken by volunteers from 12 hours before product application and during the study.
- Volunteers will not be allowed to smoke during the study.
- Volunteers must refrain from applying emollients, skin conditioning creams and/or tanning lotions to their forearms for a period of 24 hours prior to the scheduled time of product application.
- Volunteers must ensure their arm has been washed with soapy water at least 1 hour prior to the start of the study.

Volunteers will be informed of the above restrictions and each volunteer will be specifically questioned on these points prior to product application. Any deviations from the above restrictions which are made known to the investigators either voluntarily or on questioning will be recorded on the appropriate CRF (Appendix VI). A decision as to whether the affected volunteer continues with the study will be taken by the principal investigator and the supervisor.

4.4 Criteria for removal from the study

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

i. Voluntary withdrawal by the volunteer due to any reason.

ii. Illness or injury during the study if regarded as clinically significant by the principal investigator and the supervisor i.e if the principal investigator or supervisor feels that the volunteer is experiencing any medical signs or symptoms that do not have a rational explanation and that indicate that he/she may be ill or injured.

iii. Any adverse event or signs of toxicity if regarded as clinically significant by the principal investigator and the supervisor. i.e. if the principal investigator or supervisor feels that the volunteer is experiencing any medical signs or symptoms that do not have a rational explanation and that indicate that he/she may be experiencing an adverse event or toxicity

iv. Failure of the volunteer to comply with or be uncooperative towards any study requirements or restrictions if regarded as clinically significant by the principal investigator and the supervisor.

v. Volunteers withdrawn or dropped out of the study will be fully documented and accounted for in the CRF.

5 STUDY PROCEDURE

5.1 Pre- and post-study medical screening

Pre-study screening will be conducted not more than 3 days prior to the start of the study. The pre-study evaluations will be conducted as listed in the table below.

Pre-study	Post-study
Medical history Demographic data (date of birth, age, sex, origin), skin (dermatological) conditions and allergies	**Medical history** Since start of study
Dermatological assessment General assessment of the volar aspect of the forearm and any dermatological condition which may influence the barrier function of the skin.	**Dermatological assessment** General assessment of the volar aspect of the forearm
Adhesive sensitivity Assessment of volunteer's sensitivity to the tape's adhesive on application site.	

If any study related abnormalities are observed at the post-study medical, appropriate follow-up action will be taken and re-examination and re-testing conducted until the abnormality returns to normal or until the principle investigator and supervisor considers the abnormality to be clinically insignificant i.e. the clinical parameters concerned are within the normal range.

5.2 Medical tape sensitivity screening

No less than 30 days prior to the start of the study, volunteers will undergo an assessment of their tolerance to the adhesive on the medical tape used to demarcate application sites and for tape stripping procedures.

The following screening protocol will be utilised.

- Adhesion of the Opsite® (Smith-Nephew, Hull, UK) medical tape (4cm^2).
- The medical tape will be removed after four hours.
- The forearm of the volunteer will be assessed for allergies to the adhesive medical tapes.

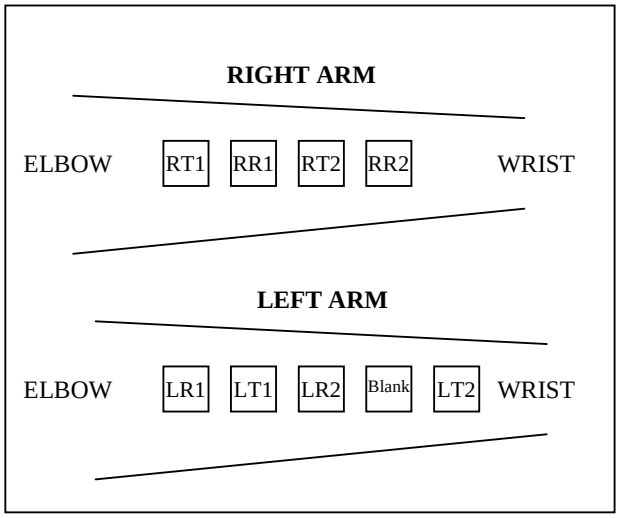
Volunteers who exhibit allergies will not be eligible to participate in this study.

5.3 Check-in and confinement

Two volunteers will be assessed per study day. Volunteers will check-in on the study day at the clinic (Room T17) in the Faculty of Pharmacy building, Rhodes University, Grahamstown 15mins before the cream is due to be applied. The principle investigator will conduct a brief medical examination, inclusion and exclusion criteria check and study restriction check. Volunteers accepted into the study will then be prepared for product application. Volunteers will remain in the study room for the entire duration of the study except when using bathroom facilities.

5.4 Study design

The study will be conducted on 10 volunteers, followed by another 10 volunteers if necessary (see section 4.1). The time between the first and last volunteer is expected to be approximately one week. For each volunteer the right arm will be used to investigate the residual method and the left arm to investigate the TS method. The investigator will work concurrently with an assistant from the principle researchers research group who has had extensive experience in dermal clinical studies . The investigator will conduct the TS procedure on the left arm and the assistant will conduct the residual method procedure on the right arm under the supervision of the investigator. A total of 8 application sites and 1 blank site will be used per volunteer. The blank site will be tape stripped and TEWL measurements taken to determine the *stratum corneum* depth. Two of the four application sites on each arm will be employed for the application of the test product and the other two sites for the reference product. The formulations will be randomized between sites and individuals. An example of an application scheme is represented below. The duration of the study for each volunteer will be approximately 2 hours.



RT1 and RT2: Right arm (residual method), test product, replicates 1 and 2 respectively
RR1 and RR2: Right arm (residual method), reference product, replicates 1 and 2 respectively
LT1 and LT2: Left arm (tape stripping method), test product, replicates 1 and 2 respectively
LR1 and LR2: Left arm (tape stripping method), reference product, replicates 1 and 2 respectively

The volunteers will be requested to wash their forearm with soap during their usual ablutions (at least an hour before the study commences) and not apply any topical products to the arm. Pre-made templates will be affixed to the left and right arm of each volunteer. The templates will be made from OpSite® (Smith-Nephew, Hull, UK) adhesive dressing reinforced with 3M Scotch® Magic tape (no.810) on the none-adhesive side. These will be used to demarcate the (2 cm x 2 cm) sites on the skin. Approximately 15mg of the formulation will be applied to each site. The application to each site will be staggered to allow time for the product removal and the tape stripping procedure. The sites on the right arm (for Residual method) will be exposed to a dose duration of 45minutes since this was the ED50 determined for the Residual method data determined from the dose duration study NP_TS1. The sites on the left arm (for the TS method) will be exposed to a dose duration of 15minutes since this was the ED50 determined for the TS data from the dose duration study NP_TS1.. The cream will be removed after the respective dose duration times by wiping with a cotton swab. For the TS

method, adhesive tapes (Sellotape® Original) will be applied to each of the sampling sites on the left arm with uniform pressure and then subsequently removed. Fifteen (15) successive strips will be made from each site. No formulation will be applied to the control site but transepidermal water loss measurements will be recorded from the site in order to determine the thickness of the *stratum cornum*.

5.5 Pre-study day activities and procedures

This study is dependent on a number of preparations prior to the study. The following is a list of items that will be made available at least 24 hours prior to the study.

- Calibration standards and mobile phase for clotrimazole analysis will be prepared.
- Templates will be prepared
- The checklist will be performed (Appendix II).
- The clinic will be set up to ensure that the volunteers are comfortable and that there is adequate space for the researcher to work.
- Volunteers will be contacted and participation confirmed

5.6 Study day activities and procedures

- The adhesive templates will be affixed onto the volunteer's forearms to delineate the application sites.
- Application and removal of the formulation (±15 mg) as well as tape stripping will take place according to the scheduled times (Appendix III)
- The cotton wool swabs will be placed in Eppendorf centrifuge tubes and the tapes strips in air tight containers until time for analysis.
- Once the formulation has been removed from all application sites and TEWL measurements have been made from the blank site the templates will be removed and the volunteer released.
- The actual time of study procedures and/or results/comments obtained during the study will be recorded on the '*Registration of Data during Tape Stripping Form*' (Appendix III).

5.8 Product application

Prior to application of the product, an Eppendorf® (0.5 ml) pipette will be filled with the formulation. The product will be dispensed twice (2 on the dial of the Eppendorf® dispenser) at each application site to allow ± 15mg of the relevant product (corresponding to ≈ 0.15mg of clotrimazole) to be applied at each site. The Eppendorf® dispenser will be used to ensure that an accurate amount of test product is dispensed to each application site for each volunteer. A pre-weighed glass rod will be used to spread the product and weighed following the spreading to determine the accurate amount of formulation applied to each site. Application of the product will be done by the principal and assistant investigator.

5.9 Posture and physical activity

Strenuous exercises will not be permitted as described in section 4.3.

5.10 Food and fluids

Volunteers will not be restricted with respect to food and fluid intake.

5.11 Volunteer monitoring

The principal investigator will be present at all times during the study. Volunteers will be asked open-ended questions about their health at the time of each assessment and any discomfort observed during the experience will be recorded in the CRF (Appendix VI).

6 SAMPLE ANALYSIS

Samples will be analysed for clotrimazole using a validated extraction procedure and HPLC analytical method. Analysis will be done within 12 hours after sample collection.

7 DATA ANALYSIS

Residual Method

For the data collected using the residual method the following analysis will be conducted:

The amount of clotrimazole in the skin will be calculated from the difference between the amount of clotrimazole applied and the amount removed using the cotton swab. For each volunteer the ratio of the amount of clotrimazole found in the skin after applying the test product to that found after applying the reference product will be calculated. A 90% confidence interval will then be constructed around the mean of this ratio to determine bioequivalence

TS Method

For the data collected using the TS method, the following analysis will be conducted:

For each volunteer the amount of clotrimazole per individual tape strip *vs*. fraction of skin thickness will be profiled. The AUC will then be calculated. The ratio of the AUC of the test product over the AUC of the reference product will then be calculated. A 90% confidence interval will then be constructed around the mean of this to determine bioequivalence.

Should any data from the analysis be omitted, justification will be necessary and suitably motivated.

7.1 Statistical analysis

Pharmacokinetic and statistical parameters will be determined by the principal investigator using MS Excel 2007 and GraphPad Prism version 4. The statistical analysis will estimate the variance associated with volunteer-to-volunteer variability. Summary statistics such as median, minimum and maximum will be given.

8 ETHICAL AND REGULATORY REQUIREMENTS

8.1 Ethical and institutional review

Approval by the Rhodes University Ethical Standards Committee (RUESC) (departmental) will be obtained before the study commences. The original signed copy of the ethical approval will be retained by the principal investigator.

The study will be conducted in accordance with the recommendations of the guidelines as set out in the Declaration of Helsinki (1964) and its amendments of Tokyo (1975), Venice (1983), Hong Kong (1989), Somerset West (1996) and Edinburgh (2000), according to ICH Good Clinical Practice (GCP) guidelines and in compliance with the Biopharmaceutics Research Group's (BRG) SOPs, RUESC requirements and guidelines on the conduct of clinical trials in South Africa.

8.2 Written informed consent

Preceding the study, the nature, purpose and risk of participating in the study will be explained to all volunteers. All volunteers will be given time to consider the information and any questions that they might have will be answered. They will also be informed that they may withdraw from the study at any time without penalty to themselves (other than a reduced remuneration) but will be encouraged to complete the study.. Each volunteer will sign a consent form in the presence of a witness. Volunteers will receive written, detailed instructions concerning the study performance and restrictions.

8.3 Case report form

The Case Report Form (CRF) used for this study will be designed and supplied by the principal investigator (Appendix VI). All case report forms will be signed off by the principle investigator and all major events such as final acceptance of a volunteer, adverse events and final release from the study will be signed by both the principal investigator and the study consultant.

8.4 Record retention

All source documents, study reports and other study documentation for which the principal investigator is responsible will be archived and retained by the Faculty of Pharmacy, Biopharmaceutics Research Group. Results will be published in the scientific journals and/or presented in a thesis submitted to Rhodes University in fulfilment of the requirement for the degree of Master of Science (Pharmacy).

8.5 Insurance

Volunteers will be insured against any permanent adverse effect on their health which may arise in connection with the conduct of the study. A copy of the insurance certificate (Appendix VII) will be provided to RUESC (departmental) as part of the application to conduct this study. Adequate insurance cover in the event of negligence on the part of the principal investigator and the supervisor will be ensured.

8.6 Termination of the study

The principal investigator reserves the right to terminate the study in the interests of volunteer welfare following consultation with the supervisor. The supervisor may terminate the study at any time for scientific or safety reasons. If the study is prematurely terminated or suspended for any reason, the principal investigator will promptly explain to the volunteers and take appropriate steps as deemed necessary under the circumstances to assure the volunteers and where applicable follow up with therapy and inform the RUESC (departmental).

8.7 Adherence to protocol

Excluding an emergency situation in which proper treatment is required for the protection, safety and well-being of study volunteers, the study will be conducted as described in the approved protocol. Any deviation from the protocol will be recorded and explained.

Should amendments to the protocol be required, the amendments will be documented and signed by the principal investigator and the supervisor. If the protocol amendment(s) has an impact on the safety of volunteers, such as a change in dosing regimen or additional formulations, the amendment will be submitted to the RUESC (departmental) for approval.

8.8 Blinding

Volunteers will not be blinded and will be informed about the products for use at the application sites.

8.9 Adverse events/Adverse drug reactions

Volunteers will be questioned by the principle investigator on their health status at check-in, during the course of the study and before leaving the clinic at the end of the study. During the study, open-ended questions will be asked. If any adverse events are reported, the principal investigator will monitor the adverse event, initiate appropriate treatment if required and decide whether or not to withdraw the volunteer from the study. Signs and symptoms of any adverse events which occur during the study will be fully documented in the appropriate CRF (Appendix VI). If necessary adverse events will be referred to a suitably qualified medical practitioner for assessment and follow up.

Adverse events (which include illnesses, volunteerive and objective signs and symptoms that have appeared or worsened during the course of the study) will be assessed by the principal investigator and the supervisor during and after the study to determine whether or not they are related to the investigational test product (i.e. ADR), to the study procedure or other. The outcome of this assessment will be recorded in the appropriate CRF (Appendix VI).

AEs classified as severe or serious will be reported to the supervisor and Rhodes University Ethical Standards Committee (departmental) within 24 hours.

ADRs classified as serious and unexpected will be volunteer to expedited reporting as detailed in the ICH E2A and E2B guidelines on Clinical Safety Data Management and Data Elements for Transmission of Individual Case Report Forms respectively.

9 REPORTS

A full report on the study will be compiled by the principal investigator in the format requested by the supervisor and submitted to the supervisor. The analytical report will include results for all standard and quality control samples. A representative number of chromatograms or other raw data will be included covering the whole concentration range for all standards and quality control samples. The validation report will also be submitted.

ABBREVIATIONS

ADR	Adverse Dug Reaction
AE	Adverse Effect/Event
AUC	Area Under the Curve
CRF	Case Report Form
RUESC	Rhodes University: Ethical Standards Committee
SOP	Standard Operating Procedure
HPLC	High Pressure Liquid Chromatography
TEWL	Transepidermal Water Loss
CLZ	clotrimazole

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APPENDIX IV

Raw data NP_TS 2.1

Raw Data NP_TS 2.1: Tape Stripping Method

Subject	Site		2	3	4	5	6	7	8	9	10	11	12	13	14	15	AUC
01	**Ref 1**	SC	8.30%	18.68%	22.83%	29.06%	33.21%	45.66%	53.96%	60.19%	64.34%	68.49%	74.72%	78.87%	85.09%	89.25%	**66.20**
		CLZ	6.47	2.15	1.15	0.61	0.29	0.32	0.18	0.08	0.02	0.01	0.02	0.01	0.00	0.01	
	Ref 2	SC	10.38%	14.53%	24.91%	35.28%	43.58%	62.26%	64.34%	74.72%	85.09%	85.09%	85.09%	85.09%	89.25%	95.47%	**61.07**
		CLZ	8.78	2.24	1.08	0.54	0.25	0.45	0.14	0.07	0.11	0.07	0.04	0.00	0.00	0.00	
	Test 1	SC	4.15%	8.30%	14.53%	24.91%	24.91%	31.13%	37.36%	45.66%	47.74%	56.04%	66.42%	68.49%	74.72%	80.94%	**29.66**
		CLZ	2.51	1.58	0.74	0.54	0.34	0.28	0.14	0.13	0.13	0.06	0.14	0.05	0.05	0.07	
	Test 2	SC	6.23%	10.38%	14.53%	16.60%	24.91%	24.91%	33.21%	37.36%	41.51%	41.51%	45.66%	45.66%	47.74%	53.96%	**32.76**
		CLZ	4.89	1.67	1.09	0.90	0.66	0.36	0.24	0.11	0.13	0.28	0.09	0.04	0.05	0.05	
02	**Ref 1**	SC	10.28%	14.13%	16.70%	17.99%	20.55%	23.12%	26.98%	28.26%	42.39%	50.10%	51.39%	52.67%	57.81%	61.66%	**37.89**
		CLZ	7.07	2.53	1.27	0.76	0.43	1.32	0.24	0.17	0.26	0.33	0.04	0.13	0.02	0.04	
	Test 2	SC	1.28%	5.14%	8.99%	11.56%	12.85%	15.42%	21.84%	28.26%	32.12%	34.69%	37.26%	37.26%	41.11%	43.68%	**26.94**
		CLZ	3.86	1.78	1.44	0.57	0.71	0.40	0.25	0.25	0.14	0.04	0.07	0.05	0.02	0.00	
	Test 1	SC	6.42%	8.99%	15.42%	16.70%	19.27%	21.84%	24.41%	26.98%	29.55%	30.83%	30.83%	32.12%	34.69%	37.26%	**20.63**
		CLZ	3.18	2.02	0.61	0.31	0.57	0.33	0.17	0.25	0.23	0.22	0.07	0.02	0.12	0.14	
	Ref 2	SC	5.14%	11.56%	19.27%	20.55%	23.12%	23.12%	26.98%	29.55%	30.83%	39.83%	44.96%	46.25%	51.39%	52.67%	**37.69**
		CLZ	6.87	error	1.57	1.16	1.10	0.68	0.41	0.31	0.21	0.06	0.00	0.00	0.04	0.00	
03	**Ref 1**	SC	9.08%	12.71%	25.41%	34.49%	34.49%	39.93%	43.56%	45.38%	50.82%	56.27%	59.90%	59.90%	59.90%	59.90%	**61.98**
		CLZ	6.37	1.91	2.04	0.82	0.51	0.62	0.39	0.19	0.27	0.24	0.22	0.18	0.13	0.16	
	Test 2	SC	5.45%	9.08%	12.71%	14.52%	18.15%	19.97%	29.04%	30.86%	34.49%	39.93%	43.56%	47.19%	49.01%	54.45%	**36.39**
		CLZ	3.23	1.80	1.37	0.89	0.69	0.72	0.54	0.68	0.30	0.38	0.40	0.39	0.29	0.22	
	Ref 2	SC	5.45%	9.08%	12.71%	12.71%	12.71%	14.52%	18.15%	21.78%	27.23%	30.86%	30.86%	30.86%	34.49%	38.12%	**45.47**
		CLZ	9.63	3.95	1.66	1.14	1.06	0.70	0.49	0.45	0.33	0.23	0.17	0.38	0.27	0.22	
	Test 1	SC	1.82%	5.45%	7.26%	9.08%	10.89%	10.89%	16.34%	16.34%	21.78%	32.67%	38.12%	43.56%	47.19%	54.45%	**19.90**
		CLZ	3.58	1.56	0.60	0.40	0.47	0.39	0.29	0.16	0.12	0.18	0.13	0.13	0.10	0.10	
04	**Test 2**	SC	3.44%	10.31%	17.19%	18.91%	22.34%	27.50%	30.94%	30.94%	30.94%	32.66%	39.53%	41.25%	41.25%	44.69%	**43.91**
		CLZ	2.92	2.43	1.75	0.75	0.67	0.45	0.31	0.19	0.25	0.22	0.19	0.11	0.06	0.09	
	Ref 1	SC	5.16%	12.03%	13.75%	17.19%	20.62%	27.50%	30.94%	32.66%	32.66%	39.53%	41.25%	42.97%	48.13%	53.28%	**66.77**
		CLZ	8.34	3.03	1.45	1.29	0.96	0.97	0.60	0.50	0.47	0.22	0.27	0.21	0.15	0.18	
	Ref 2	SC	12.03%	18.91%	22.34%	25.78%	30.94%	36.09%	36.09%	37.81%	37.81%	42.97%	44.69%	44.69%	49.84%	55.00%	**68.22**
		CLZ	8.90	2.53	1.71	1.22	1.34	0.59	1.13	0.35	0.48	0.36	0.26	0.03	0.15	0.06	
	Test 1	SC	12.03%	17.19%	20.63%	22.34%	25.78%	29.22%	34.38%	37.81%	44.69%	46.41%	53.28%	55.00%	58.44%	60.16%	**65.53**
		CLZ	9.44	3.37	2.05	1.61	0.71	0.90	0.52	0.33	0.31	0.81	0.30	0.16	0.17	0.14	
05	**Test 1**	SC	3.84%	9.61%	17.29%	21.13%	21.13%	23.06%	28.82%	30.74%	32.66%	36.51%	44.19%	46.11%	49.96%	51.88%	**40.63**
		CLZ	4.86	1.88	0.85	0.68	0.47	0.43	0.33	0.26	0.19	0.28	0.17	0.15	0.11	0.21	
	Ref 2	SC	0.00%	1.92%	3.84%	7.69%	9.61%	13.45%	17.29%	24.98%	26.90%	26.90%	30.74%	36.51%	46.11%	51.88%	**16.47**
		CLZ	3.13	0.83	0.46	0.52	0.47	0.27	0.30	0.18	0.20	0.18	0.17	0.17	0.12	0.15	

	Ref 1	SC	3.84%	9.61%	15.37%	21.13%	23.06%	26.90%	30.74%	34.58%	40.35%	40.35%	46.11%	51.88%	53.80%	57.64%	**26.90**
		CLZ	2.69	1.35	0.70	0.49	0.28	0.21	0.20	0.15	0.16	0.17	0.12	0.09	0.08	0.08	
	Test 2	SC	0.00%	5.76%	7.69%	7.69%	7.69%	9.61%	13.45%	13.45%	15.37%	17.29%	19.21%	19.21%	24.98%	30.74%	**11.20**
		CLZ	1.34	0.74	0.67	0.36	0.20	0.29	0.16	0.19	0.13	0.26	0.20	0.09	0.13	0.13	
06	**Ref 2**	SC	14.93%	26.87%	38.81%	47.76%	56.72%	62.69%	68.66%	80.60%	89.55%	95.52%	101.49%	104.48%	113.43%	119.40%	**157.41**
		CLZ	7.51	3.30	1.90	1.38	1.37	0.59	0.91	0.63	0.50	0.39	0.25	0.29	0.36	0.25	
	Test 2	SC	5.97%	8.96%	14.93%	23.88%	32.84%	50.75%	65.67%	77.61%	77.61%	77.61%	86.57%	98.51%	101.49%	104.48%	**102.76**
		CLZ	8.47	3.74	2.63	1.52	1.13	0.66	0.44	0.37	0.19	0.18	0.23	0.32	0.11	0.07	
	Ref 1	SC	11.94%	23.88%	56.72%	62.69%	68.66%	74.63%	83.58%	92.54%	98.51%	101.49%	104.48%	104.48%	104.48%	113.43%	**151.53**
		CLZ	5.67	3.10	1.17	1.17	0.79	0.57	0.15	0.45	0.45	0.24	0.35	0.27	0.22	0.17	
	Test 1	SC	8.96%	14.93%	23.88%	26.87%	26.87%	32.84%	35.82%	35.82%	38.81%	44.78%	44.78%	50.75%	50.75%	53.73%	**63.47**
		CLZ	6.85	2.97	1.42	0.81	0.96	0.63	0.33	0.26	0.24	0.34	0.33	0.35	0.07	0.27	
07	**Test 1**	SC	2.25%	6.76%	13.53%	18.04%	18.04%	18.04%	24.80%	31.56%	33.82%	36.07%	42.84%	47.35%	49.60%	54.11%	**55.36**
		CLZ	5.32	2.30	1.91	1.26	0.60	0.58	0.70	0.74	0.32	0.49	0.31	0.31	0.21	0.13	
	Test 2	SC	4.51%	9.02%	9.02%	11.27%	11.27%	18.04%	18.04%	22.55%	22.55%	27.06%	27.06%	36.07%	36.07%	38.33%	**32.35**
		CLZ	5.22	2.84	1.32	0.89	1.06	0.60	0.40	0.39	0.41	0.38	0.24	0.23	0.21	0.16	
	Ref 1	SC	4.51%	4.51%	6.76%	13.53%	13.53%	15.78%	22.55%	27.06%	36.07%	40.58%	45.09%	49.60%	51.86%	58.62%	**28.96**
		CLZ	4.47	2.50	1.33	1.03	0.65	0.65	0.59	0.36	0.37	0.27	0.19	0.40	0.19	0.13	
	Ref 2	SC	6.76%	13.53%	20.29%	22.55%	29.31%	38.33%	47.35%	47.35%	51.86%	51.86%	51.86%	51.86%	56.37%	56.37%	**41.64**
		CLZ	3.91	1.51	0.95	0.67	0.42	0.42	0.39	0.22	0.28	0.21	0.23	0.26	0.17	0.20	
08	**Test 2**	SC	6.75%	15.75%	15.75%	22.50%	22.50%	29.25%	29.25%	38.25%	40.49%	47.24%	56.24%	56.24%	62.99%	65.24%	**43.65**
		CLZ	5.14	1.54	0.90	0.66	0.39	0.27	0.36	0.16	0.21	0.15	0.16	0.08	0.09	0.06	
	Test 1	SC	11.25%	13.50%	15.75%	18.00%	22.50%	27.00%	31.50%	36.00%	40.49%	44.99%	49.49%	49.49%	49.49%	53.99%	**34.52**
		CLZ	6.71	2.46	1.21	0.96	0.84	0.59	0.54	0.46	0.50	0.27	0.14	0.12	0.11	0.25	
	Ref 1	SC	4.50%	13.50%	18.00%	18.00%	18.00%	20.25%	22.50%	24.75%	24.75%	31.50%	31.50%	33.75%	40.49%	44.99%	**43.85**
		CLZ	4.00	2.63	1.07	1.09	0.44	0.23	0.37	0.41	0.24	0.23	0.13	0.19	0.09	0.13	
	Ref 2	SC	11.25%	18.00%	22.50%	22.50%	38.25%	38.25%	38.25%	40.49%	53.99%	89.99%	101.24%	105.74%	107.99%	114.74%	**56.26**
		CLZ	5.09	1.79	0.83	0.64	0.59	0.54	0.35	0.55	0.17	0.19	0.32	0.11	0.11	0.16	
09	**Test 1**	SC	21.52%	43.04%	53.80%	64.56%	75.32%	82.49%	89.66%	96.84%	107.59%	118.35%	121.94%	136.29%	139.87%	147.05%	**247.47**
		CLZ	11.87	4.41	1.86	1.10	0.65	0.51	0.26	0.15	0.15	0.10	0.06	0.08	0.06	0.07	
	Ref 2	SC	17.93%	32.28%	43.04%	53.80%	64.56%	78.90%	86.08%	86.08%	93.25%	104.01%	111.18%	114.77%	125.53%	125.53%	**109.73**
		CLZ	6.19	2.60	1.21	0.68	0.36	0.30	0.37	0.17	0.14	0.06	0.08	0.05	0.02	0.03	
	Test 2	SC	10.76%	21.52%	28.69%	39.45%	46.62%	53.80%	57.38%	64.56%	78.90%	86.08%	93.25%	104.01%	107.59%	114.77%	**67.66**
		CLZ	4.33	2.20	0.99	0.47	0.26	0.35	0.23	0.16	0.09	0.17	0.08	0.10	0.11	0.17	
	Ref 1	SC	17.93%	35.86%	46.62%	60.97%	71.73%	82.49%	93.25%	104.01%	118.35%	121.94%	121.94%	125.53%	132.70%	139.87%	**110.76**
		CLZ	3.72	2.50	0.96	0.79	0.63	0.40	0.20	0.13	0.29	0.11	0.08	0.05	0.19	0.08	
10	**Ref 2**	SC	13.89%	20.84%	31.25%	41.67%	45.15%	52.09%	55.56%	59.04%	69.46%	72.93%	76.40%	86.82%	97.24%	100.71%	**84.75**
		CLZ	5.34	2.38	1.47	1.44	0.66	0.72	0.44	0.41	0.26	0.24	0.20	0.34	0.13	0.10	
	Test 1	SC	24.31%	24.31%	31.25%	38.20%	45.15%	45.15%	55.56%	65.98%	76.40%	83.35%	93.76%	100.71%	107.66%	118.07%	**53.78**
		CLZ	6.09	2.11	0.99	1.12	0.96	0.56	0.40	1.03	0.33	0.26	0.17	0.22	0.19	0.17	

Test 2	SC	6.95%	6.95%	13.89%	20.84%	24.31%	34.73%	38.20%	48.62%	59.04%	69.46%	72.93%	76.40%	86.82%	104.18%	**56.60**
	CLZ	4.75	2.21	1.82	1.21	0.50	0.68	0.38	0.46	0.41	0.35	0.33	0.24	0.23	0.19	
Ref 1	SC	10.42%	17.36%	27.78%	38.20%	38.20%	41.67%	48.62%	52.09%	59.04%	65.98%	72.93%	76.40%	76.40%	86.82%	**101.04**
	CLZ	6.00	2.96	2.36	1.22	1.01	0.40	0.56	0.76	0.54	0.46	0.35	0.68	0.27	0.29	

Raw Data NP_TS 2.1: Residual Method

Subject	Site	Formulation applied	CLZ applied	CLZ removed	CLZ assumed to be in the skin
01	**Test 1**	14.9	149	122.61	**26.39**
	Test 2	14.3	143	140.97	**2.03**
	Ref 1	14.9	149	123.34	**25.66**
	Ref 2	14.1	141	107.72	**33.28**
02	**Test 1**	error	error	error	**error**
	Ref 1	13.3	133	95.38	**37.62**
	Test 2	14.5	145	107.70	**37.30**
	Ref 2	13.4	134	99.66	**34.34**
03	**Test 1**	14.4	144	96.91	**47.09**
	Ref 1	14.9	149	105.47	**43.53**
	Test 2	13.8	138	95.69	**42.31**
	Ref 2	14.4	144	112.80	**31.20**
04	**Ref 2**	14.1	141	108.75	**32.25**
	Test 1	14.5	145	91.23	**53.77**
	Test 2	14.6	146	127.20	**18.80**
	Ref 1	14.4	144	108.30	**35.70**
05	**Ref 1**	14.2	142	118.67	**23.33**
	Test 1	14.7	147	140.97	**6.03**
	Ref 2	14.6	146	126.40	**19.60**
	Test 2	14.4	144	118.56	**25.44**
06	**Ref 1**	14.8	148	106.94	**41.06**
	Test 2	14.1	141	102.80	**38.20**
	Test 1	14.4	144	115.36	**28.64**
	Ref 2	14.3	143	92.91	**50.09**
07	**Test 2**	14.9	149	109.42	**39.58**
	Ref 2	14.2	142	110.64	**31.36**
	Test 1	14.5	145	88.49	**56.51**
	Ref 1	14.2	142	114.15	**27.85**
08	**Ref 2**	14.6	146	105.85	**40.15**
	Ref 1	15.0	150	106.13	**43.87**
	Test 2	14.6	146	86.59	**59.41**
	Test 1	14.6	146	114.89	**31.11**
09	**Ref 2**	14.4	144	111.93	**32.07**
	Test 2	13.9	139	105.09	**33.91**
	Ref 1	14.3	143	120.70	**22.30**
	Test 1	14.9	149	114.54	**34.46**
10	**Test 2**	14.5	145	120.27	**24.73**
	Ref 2	13.8	138	101.80	**36.20**
	Ref 1	13.3	133	118.52	**14.48**
	Test 1	14.0	140	120.29	**19.71**

APPENDIX V

Amendments to protocol NP_TS 2.1

11 Jan. 10. 09

Dear Dr. C. Oltmann

Amendments to protocol NP_TS2

I would like to request that the Faculty of Pharmacy Internal Ethics Committee consider the minor amendments I have made to the originally approved protocol NP_TS2. I have attached the changes with an explanation as to why they were carried out. All changes have been looked at and approved by my supervisor Prof. I. Kanfer.

Regards,

Natalie Parfitt

Amendments to
Protocol NP_TS2

ASSESSMENT OF THE RESIDUAL AND TAPE STRIPPING METHODS AS TOOLS FOR THE DETERMINATION OF BIOEQUIVALENCE OF A CLOTRIMAZOLE 1% CREAM

October 2009

Introduction

The study described in protocol NP_TS2 was carried out on 10 volunteers. However, although the same formulation was used as the test and reference product, the 90% confidence interval did not fall within the BE acceptance range of 0.8 – 0.25. Subsequent investigations indicated that the variability was most likely due to contamination of the template with the formulation. Various measures have now been put in place to prevent this happening in the add-on study. These measures include:

1) New template design
2) New order of product application
3) Amended data recording sheet

1) New template design

Two templates will be utilised. The first template (Fig1(i)) will be made from an adhesive label (Tower®) and will be designed to expose a 2.1 x 2.1cm site. A permanent marker will then be used to mark the corners of each of the four sites (Fig1(ii)) and the template will be removed. The formulation will be applied within the boundaries marked on the skin (Fig1(iii)) and allowed to penetrate for 15minutes. The formulation will then be removed (Fig1(iv)) using a cotton swab. The second template (Fig1(v)) will then be applied. It will be made from Opsite® reinforced with 3M Magic Tape. This template will be designed to expose a 2x2cm site and will be placed directly over the markings drawn on the skin. An individual template will be used for each site. Each site will be tape stripped as described in protocol NP_TS2.

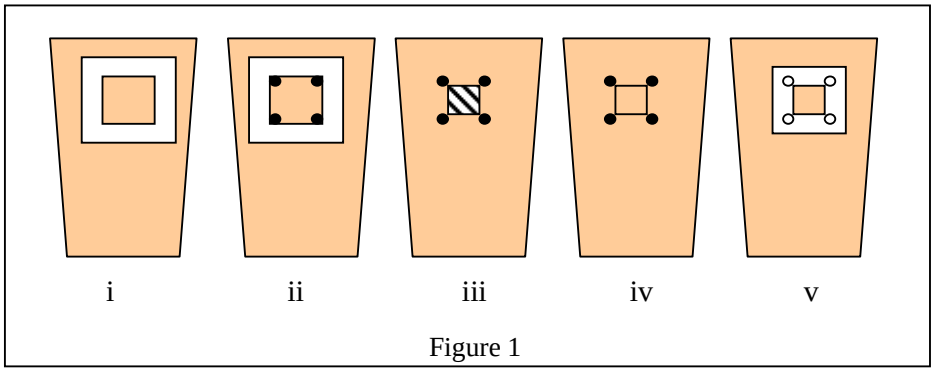


Figure 1

2) New order of product application

The product applied to each site will be randomised but the order in which the sites are tape stripped will be from A – E (E being at the elbow and A being at the

wrist).Note: if any other site besides E happens to be the blank that site will be skipped and done at the end.

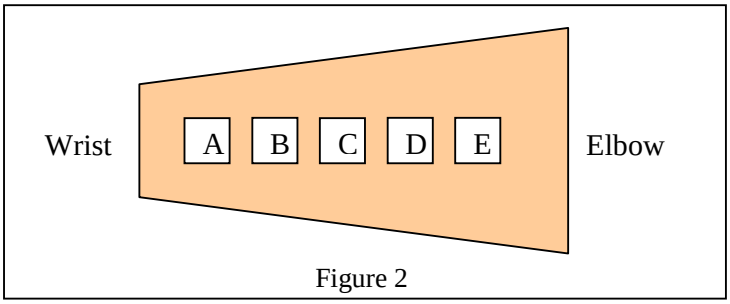


Figure 2

3) Amended data recording sheet
Right arm and Left arm data recording sheet are attached as appendix 1.

All changes have been approved by the principle investigator (Natalie Parfitt) and supervisor (Prof. I. Kanfer).

_____ Date:_____
Prof. I. Kanfer
Supervisor

_____ Date:_____
N.Parfitt
Principle investigator

Appendix 1

LEFT ARM

(First Volunteer)

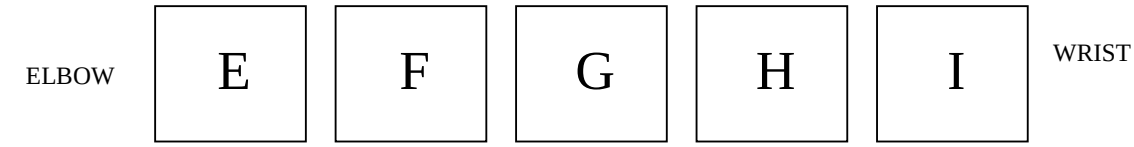
Volunteer Reference	
Procedure	
Investigator	

Time Schedule:

LEFT ARM : TS METHOD					
		Start time		**Stop time**	
Activity	**Site**	**Scheduled**	**Actual**	**Scheduled**	**Actual**
Arrival	N/A	7:00		7:05	
Template 1 application and marking	N/A	7:05		7:10	
Apply site A		7:10		**7:15**	
Apply site B		7:25		**7:30**	
Remove site A, apply template 2 + TS		**7:30**		7:45	
Remove site B, apply template 2 + TS		**7:45**		8:00	
Apply site C		8:00		**8:05**	
Apply site D		8:15		**8:20**	
Remove site C, apply template 2 + TS		**8:20**		8:35	
Remove site D, apply template 2 + TS		**8:35**		8:50	
TEWL		8:50		9:05	
Template removal and Release	N/A	9:05		9:10	

Application:

Mass before spreading: _____g



Code: _____ _____ _____ _____ _____

Mass after spreading: _____g _____g _____g _____g _____g

RIGHT ARM

(First Volunteer)

Volunteer Reference	
Procedure	
Investigator	

Time Schedule:

RIGHT ARM : RESIDUAL METHOD					
		Start time		**Stop time**	
Activity	**Site**	**Scheduled**	**Actual**	**Scheduled**	**Actual**
Arrival	N/A	7:00		7:05	
Template 1 application and marking	N/A	7:05		7:10	
Apply site A		7:10		**7:15**	
Apply site B		7:15		**7:20**	
Apply site C		7:20		**7:25**	
Apply site D		7:25		**7:30**	
Remove site A		**8:00**		8:05	
Remove site B		**8:05**		8:10	
Remove site C		**8:10**		8:15	
Remove site D		**8:15**		8:20	
Template removal and Release	N/A	8:20		8:25	

Application:

Mass before spreading: _____g

ELBOW | A | B | C | D | WRIST

Code: _____ _____ _____ _____

Mass after spreading: _____g _____g _____g _____g

APPENDIX VI

Raw data NP_TS 2.2

Subject	Site		2	3	4	5	6	7	8	9	10	11	12	13	14	15	AUC
01	**Ref**	SC	3.73%	8.76%	11.35%	15.57%	19.46%	24.00%	28.87%	34.71%	37.95%	42.98%	46.38%	48.98%	52.71%	56.27%	**32.95**
		CLZ	3.47	1.28	0.87	0.72	0.49	0.45	0.27	0.24	1.19	0.23	0.09	0.06	0.08	0.03	
	Test	SC	4.05%	6.65%	9.73%	13.95%	20.11%	25.30%	31.14%	33.89%	39.90%	42.33%	46.06%	53.03%	59.52%	67.14%	**21.69**
		CLZ	4.52	1.41	0.85	0.55	0.45	0.24	0.14	0.05	0.04	0.06	0.06	0.06	0.00	0.00	
	Ref	SC	5.51%	9.89%	14.43%	20.27%	24.98%	36.00%	40.22%	44.60%	50.60%	54.00%	58.22%	63.41%	72.82%	81.25%	**28.08**
		CLZ	3.58	1.81	0.55	0.77	0.33	0.18	0.07	0.07	0.02	0.02	0.02	0.02	0.02	0.05	
	Test	SC	5.51%	7.95%	13.95%	18.00%	25.30%	30.49%	36.49%	42.65%	46.22%	48.98%	54.65%	58.22%	60.65%	68.76%	**48.45**
		CLZ	3.85	0.11	2.24	0.95	1.91	0.48	1.09	0.28	0.16	0.12	0.47	0.09	0.10	0.02	
02	**Ref**	SC	4.22%	5.20%	8.94%	13.16%	16.90%	20.47%	21.12%	24.86%	27.78%	31.19%	34.28%	35.74%	42.89%	45.98%	**18.40**
		CLZ	3.99	1.49	1.43	0.57	0.49	0.26	0.21	0.19	0.13	0.00	0.00	0.00	0.20	0.07	
	Test	SC	3.09%	7.64%	14.13%	16.08%	19.50%	23.39%	24.69%	24.69%	27.13%	28.43%	30.54%	30.54%	33.95%	36.23%	**18.83**
		CLZ	2.50	1.43	0.74	0.38	0.19	0.07	0.11	0.11	0.00	0.00	0.00	0.00	0.00	0.00	
	Test	SC	3.09%	5.52%	9.10%	12.35%	16.08%	16.90%	18.85%	20.80%	23.88%	25.83%	27.46%	27.94%	33.63%	36.72%	**9.84**
		CLZ	2.40	0.77	0.71	0.25	0.33	0.20	0.02	0.00	0.04	0.01	0.00	0.00	0.03	0.00	
	Ref	SC	3.41%	6.82%	8.94%	11.37%	13.65%	15.92%	19.66%	21.77%	23.88%	26.16%	27.29%	29.08%	32.82%	34.44%	**20.34**
		CLZ	3.81	1.62	1.06	0.52	0.43	0.47	0.70	0.15	0.18	0.09	0.04	0.04	0.07	0.04	
03	**Ref**	SC	6.10%	9.87%	14.72%	14.72%	18.84%	21.89%	24.94%	28.17%	33.38%	36.79%	44.33%	44.33%	48.45%	53.84%	**18.58**
		CLZ	3.33	1.03	0.97	0.81	0.45	0.26	0.16	0.13	0.00	0.00	0.05	0.00	0.04	0.00	
	Ref	SC	5.20%	11.49%	11.49%	13.10%	13.82%	17.59%	17.59%	21.18%	27.82%	32.30%	36.43%	38.76%	42.53%	48.81%	**38.85**
		CLZ	5.22	4.26	1.55	1.00	0.50	0.26	0.43	0.32	0.38	0.11	0.02	0.00	0.00	0.00	
	Test	SC	2.33%	2.69%	5.38%	7.36%	9.69%	12.92%	14.72%	18.30%	23.69%	27.64%	33.38%	39.30%	48.09%	53.48%	**18.67**
		CLZ	3.08	1.99	1.80	0.76	0.50	0.50	0.44	0.41	0.28	0.22	0.07	0.11	0.00	0.00	
	Test	SC	6.10%	9.15%	10.77%	13.28%	18.66%	21.36%	24.23%	24.23%	27.10%	28.17%	31.23%	33.74%	35.71%	37.33%	**34.95**
		CLZ	7.23	2.55	1.82	1.20	1.01	1.01	0.47	0.34	0.05	0.35	0.16	0.05	0.00	0.04	
04	**Test**	SC	9.90%	16.72%	22.22%	26.62%	33.22%	38.50%	41.80%	45.98%	49.50%	53.24%	56.54%	62.04%	66.44%	72.38%	**36.09**
		CLZ	4.57	1.42	0.77	0.45	0.44	0.33	0.08	0.12	0.08	0.06	0.03	0.01	0.00	0.01	
	Ref	SC	9.02%	15.40%	20.90%	25.08%	29.04%	33.00%	37.18%	42.24%	45.32%	48.84%	52.36%	56.54%	60.50%	64.24%	**34.27**
		CLZ	4.20	1.73	0.92	0.46	0.26	0.38	0.17	0.09	0.01	0.05	0.05	0.00	0.03	0.00	
	Test	SC	7.92%	13.64%	19.36%	23.10%	27.94%	33.22%	37.62%	41.14%	44.22%	48.62%	52.58%	55.22%	58.74%	62.70%	**36.93**
		CLZ	4.21	2.03	1.25	0.57	0.42	0.34	0.15	0.08	0.03	0.03	0.02	0.00	0.00	0.00	
	Ref	SC	9.24%	15.84%	22.44%	26.40%	31.68%	35.20%	39.82%	42.46%	45.32%	47.52%	51.92%	54.78%	57.64%	58.52%	**51.43**

		CLZ	4.63	2.23	1.83	0.67	0.97	0.25	0.60	0.18	0.14	0.05	0.05	0.00	0.00	0.00	
05	**Test**	SC	6.71%	10.55%	15.34%	22.37%	26.85%	34.20%	39.95%	47.94%	52.73%	62.32%	69.67%	76.38%	85.65%	89.81%	**12.24**
		CLZ	1.35	0.61	0.36	0.23	0.08	0.25	0.12	0.10	0.00	0.00	0.00	0.00	0.00	0.00	
	Ref	SC	6.39%	12.14%	19.50%	25.89%	32.28%	37.39%	44.42%	58.80%	68.07%	82.45%	88.53%	95.56%	101.63%	106.74%	**14.79**
		CLZ	1.85	0.60	0.41	0.15	0.13	0.10	0.01	0.03	0.00	0.00	0.00	0.00	0.00	0.00	
	Ref	SC	5.43%	11.19%	15.34%	21.09%	27.48%	31.96%	36.43%	45.70%	55.29%	63.28%	69.03%	71.91%	79.90%	83.41%	**14.85**
		CLZ	1.49	0.77	0.37	0.36	0.13	0.05	0.09	0.10	0.03	0.00	0.00	0.00	0.00	0.00	
	Test	SC	5.11%	8.63%	15.02%	19.18%	25.89%	30.04%	34.52%	38.35%	43.46%	47.94%	53.69%	53.69%	58.49%	59.76%	**20.17**
		CLZ	2.72	0.98	0.63	0.44	0.47	0.16	0.18	0.12	0.06	0.00	0.02	0.00	0.00	0.00	
06	**Test**	SC	4.48%	6.38%	9.14%	11.21%	13.28%	16.21%	18.46%	21.22%	23.11%	26.39%	29.67%	33.12%	37.26%	40.02%	**19.33**
		CLZ	4.08	1.74	1.12	0.65	0.58	0.63	0.36	0.26	0.27	0.20	0.10	0.15	0.09	0.11	
	Test	SC	6.90%	13.97%	19.15%	21.73%	25.53%	29.32%	32.26%	34.67%	37.08%	41.40%	42.26%	45.54%	48.81%	52.09%	**55.62**
		CLZ	5.07	3.43	1.77	1.02	0.61	0.47	0.23	0.18	0.12	0.23	0.06	0.06	0.04	0.09	
	Ref	SC	6.38%	12.76%	17.77%	21.22%	25.18%	28.29%	32.77%	35.19%	38.81%	41.40%	45.19%	48.30%	51.75%	55.20%	**48.47**
		CLZ	4.39	3.00	1.81	1.02	0.58	0.42	0.30	0.22	0.14	0.03	0.03	0.00	0.00	0.00	
	Ref	SC	5.17%	10.18%	15.18%	18.46%	21.04%	23.98%	27.77%	29.84%	32.43%	33.81%	35.19%	36.22%	38.29%	50.88%	**27.53**
		CLZ	4.27	2.09	0.72	0.46	0.29	0.13	0.16	0.03	0.12	0.00	0.04	0.01	0.00	0.00	
07	**Ref**	SC	4.69%	10.03%	14.24%	16.99%	21.36%	27.19%	44.19%	50.98%	56.65%	63.45%	66.68%	69.92%	73.97%	75.59%	**28.97**
		CLZ	3.53	1.44	0.64	0.36	0.25	0.36	0.19	0.10	0.08	0.06	0.00	0.00	0.00	0.00	
	Test	SC	3.40%	7.45%	9.39%	12.95%	16.67%	19.91%	22.82%	26.71%	30.27%	33.50%	36.90%	39.65%	43.21%	45.00%	**15.00**
		CLZ	1.73	1.15	0.48	0.54	0.37	0.32	0.21	0.12	0.11	0.21	0.04	0.00	0.05	0.00	
	Ref	SC	3.88%	7.61%	11.33%	14.57%	17.32%	20.72%	23.47%	25.73%	28.97%	32.21%	35.61%	38.36%	40.46%	44.02%	**14.64**
		CLZ	2.30	0.93	0.65	0.36	0.27	0.31	0.12	0.08	0.11	0.13	0.11	0.04	0.00	0.03	
	Test	SC	4.53%	7.77%	12.14%	14.24%	17.64%	21.04%	24.12%	27.51%	30.27%	33.67%	33.67%	36.09%	74.13%	76.56%	**18.51**
		CLZ	3.14	1.14	0.83	0.48	0.39	0.28	0.20	0.11	0.11	0.11	0.08	0.03	0.02	0.04	
08	**Ref**	SC	4.08%	10.09%	15.88%	21.46%	28.12%	34.56%	40.14%	45.08%	50.66%	55.81%	59.89%	64.39%	69.55%	75.13%	**8.01**
		CLZ	1.07	0.32	0.23	0.08	0.10	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
	Test	SC	3.01%	6.87%	11.59%	16.31%	21.89%	27.69%	31.98%	37.78%	42.07%	47.87%	56.02%	60.10%	65.04%	70.40%	**11.70**
		CLZ	1.26	0.63	0.59	0.18	0.35	0.13	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
	Test	SC	4.94%	9.02%	13.52%	16.53%	24.04%	27.05%	31.12%	36.28%	42.07%	46.79%	52.59%	56.45%	61.82%	65.47%	**13.26**
		CLZ	2.00	0.63	0.54	0.19	0.51	0.05	0.09	0.00	0.03	0.00	0.00	0.00	0.00	0.00	
	Ref	SC	3.86%	7.30%	12.45%	15.24%	18.67%	22.32%	26.83%	29.84%	34.77%	39.71%	43.57%	48.08%	52.37%	55.16%	**5.97**

		CLZ	0.95	0.34	0.32	0.16	0.14	0.02	0.12	0.00	0.01	0.00	0.00	0.00	0.00	0.00	
09	**Ref**	SC	5.66%	10.76%	14.91%	19.25%	23.97%	27.93%	33.03%	37.37%	41.33%	46.43%	55.86%	62.84%	67.00%	69.83%	**18.95**
		CLZ	2.38	0.98	0.39	0.31	0.18	0.16	0.17	0.17	0.14	0.12	0.09	0.00	0.00	0.00	
	Ref	SC	8.12%	15.66%	21.70%	26.61%	30.57%	35.67%	39.25%	43.60%	46.80%	50.39%	53.97%	57.37%	59.83%	62.84%	**29.52**
		CLZ	3.23	1.40	0.58	0.30	0.17	0.16	0.16	0.12	0.13	0.00	0.09	0.00	0.00	0.00	
	Test	SC	8.12%	13.59%	18.12%	21.89%	25.48%	28.87%	33.22%	35.67%	40.39%	43.97%	47.56%	50.77%	53.41%	56.05%	**26.26**
		CLZ	3.15	1.72	0.68	0.44	0.28	0.19	0.26	0.15	0.12	0.09	0.09	0.09	0.00	0.00	
	Test	SC	8.30%	12.64%	16.99%	22.27%	26.23%	29.63%	34.16%	38.31%	42.27%	50.01%	54.16%	56.43%	62.28%	65.11%	**21.52**
		CLZ	2.98	1.23	0.27	0.75	0.33	0.20	0.15	0.11	0.15	0.11	0.10	0.00	0.00	0.00	
10	**Test**	SC	6.21%	9.97%	13.55%	17.31%	19.94%	25.78%	28.60%	32.17%	36.31%	39.70%	41.58%	47.79%	50.80%	55.88%	**20.96**
		CLZ	2.41	1.13	0.62	0.77	0.28	0.91	0.18	0.32	0.09	0.12	0.00	0.01	0.00	0.00	
	Ref	SC	4.14%	9.03%	14.11%	15.05%	19.19%	23.52%	25.21%	27.66%	30.86%	33.68%	42.71%	47.22%	51.36%	53.43%	**18.00**
		CLZ	2.59	1.18	0.90	0.31	0.30	0.20	0.12	0.06	0.00	0.00	0.00	0.00	0.00	0.00	
	Test	SC	3.20%	6.77%	10.16%	13.55%	18.25%	21.26%	25.21%	25.21%	28.97%	32.17%	35.75%	38.95%	40.64%	43.46%	**16.38**
		CLZ	3.12	1.21	0.70	0.35	0.41	0.20	0.16	0.00	0.02	0.04	0.00	0.00	0.00	0.00	
	Ref	SC	5.46%	10.35%	15.99%	22.20%	27.85%	30.67%	36.88%	39.51%	41.20%	44.03%	47.79%	49.86%	52.68%	55.32%	**33.59**
		CLZ	3.06	1.50	1.94	0.42	0.62	0.10	0.24	0.01	0.04	0.00	0.00	0.00	0.00	0.00	
11	**Ref**	SC	2.96%	9.16%	11.86%	18.32%	23.17%	24.52%	24.52%	25.33%	30.45%	36.38%	42.31%	47.16%	52.54%	57.40%	**22.28**
		CLZ	3.09	1.15	0.84	0.50	0.19	0.18	0.03	0.05	0.00	0.01	0.00	0.00	0.00	0.00	
	Ref	SC	7.54%	7.81%	14.28%	18.86%	23.17%	27.75%	32.34%	36.38%	39.07%	42.57%	47.43%	50.66%	51.47%	53.35%	**13.08**
		CLZ	2.71	1.25	0.76	0.41	0.14	0.27	0.09	0.03	0.00	0.02	0.00	0.00	0.00	0.00	
	Test	SC	7.01%	7.54%	7.54%	11.86%	17.51%	21.56%	23.71%	29.10%	29.91%	32.07%	34.22%	34.22%	37.99%	43.92%	**9.39**
		CLZ	2.98	1.67	1.01	0.50	0.45	0.15	0.14	0.05	0.00	0.06	0.00	0.00	0.00	0.00	
	Test	SC	6.47%	12.66%	15.63%	15.63%	22.37%	27.49%	32.60%	36.65%	40.42%	44.73%	50.93%	50.93%	53.08%	53.08%	**32.92**
		CLZ	3.02	1.62	2.00	0.70	0.91	0.46	0.33	0.15	0.05	0.02	0.19	0.00	0.04	0.02	
12	**Test**	SC	5.98%	8.97%	11.08%	13.01%	14.77%	14.77%	19.34%	20.05%	22.86%	28.13%	31.83%	32.53%	35.52%	39.56%	**13.95**
		CLZ	2.90	1.58	0.65	0.47	0.32	0.46	0.19	0.06	0.10	0.10	0.08	0.08	0.07	0.02	
	Test	SC	6.15%	10.20%	13.54%	13.72%	13.72%	15.83%	18.81%	18.81%	21.98%	24.27%	29.01%	31.30%	33.59%	37.81%	**21.30**
		CLZ	3.98	2.08	1.42	0.49	0.31	0.33	0.22	0.15	0.10	0.06	0.08	0.06	0.07	0.06	
	Ref	SC	3.69%	8.97%	12.31%	14.77%	16.35%	20.40%	24.44%	26.02%	28.66%	32.35%	36.75%	39.04%	41.85%	43.78%	**22.87**
		CLZ	2.82	1.78	0.93	0.47	0.33	0.34	0.21	0.08	0.15	0.07	0.06	0.02	0.02	0.02	
	Ref	SC	5.28%	9.32%	14.42%	17.94%	20.57%	21.63%	24.62%	24.97%	27.96%	32.00%	35.70%	37.98%	40.62%	43.26%	**28.29**

		CLZ	4.03	1.90	1.27	0.39	0.91	0.18	0.30	0.05	0.24	0.11	0.17	0.06	0.07	0.02	
13	**Test**	SC	7.63%	10.69%	13.74%	16.79%	20.50%	23.77%	26.39%	27.48%	30.10%	33.15%	36.42%	39.69%	42.97%	47.55%	**13.80**
		CLZ	4.46	1.08	0.46	0.25	0.26	0.14	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
	Ref	SC	6.98%	12.43%	17.01%	26.83%	29.88%	33.59%	35.99%	37.95%	42.53%	44.93%	46.67%	50.60%	54.09%	56.92%	**20.33**
		CLZ	3.51	1.03	0.52	0.21	0.14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
	Test	SC	7.42%	12.87%	19.63%	24.21%	27.92%	32.06%	36.64%	41.22%	45.15%	51.04%	55.62%	58.45%	62.38%	66.74%	**21.78**
		CLZ	3.88	1.07	0.47	0.35	0.04	0.09	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
	Ref	SC	9.81%	15.49%	20.07%	25.95%	31.41%	36.42%	40.79%	45.80%	48.85%	54.31%	58.23%	62.60%	65.43%	72.19%	**50.11**
		CLZ	5.97	2.69	1.53	0.68	0.47	0.30	0.33	0.27	0.00	0.22	0.00	0.00	0.00	0.00	

APPENDIX VII

Protocol NP_TS 3

1 BACKGROUND INFORMATION

1.1 Clotrimazole

1.1.1 Molecular structure

Clotrimazole is a widely used imidazole antifungal agent that was first synthesised by Bayer in 1967 [1, 2]. It has a molecular weight of 344.8u and is found as a white crystalline powder [3]. The molecular structure of clotrimazole is shown in Figure 1 below and has the empirical formula, $C_{22}H_{17}ClN_2$ [3].

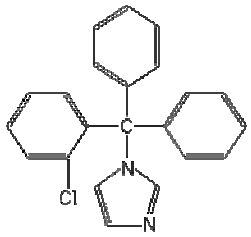


Fig. 1 Chemical structure of clotrimazole [4]

1.1.4 Mode of action and uses

Ergosterol is an important component of the fungal cell membrane and helps maintain the cells integrity in a similar way to cholesterol found in animal cells [5]. Ergosterol is synthesised in the fungal cells from lanosterol. The enzyme responsible for converting lanosterol to ergosterol is the CYP450 enzyme, 14–α–demethylase. Clotrimazole interacts with this enzyme thereby inhibiting ergosterol synthesis [2, 5]. The lack of ergosterol alters the fungal cell membrane causing an increase in cellular permeability. This causes the contents to leak out of the fungal cells thereby destroying them. The action of clotrimazole is specific to the particular pathogen and will not affect the host cells [6].

Clotrimazole is available in topical preparations (as a cream, lotion, spray, solution and powder), vaginal preparations (as a vaginal tablet and vaginal creams) and lozenges. Clotrimazole can also be administered orally but it has been largely superseded by other oral azoles [7]. The 1% topical cream that will be used in this study has the following indications: treatment of cutaneous candidiasis (yeast infection or thrush which is a fungal infection produced by the *Candida* species, of which *Candida albicans* is the most common) and dermatophytoses (fungal infections of keratin in the skin and nails). It may also be useful in the treatment of *tinea vesicolor* (a common skin infection caused by the yeast *Malassezia furfur* which is normally found on the human skin and only becomes troublesome under certain conditions) and *Corynebacterium minutissimum* (a bacterium which causes a superficial skin infection marked by reddish brown scaly patches known as erythrasma.) [8].

1.1.3 Pharmacokinetics

A topical application of clotrimazole will penetrate the epidermis but there is little if any systemic absorption [7]. This is desirable since the *stratum corneum* is considered to be the site of action (dermatophytes that infect the skin are only able to survive in the dead keratinised cells of the *stratum corneum*). After vaginal use, however, between 3% and 10% of a dose has been reported in the

systemic circulation [7]. Clotrimazole is metabolised by liver enzymes to form inactive compounds that are excreted in the faeces and urine [7].

1.1.4 Adverse effects

Local reactions including skin irritation and a burning sensation may occur following topical treatment. Contact allergic dermatitis has been reported. In cases of systemic absorption, lower abdominal cramps, increase in urinary frequency or skin rash may occur [7, 8]. Nausea, vomiting, unpleasant mouth sensations, and pruritus have been reported after the use of oral clotrimazole lozenges. Raised liver enzyme values have also occurred [7]. Intravaginal preparations of clotrimazole may damage latex contraceptives and additional contraceptive measures are therefore necessary during local application. Gastrointestinal disturbance, elevation of liver enzymes, dysuria, and mental depression have been reported after oral clotrimazole [7, 8].

1.1.5 Contraindications

Hypersensitivity to clotrimazole [8].

Copies of the product information leaflets of the topical formulations to be used in this study are located in Appendix I.

1.2 The Tape Stripping (TS) Method

1.2.1 Overview of tape stripping

Tape stripping is a non-invasive technique employed to study the penetration, the distribution and the dermatopharmacokinetics of topically applied drugs and cosmetics products [10-13] within the *stratum corneum*. It is commonly used to disrupt the epidermal barrier to enhance the delivery of drugs *in vivo* [14] and to obtain information about *stratum corneum* function [12]. Tape stripping has been used extensively in dermatological and pharmaceutical research to measure the *stratum corneum* mass and thickness, to collect *stratum corneum* lipids and protein samples, detect proteolytic activity associated with the *stratum corneum*, quantitatively estimate enzyme levels and activities in the *stratum corneum* and allow the detection of metal in the *stratum corneum* [12]. Although in use for over five decades, there are no universally accepted protocols for tape stripping [15]. However, TS has previously been identified to be of sufficient utility to have been proposed by the FDA as a method to evaluate the bioequivalence of topical dermatological dosage forms [16] and a preliminary guidance, which was subsequently withdrawn due to issues of variability, was issued by the US FDA.

1.2.2 Principles of tape stripping

Tape stripping involves the sequential removal of microscopic layers (typically 0.5 - 1 μm) of the *stratum corneum* [11, 17] by placing a strip of adhesive tape onto the skin surface with uniform pressure, which is subsequently removed [10,11]. Drug uptake into the *stratum corneum in vivo* can be measured by harvesting the *stratum corneum* previously exposed to a topical product and removed

with adhesive tapes. The latter are extracted and quantified for drug concentration by a validated analytical method [18]. The number of tape strips needed to remove the *stratum corneum* varies with age, gender, anatomical site, skin condition and possibly ethnicity [12]. Tape stripping is putatively simple, inexpensive and has been described as a minimally invasive technique [10].

1.2.3 Invasiveness of tape stripping

Although tape-stripping is considered to be essentially non-invasive, stripped sites in certain dark-skinned individuals may remain hyperpigmented for several months after healing [11]. This effect will be communicated to the human volunteers prior to entering the study.

1.2.4 Measurement of Transepidermal Water Loss (TEWL)

TEWL has been shown to inversely correlate with *stratum corneum* depth [19]. Therefore, TEWL measurements in conjunction with the mass of *stratum corneum* harvested can be used to determine the *stratum corneum* thickness for each volunteer. This information will enable the tape stripping data to be normalised. TEWL readings will be measured at the control site only. Readings will be taken after the removal of each tape strip. A Delfin® vapometer will be used to measure the TEWL. The instrument is placed on the surface of the skin, slight pressure is applied to ensure the chamber is sealed and a reading is obtained within 10 to 20 seconds.

2 STUDY PRODUCTS

2.1 Description

	Innovator product	**Test product**
Commercial name	Canesten® topical cream	Candid® topical gel
Generic name	Clotrimazole cream	Clotrimazole gel
Dosage form	Cream	Gel
Strength	1% m/m	1% m/m
Manufacturer	Bayer, Germany	Glenmark, India
MCC registration details	E/20.2.2/49	N/A
Expiry date	06/2013	11/2012
Description	A soft, white cream	A white, slightly viscous lotion
Dosage	± 4 mg per $1cm^2$ application site	± 4 mg per $1cm^2$ application site

2.2 Supply, storage and use

Sufficient Canesten® Topical Cream and Candid® Topical Gel for use in the study will be timeously supplied and logged into a record book by the principle investigator. The products are for external use only. The products will be stored below 25° C and kept out of reach of children [8]. Dispensing and

administration of the products will be recorded and administered only to volunteers participating in this study.

3 OBJECTIVES

The objective of this study is to assess whether the TS method can be used to determine bioequivalence and also if it has the sensitivity to pick up bioinequivalence between topical products. Therefore, the innovator product will be compared to a product known to be bioequivalent i.e. Canesten cream® vs Canesten cream® and also compared to a product known to be inequivalent i.e. Canesten Cream® vs Candid® Gel.

4 STUDY POPULATION AND MEDICAL ASSESSMENT

4.1 Number of volunteers

Previous studies involved between 6 - 18 human volunteers to study the skin penetration of some topical products [10-15]. This study will make use of up to 7 healthy human volunteers and will include both male and female volunteers. Three application sites plus one blank site will be used on the left arm. Statistical analysis (Section 8) will be performed on all volunteers completing the study. If bioequivalence cannot be demonstrated because of a result of a larger than expected random variation or a relative difference, an add-on volunteer study using up to 10 additional volunteers will be conducted. If no further study is required, preliminary statistical analysis will be finalised and presented in the final report. If an add-on study is conducted, data for all evaluable volunteers from both Group 1 and Group 2 will be combined and used in the preparation of the final report.

4.2 Inclusion/Exclusion criteria

Inclusion criteria

Only those volunteers meeting the following criteria will be included in the study:

vii. Volunteers who between the ages of 18 and 50.

viii. Volunteers who are in general good health.

ix. Volunteers who will be available for the entire study period.

Exclusion criteria

Volunteers meeting the following criteria will be excluded from the study.

xxv. Female volunteers who are pregnant, possibly pregnant or breast feeding.

xxvi. Volunteers who have a known allergy/hypersensitivity to clotrimazole or any fungicides.

xxvii. Volunteers who have any history of drug or alcohol abuse.

xxviii. Volunteers who have any mental deficiency or handicap.

xxix. Volunteers who have hairy ventral forearm surfaces and/or abrasions, scares, marks etc on the underside of their forearms.

xxx. Volunteers who regularly use cosmetic procedures on their forearms (e.g. shaving or waxing of arms, intense exfoliation, intense moisturisation, sunbed, spa or beauty treatments)

xxxi. Volunteers who have engaged in any sun-tanning or taken any sunny vacations within the last month.

xxxii. Volunteers who have participated in another dermal study within 2 months of the study date.

xxxiii. Volunteers who have used any topical fungicides within the last three months.

xxxiv. Volunteers who suffer from any allergic conditions (hayfever, allergic rashes, asthma or childhood eczema).

xxxv. Volunteers who suffer from any skin disorder such as psoriasis, eczema or other relevant skin disorder.

xxxvi. Volunteers who take regular medicine or tablets or who have used any medicated creams within one week (contraceptive pills excluded) prior to the study commencement.

4.3 Volunteer restrictions

- No prescription medication and OTC medication (particularly any medicated creams, e.g. corticosteroids, high dose vitamin A, Roaccutane® or its generics) will be allowed for at least one week prior to the study.
- With the exception of study product no concomitant medication may be taken by volunteers during the study.
- No strenuous physical activity may be undertaken by volunteers from 12 hours before product application and during the study.
- Volunteers will not be allowed to smoke during the study.
- Volunteers must refrain from applying emollients, skin conditioning creams and/or tanning lotions to their forearms for a period of 24 hours prior to the scheduled time of product application.
- Volunteers must ensure their arm has been washed with soapy water at least 1 hour prior to the start of the study.

Volunteers will be informed of the above restrictions and each volunteer will be specifically questioned on these points prior to product application. Any deviations from the above restrictions which are made known to the investigators either voluntarily or on questioning will be recorded on the appropriate CRF (Appendix VI). A decision as to whether the affected volunteer continues with the study will be taken by the principal investigator and the supervisor.

4.4 Criteria for removal from the study

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

vi. Voluntary withdrawal by the volunteer due to any reason.

vii. Illness or injury during the study if regarded as clinically significant by the principal investigator and the supervisor i.e if the principal investigator or supervisor feels that the volunteer is experiencing any medical signs or symptoms that do not have a rational explanation and that indicate that he/she may be ill or injured.

viii. Any adverse event or signs of toxicity if regarded as clinically significant by the principal investigator and the supervisor. i.e. if the principal investigator or supervisor feels that the volunteer is experiencing any medical signs or symptoms that do not have a rational explanation and that indicate that he/she may be experiencing an adverse event or toxicity

ix. Failure of the volunteer to comply with or be uncooperative towards any study requirements or restrictions if regarded as clinically significant by the principal investigator and the supervisor.

x. Volunteers withdrawn or dropped out of the study will be fully documented and accounted for in the CRF.

5 STUDY PROCEDURE

5.1 Pre- and post-study medical screening

Pre-study screening will be conducted not more than 3 days prior to the start of the study. The pre-study evaluations will be conducted as listed in the table below.

Pre-study	Post-study
Medical history Demographic data (date of birth, age, sex, origin), skin (dermatological) conditions and allergies	**Medical history** Since start of study
Dermatological assessment General assessment of the volar aspect of the forearm and any dermatological condition which may influence the barrier function of the skin.	**Dermatological assessment** General assessment of the volar aspect of the forearm
Adhesive sensitivity Assessment of volunteer's sensitivity to the tape's adhesive on application site.	

If any study related abnormalities are observed at the post-study medical, appropriate follow-up action will be taken and re-examination and re-testing conducted until the abnormality returns to normal or until the principle investigator and supervisor considers the abnormality to be clinically insignificant i.e. the clinical parameters concerned are within the normal range.

5.2 Medical tape sensitivity screening

No less than 30 days prior to the start of the study, volunteers will undergo an assessment of their tolerance to the adhesive on the medical tape used to demarcate application sites and for tape stripping procedures.

The following screening protocol will be utilised.

- Adhesion of the Opsite® (Smith-Nephew, Hull, UK) medical tape (4cm^2).

- The medical tape will be removed after four hours.
- The forearm of the volunteer will be assessed for allergies to the adhesive medical tapes.

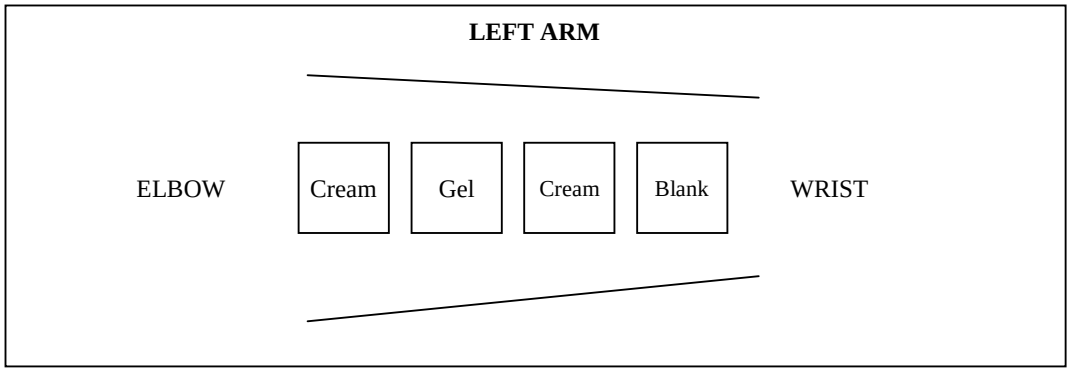
Volunteers who exhibit allergies will not be eligible to participate in this study.

5.3 Check-in and confinement

One volunteer will be assessed per study day. Volunteers will check-in on the study day at Room T14 in the Faculty of Pharmacy building, Rhodes University, Grahamstown 15mins before the start of the study. The principle investigator will conduct a brief medical examination, inclusion and exclusion criteria check and study restriction check. Volunteers accepted into the study will then be prepared for product application. Volunteers will remain in the study room for the entire duration of the study except when using bathroom facilities.

5.4 Study design

The study will be conducted on 7 volunteers, followed by another 10 volunteers if necessary (see section 4.1). The time between the first and last volunteer is expected to be 2 weeks at the most. A total of 3 application sites and 1 blank site will be used per volunteer. The blank site will be tape stripped and TEWL measurements taken to determine the *stratum corneum* depth. Two of the three application sites on each arm will be employed for the application of the innovator product and the other site for the test product. The formulations will be randomized between sites. An example of an application scheme is represented below. The duration of the study for each volunteer will be approximately 3 hours.



Cream: Canesten® Topical Cream (Reference/ innovator product)
Gel: Candid® Gel (Test product)
Blank: Site used for TEWL measurements

The volunteers will be requested to wash their forearm with soap during their usual ablutions (at least an hour before the study commences) and not apply any topical products to the arm. Two templates will be utilised. The first template (Fig1(i)) will be made from an adhesive label (Tower®) and will be designed to expose a 2.1 x 2.1cm site. A permanent marker will then be used to mark the corners of each of the four sites (Fig1(ii)) and the template will be removed. The formulation will be applied

within the boundaries marked on the skin (Fig1(iii)) and allowed to penetrate for 15minutes. The formulation will then be removed (Fig1(iv)) using a cotton swab. The second template (Fig1(v)) will then be applied. It will be made from Opsite® reinforced with 3M Magic Tape. This template will be designed to expose a 2x2cm site and will be placed directly over the markings drawn on the skin. An individual template will be used for each site.

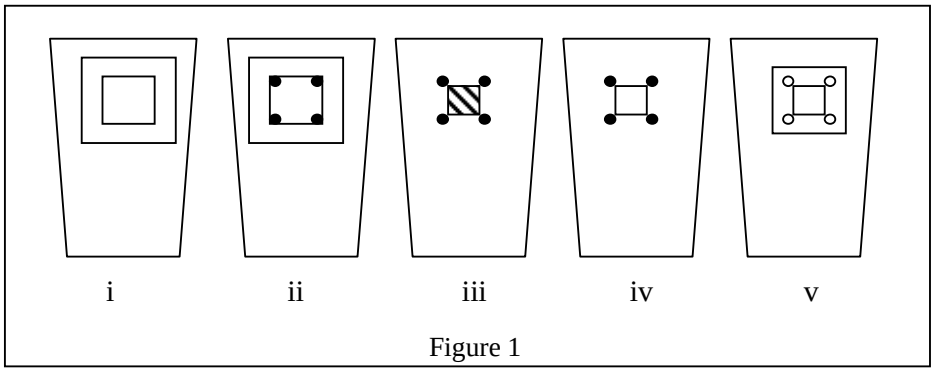


Figure 1

Approximately 15mg of the formulation will be applied to each site. The application to each site will be staggered to allow time for the product removal and the tape stripping procedure. The application sites will be exposed to the products for a dose duration of 15minutes since this was the ED50 determined for the TS data from the dose duration study NP_TS1. Adhesive tapes (Sellotape® Original) will be applied to each of the clean sampling sites with uniform pressure and then subsequently removed. Fifteen (15) successive strips will be made from each site. No formulation will be applied to the control site but transepidermal water loss measurements will be recorded from the site in order to determine the thickness of the *stratum cornum*.

5.5 Pre-study day activities and procedures

This study is dependent on a number of preparations prior to the study. The following is a list of items that will be made available at least 24 hours prior to the study.

- Calibration standards and mobile phase for clotrimazole analysis will be prepared.
- Templates will be prepared
- The checklist will be performed (Appendix II).
- The clinic will be set up to ensure that the volunteers are comfortable and that there is adequate space for the researcher to work.
- Volunteers will be contacted and participation confirmed

5.6 Study day activities and procedures

- The adhesive templates will be affixed onto the volunteer's forearms to delineate the application sites.
- The sites will be marked and the template removed

- Application and removal of the formulations (±15 mg) as well as tape stripping will take place according to the scheduled times (Appendix III)
- The cotton wool swabs will be placed in Eppendorf centrifuge tubes and the tapes strips in air tight containers until time for analysis.
- Once the formulation has been removed from all application sites and TEWL measurements have been made from the blank site the templates will be removed and the volunteer released.
- The actual time of study procedures and/or results/comments obtained during the study will be recorded on the '*Registration of Data during Tape Stripping Form*' (Appendix III).

5.8 Product application

Prior to application of the product, an Eppendorf® (0.5 ml) pipette will be filled with the formulation. The product will be dispensed twice (2 on the dial of the Eppendorf® dispenser) at each application site to allow ± 15mg of the relevant product (corresponding to ≈ 0.15mg of clotrimazole) to be applied at each site. The Eppendorf® dispenser will be used to ensure that an accurate amount of test product is dispensed to each application site for each volunteer. A pre-weighed glass rod will be used to spread the product and weighed following the spreading to determine the accurate amount of formulation applied to each site. Application of the product will be done by the principal and assistant investigator.

5.9 Posture and physical activity

Strenuous exercises will not be permitted as described in section 4.3.

5.10 Food and fluids

Volunteers will not be restricted with respect to food and fluid intake.

5.11 Volunteer monitoring

The principal investigator will be present at all times during the study. Volunteers will be asked open-ended questions about their health at the time of each assessment and any discomfort observed during the experience will be recorded in the CRF (Appendix VI).

6 SAMPLE ANALYSIS

Samples will be analysed for clotrimazole using a validated extraction procedure and HPLC analytical method. Analysis will be done within 12 hours after sample collection.

7 DATA ANALYSIS

TS Method

For the data collected using the TS method, the following analysis will be conducted:

For each volunteer the amount of clotrimazole per individual tape strip *vs*. fraction of skin thickness will be profiled. The AUC will then be calculated. The ratio of the AUC of the test product over the

AUC of the reference product will then be calculated. A 90% confidence interval will then be constructed around the mean of this to determine bioequivalence.

Should any data from the analysis be omitted, justification will be necessary and suitably motivated.

7.1 Statistical analysis

Pharmacokinetic and statistical parameters will be determined by the principal investigator using MS Excel 2007 and GraphPad Prism version 4. The statistical analysis will estimate the variance associated with volunteer-to-volunteer variability. Summary statistics such as median, minimum and maximum will be given.

8 ETHICAL AND REGULATORY REQUIREMENTS

8.1 Ethical and institutional review

Approval by the Rhodes University Ethical Standards Committee (RUESC) (departmental) will be obtained before the study commences. The original signed copy of the ethical approval will be retained by the principal investigator.

The study will be conducted in accordance with the recommendations of the guidelines as set out in the Declaration of Helsinki (1964) and its amendments of Tokyo (1975), Venice (1983), Hong Kong (1989), Somerset West (1996) and Edinburgh (2000), according to ICH Good Clinical Practice (GCP) guidelines and in compliance with the Biopharmaceutics Research Group's (BRG) SOPs, RUESC requirements and guidelines on the conduct of clinical trials in South Africa.

8.2 Written informed consent

Preceding the study, the nature, purpose and risk of participating in the study will be explained to all volunteers. All volunteers will be given time to consider the information and any questions that they might have will be answered. They will also be informed that they may withdraw from the study at any time without penalty to themselves (other than a reduced remuneration) but will be encouraged to complete the study.. Each volunteer will sign a consent form in the presence of a witness. Volunteers will receive written, detailed instructions concerning the study performance and restrictions.

8.3 Case report form

The Case Report Form (CRF) used for this study will be designed and supplied by the principal investigator (Appendix VI). All case report forms will be signed off by the principle investigator and all major events such as final acceptance of a volunteer, adverse events and final release from the study will be signed by both the principal investigator and the study consultant.

8.4 Record retention

All source documents, study reports and other study documentation for which the principal investigator is responsible will be archived and retained by the Faculty of Pharmacy, Biopharmaceutics Research

Group. Results will be published in the scientific journals and/or presented in a thesis submitted to Rhodes University in fulfilment of the requirement for the degree of Master of Science (Pharmacy).

8.5 Insurance

Volunteers will be insured against any permanent adverse effect on their health which may arise in connection with the conduct of the study. A copy of the insurance certificate (Appendix VII) will be provided to RUESC (departmental) as part of the application to conduct this study. Adequate insurance cover in the event of negligence on the part of the principal investigator and the supervisor will be ensured.

8.6 Termination of the study

The principal investigator reserves the right to terminate the study in the interests of volunteer welfare following consultation with the supervisor. The supervisor may terminate the study at any time for scientific or safety reasons. If the study is prematurely terminated or suspended for any reason, the principal investigator will promptly explain to the volunteers and take appropriate steps as deemed necessary under the circumstances to assure the volunteers and where applicable follow up with therapy and inform the RUESC (departmental).

8.7 Adherence to protocol

Excluding an emergency situation in which proper treatment is required for the protection, safety and well-being of study volunteers, the study will be conducted as described in the approved protocol. Any deviation from the protocol will be recorded and explained.

Should amendments to the protocol be required, the amendments will be documented and signed by the principal investigator and the supervisor. If the protocol amendment(s) has an impact on the safety of volunteers, such as a change in dosing regimen or additional formulations, the amendment will be submitted to the RUESC (departmental) for approval.

8.8 Blinding

Volunteers will not be blinded and will be informed about the products for use at the application sites.

8.9 Adverse events/Adverse drug reactions

Volunteers will be questioned by the principle investigator on their health status at check-in, during the course of the study and before leaving the clinic at the end of the study. During the study, open-ended questions will be asked. If any adverse events are reported, the principal investigator will monitor the adverse event, initiate appropriate treatment if required and decide whether or not to withdraw the volunteer from the study. Signs and symptoms of any adverse events which occur during the study will be fully documented in the appropriate CRF (Appendix VI). If necessary adverse events will be referred to a suitably qualified medical practitioner for assessment and follow up.

Adverse events (which include illnesses, volunteerive and objective signs and symptoms that have appeared or worsened during the course of the study) will be assessed by the principal investigator and the supervisor during and after the study to determine whether or not they are related to the investigational test product (i.e. ADR), to the study procedure or other. The outcome of this assessment will be recorded in the appropriate CRF (Appendix VI).

AEs classified as severe or serious will be reported to the supervisor and Rhodes University Ethical Standards Committee (departmental) within 24 hours.

ADRs classified as serious and unexpected will be volunteer to expedited reporting as detailed in the ICH E2A and E2B guidelines on Clinical Safety Data Management and Data Elements for Transmission of Individual Case Report Forms respectively.

9 REPORTS

A full report on the study will be compiled by the principal investigator in the format requested by the supervisor and submitted to the supervisor. The analytical report will include results for all standard and quality control samples. A representative number of chromatograms or other raw data will be included covering the whole concentration range for all standards and quality control samples. The validation report will also be submitted.

ABBREVIATIONS

ADR	Adverse Dug Reaction
AE	Adverse Effect/Event
AUC	Area Under the Curve
CRF	Case Report Form
RUESC	Rhodes University: Ethical Standards Committee
SOP	Standard Operating Procedure
HPLC	High Pressure Liquid Chromatography
TEWL	Transepidermal Water Loss
CLZ	clotrimazole

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APPENDIX VIII

Raw data NP_TS 3

Subject	Site		2	3	4	5	6	7	8	9	10	11	12	13	14	15	AUC
01	**T_1**	SC	6.41%	11.63%	16.85%	21.12%	32.76%	37.50%	44.39%	50.32%	52.22%	58.15%	62.90%	65.51%	70.02%	75.48%	**22.99**
		CLZ	2.46	1.10	0.78	0.45	0.27	0.15	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
	R	SC	6.17%	13.05%	23.50%	30.62%	37.03%	43.67%	47.71%	50.79%	56.49%	61.48%	66.93%	70.73%	75.01%	84.50%	**23.15**
		CLZ	2.17	0.94	0.65	0.11	0.14	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
	T_2	SC	5.22%	5.46%	13.53%	19.23%	24.92%	29.43%	34.89%	50.08%	54.59%	54.59%	55.78%	61.71%	63.85%	67.17%	**26.57**
		CLZ	4.06	1.72	1.90	0.41	0.33	0.15	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
02	**R**	SC	5.88%	6.83%	8.16%	12.71%	17.46%	20.68%	23.34%	24.85%	26.37%	29.60%	32.82%	34.53%	40.22%	45.35%	**2.33**
		CLZ	1.28	0.63	0.16	0.08	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
	T_2	SC	1.33%	8.16%	10.44%	14.04%	14.23%	21.44%	27.51%	27.70%	28.27%	33.58%	40.98%	41.17%	41.17%	44.02%	**20.42**
		CLZ	3.96	0.93	0.45	0.21	0.23	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
	T_1	SC	1.90%	5.50%	5.50%	6.07%	7.40%	8.54%	14.99%	29.03%	44.78%	47.05%	49.52%	49.52%	49.52%	50.09%	**6.13**
		CLZ	2.40	0.81	0.39	0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
03	**T_2**	SC	25.02%	31.49%	37.38%	58.28%	59.16%	67.69%	74.76%	78.00%	78.00%	78.00%	85.94%	90.95%	95.07%	105.66%	**90.52**
		CLZ	6.05	3.41	1.58	0.60	0.97	1.10	0.34	0.48	0.33	0.34	0.27	0.17	0.22	0.20	
	T_1	SC	5.59%	10.30%	17.36%	22.96%	27.08%	27.08%	35.91%	41.79%	48.56%	52.39%	59.75%	63.28%	67.40%	72.70%	**18.11**
		CLZ	2.34	0.93	0.63	0.29	0.37	0.15	0.04	0.01	0.01	0.00	0.00	0.00	0.00	0.00	
	R	SC	9.42%	16.48%	23.25%	28.25%	32.38%	38.85%	44.44%	49.45%	57.98%	65.05%	72.11%	80.64%	85.65%	91.24%	**61.57**
		CLZ	7.62	2.07	1.15	0.59	0.47	0.40	0.40	0.14	0.26	0.03	0.06	0.01	0.01	0.00	
04	**R**	SC	6.26%	13.35%	18.08%	22.33%	26.23%	30.72%	34.86%	38.29%	41.83%	45.61%	48.33%	50.81%	50.81%	53.77%	**51.95**
		CLZ	5.57	3.20	1.18	0.72	0.42	0.42	0.22	0.07	0.08	0.00	0.02	0.00	0.04	0.00	
	T_2	SC	6.50%	11.11%	15.84%	18.91%	22.57%	26.23%	29.66%	34.51%	37.58%	40.06%	44.91%	49.04%	52.00%	54.36%	**48.10**
		CLZ	10.09	2.77	1.28	0.75	0.44	0.24	0.14	0.06	0.00	0.11	0.04	0.06	0.12	0.00	
	T_1	SC	4.37%	9.69%	13.00%	17.49%	19.97%	23.16%	27.65%	29.42%	32.62%	34.98%	40.06%	42.42%	46.09%	48.92%	**31.14**
		CLZ	4.38	2.27	1.32	0.56	0.39	0.26	0.09	0.06	0.04	0.00	0.00	0.00	0.00	0.00	
05	**T_2**	SC	5.60%	10.81%	11.41%	18.61%	22.42%	24.62%	24.62%	25.42%	28.02%	30.62%	32.62%	35.62%	39.63%	42.43%	**47.20**
		CLZ	7.70	3.67	2.11	0.88	0.53	0.30	0.55	0.24	0.06	0.01	0.17	0.02	0.07	0.00	
	T_1	SC	4.40%	10.01%	14.81%	16.61%	19.01%	19.21%	22.22%	22.62%	23.02%	23.02%	24.82%	28.42%	31.22%	34.62%	**49.69**
		CLZ	8.62	3.10	1.65	0.51	0.68	0.77	0.30	0.17	0.14	0.03	0.06	0.00	0.00	0.00	

	R	SC	7.00%	9.61%	16.81%	21.61%	27.42%	27.42%	29.02%	31.02%	31.42%	32.62%	35.02%	36.82%	40.03%	43.23%	**55.88**
		CLZ	8.59	1.12	2.75	2.16	1.46	2.26	1.23	0.17	0.12	0.32	0.38	0.00	0.32	0.14	
06	**T_1**	SC	5.61%	10.82%	11.42%	18.64%	22.45%	24.65%	25.85%	26.65%	29.26%	31.86%	33.87%	36.88%	40.88%	43.69%	**43.11**
		CLZ	6.10	3.40	1.54	0.53	0.92	1.05	0.28	0.41	0.26	0.27	0.21	0.10	0.15	0.13	
	R	SC	4.41%	10.02%	14.83%	16.63%	19.04%	19.04%	22.04%	22.45%	22.85%	22.85%	24.65%	28.26%	31.06%	34.47%	**13.87**
		CLZ	2.31	0.87	0.57	0.23	0.30	0.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
	T_2	SC	7.01%	9.62%	16.83%	21.64%	27.46%	42.09%	43.69%	45.69%	46.09%	47.30%	49.70%	51.50%	54.71%	57.92%	**37.25**
		CLZ	7.70	2.03	1.10	0.53	0.41	0.34	0.33	0.07	0.20	0.00	0.00	0.00	0.00	0.00	
07	**T_2**	SC	9.45%	16.60%	24.67%	30.20%	35.04%	41.04%	47.49%	53.72%	55.56%	58.79%	59.02%	63.17%	65.47%	70.78%	**118.20**
		CLZ	6.38	5.49	2.74	2.00	2.15	1.36	0.45	0.20	0.13	0.10	0.09	0.03	0.00	0.00	
	R	SC	2.77%	7.61%	10.84%	11.99%	14.99%	17.29%	24.90%	27.43%	32.97%	44.26%	48.18%	54.41%	64.32%	error	**23.31**
		CLZ	2.92	1.11	0.58	0.83	0.50	0.52	0.22	0.22	0.19	0.14	0.04	0.00	0.00	error	
	T_1	SC	5.53%	9.45%	11.99%	14.29%	17.29%	21.90%	24.90%	28.36%	29.74%	31.58%	35.04%	39.19%	64.78%	69.62%	**27.28**
		CLZ	5.26	2.40	1.06	0.36	0.55	0.35	0.22	0.13	0.25	0.13	0.14	0.00	0.00	0.00	