EVALUATION OF THE PHARMACEUTICAL AVAILABILITY OF ERYTHROMYCIN FROM TOPICAL FORMULATIONS

by

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ABSTRACT

Erythromycin (ERY) is a macrolide antibiotic which is used in the treatment of *acne vulgaris*. Acne is a common skin condition that occurs when the sebaceous glands and hair shafts become infected by the bacteria *Propionibacterium acnes*. Acne is a chronic condition that may last for years and the severity of the effects of the disease on patients is often undermined especially in third world countries where more emphasis is placed on other more life-threatening diseases. It may cause considerable physical and emotional distress to sufferers along with the possibility of permanent scarring. Although use of topical ERY formulations is not the first line of treatment it has proven to be effective in treating inflammation of skin and skin structures cause by the responsible bacteria.

To-date there are a variety of vehicles which are used in preparing topical ERY formulations namely ointment and gel bases, alcoholic solutions and pledgets. All the gel formulations on the market contain hydroxypropyl cellulose, alcohol and water along with the active ingredient(s). However, some gel formulations contain propylene glycol in addition to these excipients an example being Emgel®. Propylene glycol has been shown to affect the penetration of topically applied drugs through the skin suggesting that it would be highly likely that those formulations which contain propylene glycol may release more ERY into the skin following application. With this in mind, two ERY gel formulations were produced which contained different percentages of propylene glycol.

According to the FDA guidelines, pharmacokinetic measurements in blood, plasma and/or urine of topical dermatological drug products are not feasible to document bioequivalence since the active ingredient(s) in topical formulations is/are not intended to be absorbed into the systemic circulation and in addition, concentrations in extracutaneous biological tissues would generally not be measurable. This limits determination of bioavailability and assessment of bioequivalence of such products to pharmacodynamic measurements, clinical trials and dermatopharmacokinetic (DPK) measurements such as tape stripping (TS) and microdialysis (MD).

TS is a sampling technique which involves sequential removal of layers of the *stratum corneum* using strips of adhesive tape. This technique has found increasing use in DPK studies for investigation of drug kinetics in the skin following the application of a topical formulation. The technique has also been used as a diagnostic tool in assessing the quality of the *stratum corneum* in diseased skin. In the current research study, the tape stripping technique was used to investigate the pharmaceutical/biological availability of topical gel formulations containing ERY.

MD is another DPK sampling technique which has been used to determine the amount of a topically applied drug that penetrates through the *stratum corneum* to reach deeper tissues of the skin. The *in vivo* sampling technique involves the insertion of microdialysis probes beneath the skin surface in the dermal tissue and allows for real-time sampling of the analyte at its target site. Recently *in vitro* MD has also been successfully used to assess the pharmaceutical availability of a topical corticosteroid, mometesone furoate, from topical formulations. Based on this work, microdialysis was used to determine the pharmaceutical availability of ERY from gel formulations which were developed for use in this research.

The results of the pharmaceutical availability of ERY from *in vivo* tape stripping studies and the *in vitro* microdialysis studies were compared to establish correlation between the data. Pharmaceutical equivalence and bioequivalence data obtained from the respective studies on the gel formulations were investigated by statistical analysis of the data generated from both the *in vitro* and *in vivo* experiments.

In summary the objectives of this research were:

1. To develop and validate a high performance liquid chromatography method suitable to analyse ERY concentrations obtained from *in vitro* microdialysis studies and *in vivo* tape stripping studies.
2. To prepare two different ERY gel formulations with different percentage content of propylene glycol.
3. To determine the pharmaceutical availability of ERY from two different gel formulations using *in vitro* microdialysis.

4. To develop and validate a tape stripping technique which could be used to determine percutaneous penetration and bioequivalence of the gel formulations.
5. To compare *in vitro* microdialysis and *in vivo* tape stripping data and attempt to establish a correlation between the two different approaches.

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LIST OF ABBREVIATIONS

α	Selectivity factor
x	Thickness of the SC removed with each tape strip
w/o	Water-in-oil
UV	Ultraviolet
TS	Tape stripping
TLC	Thin layer chromatography
TEWL	Transepidermal water loss
TBA	Tetrabutylammonium hydrogen sulphate
T	Test product
SOLV	4 % solution of dextran 70 in normal saline with 5 % ethanol
SC	Stratum Corneum
S/N	Signal-to-noise
S	Slope
Rt	Retention time
RP	Reverse phase
R	Resolution
R	Reference product
QC	Quality control
PG	Propylene glycol
P. acnes	*Propionibacterium acnes*
NS	5 % ethanol in normal saline
N	Number of theoretical plates
MF	Mometasone furoate
MD	Microdialysis
M2	Membrane 2
M1	Membrane 1
LOQ	Limit of Quantification
LOD	Limit of Detection
LC-MS-MS	Liquid chromatography mass spectrometry

L	Apparent thickness of the stratum corneum
kα	Capacity factor
K	Partition coefficient
ICH	International Conference of Harmonisation
I.V.	Intravenous
HPLC	High performance liquid Chromatography
HPC	Hydroxypropyl cellulose
HETP/H	Height Equivalent to a Theoretic Plate
GC	Gas chromatography
G2	Gel 2
G1	Gel 1
F.D.A.	Food and Drug Administration
ERY	Erythromycin
ECF	extracellular fluid
ECD	Electrochemical detection
DPK	Dermatopharmacokinetics
D	Diffusion coefficient
CZE	Capillary zone electrophoresis
CV %	Percentage coefficient of variation
CLM	Clarithromycin
CDER	Centre for Drug Evaluation and Research
BE	Bioequivalence
BA	Bioavailability
AUC	Area under the curve
% RSD	Percentage relative standard deviation
% recovery	Percentage recovery
% loss	Percentage loss
% gain	Percentage gain

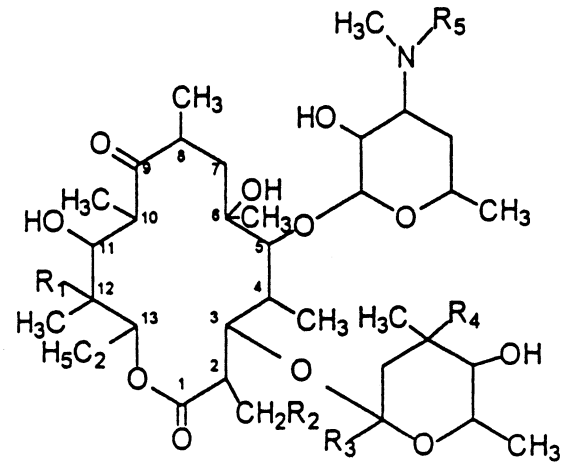
CHAPTER 1

ERYTHROMYCIN

1. INTRODUCTION

1.1 ISOLATION AND STRUCTURE OF ERYTHROMYCIN

Erythromycin (ERY) is a macrolide antibiotic produced by a strain of *Streptomyces erythreus* now reclassified as *Saccharopolyspora erythea* [1]. It was first discovered and isolated by McGuire *et al* in 1952 [2]. The strain produces a mixture of various forms of the macrolide which are ERY A, ERY B, ERY C, ERY D, ERY E, ERY F and ERY G as shown in Figure 1.1. The main component in the mixture is ERY A [1;3;4]. ERY E and ERY F are metabolites of ERY A, the other components are intermediates in the biosynthesis of ERY A. The sum of percentages of these components is not less than 85.0 percent and not more than 100.5 percent calculated on the anhydrous basis [5].



	R_1	R_2	R_3	R_4	R_5
ERY A	OH	H	H	OCH_3	CH_3
ERY B	H	H	H	OCH_3	CH_3
ERY C	OH	H	H	OH	CH_3
ERY D	H	H	H	OH	CH_3
ERY E	OH	-O-		OCH_3	CH_3
ERY F	OH	OH	H	OCH_3	CH_3
ERY G	H	OH	H	CH_3	CH_3

Figure 1.1 Chemical structures of erythromycin and its related substances

The aglycone portion of ERY has a characteristic 14 membered macrocylic lactone ring structure which has two sugar groups attached to it, an amino sugar D-desosamine at C-5 and a neutral sugar at C-3. The amino group of the D-desosamine sugar confers basic properties on the molecule. The neutral sugar entity is the L-cladinose sugar group except for ERY C and ERY D which contain L-mycarose. Both sugars are attached to the lactone ring structure through β-glycosidic linkages [4]. ERY is a broad spectrum antibiotic which has a variety of uses such as the treatment of upper respiratory and soft tissue infections and more importantly in the treatment of *acne vulgaris* [2,3].

The chemical name of ERY, Figure 1.1, (CAS 114-07-8) is 3R*,4S*,5S*,6R*,7R*,9R*, 11R*,12R*,13S*,14R*-4-[(2,6-Dideoxy-3-C-methyl-3-O-methyl-L-ribo-hexopyranosyl)oxy] -14-ethyl-7,12,13-trihydroxy-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino) -D-xylo-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione[6]. It has the empirical formula $C_{37}H_{67}NO_{13}$ and a molecular weight of 733.94 [5;6]. ERY A base is a white or slightly yellow crystalline powder, practically odourless with a bitter taste [7].

It has been reported that there is a significant loss of activity if the macrocyclic lactone ring structure is cleaved [8]. The neutral L-cladinose moiety has been associated with efflux resistance and modifications to the ERY structure to overcome this issue have lead to the development of the ketolide antibiotics. These analogues maintain the same therapeutic efficacy but do not possess the same problems with resistance associated with ERY. The tertiary amine on the cladinose sugar is necessary for binding of ERY to its ribosomal target in the bacteria. The four main active sites on the ERY structure are the C-9 ketone, 6-OH, 12-OH and 8-H [2]. Modification of these sites has been shown to yield analogues with expanded gram negative activity, acidic stability and improved tolerance [2;9].

1.2 PHYSICO-CHEMICAL PROPERTIES

1.2.1 Melting Point

The melting point of anhydrous ERY A crystalline powder is 190 – 193 ºC [10]. The monohydrate of ERY A has a melting point of 137 -140 ºC [11].

1.2.2 Solubility

ERY A is slightly soluble in water, 1-1000 parts of water [10]. The aqueous solubility decreases with an increase in temperature. ERY A is freely soluble in alcohol, soluble in methanol, ether and chloroform [7;10].

1.2.3 Dissociation Constant

The dissociation constant is a measure of the extent of ionization of a drug molecule. Due to the presence of the dimethylamino desosamine sugar group ERY A is weakly basic with a pKa of 8.8 [11]. A saturated solution of ERY A in water (0.067 %) is alkaline with a pH range of 8.0 - 10.5 [4].

1.2.4 Partition Coefficient

The partition coefficient, otherwise known as the Log P value, is a measure of the lipophilicity of a drug molecule. It is a logarithm of the partition ratio of a drug between octanol and water. The partition coefficient of ERY A is 3.1 [12]. It is a useful indicator of absorption, permeation, distribution and transportation of a drug molecule in biological fluids. It has been reported that drugs which exhibit optimum permeation through the skin have a partition coefficient between 2 and 3 [13].

1.2.5 Ultraviolet Absorption Spectrum

The characteristic 14-membered macrocyclic lactone ring structure of ERY A does not have good light absorbing chromophores. As a result ERY A has a low molar absorptivity [14]. However, it has been reported that macrolides absorb UV radiation at very low UV wavelengths thereby allowing analysis by UV detection methods. The wavelength, 215 nm has proved to be the most useful and is the most commonly used wavelength for UV analysis [14-16].

1.2.6 Stability

Storage conditions for ERY topical products should preferably be kept between 15 – 30 ºC in air tight containers. The ointment and gel formulations should be protected from freezing [17].

1.3. PHARMACOLOGY

1.3.1 Classification

ERY is classified as a broad spectrum antibiotic for systemic use and also as an anti-acne agent when used topically. The available topical dosage forms for ERY depend on the country where the formulation is marketed (See Table 1.2). Generally, it is available in 2 to 4% m/m gel or solution formulations either alone or in combination preparations with benzoyl peroxide, ichthammol, isotretinoin, tretinoin, and zinc acetate [4].

1.3.2 Indications

ERY is indicated for use in the treatment of infections caused by susceptible strains of microorganisms. It has found use in the topical treatment of *acne vulgaris* as the microorganism which causes acne, *Propionibacterium acnes*, is susceptible to ERY [18]. It may be effective in treating grades II and III acne which are characterised by inflammatory lesions such as papules and pustules [17]. ERY topical preparations are used as a second line topical treatment for acne following failure of non-antibiotic topical preparations to treat the condition [19].

ERY ointment is specifically indicated for topical prophylaxis and treatment of superficial pyogenic infections of the skin [17].

1.3.3 Dosage

The dosage for the gel, ointment and solution dosage forms require application of a thin film or layer to the affected areas once or twice daily after the skin has been thoroughly cleansed and dried [20].

When using the topical solution, if excessive dryness or irritation becomes a problem the solution dosage may be reduced to once a day or less until the symptoms have subsided. For the pledgets, the recommended application is twice daily to the affected skin areas [17].

1.3.4 Mode of Action

ERY binds reversibly to the 50 S subunit of the microbial ribosome thereby inhibiting the binding of peptide chains during translocation. This inhibits the transpeptidation or translocation reactions and thus prevents protein synthesis in the bacteria [8;21]. This reversible binding of ERY to the 50 S subunit ultimately results in the premature detachment of incomplete peptide chains which causes inhibition of cell growth and proliferation subsequently resulting in cell death [4;8]. . ERY is

mainly bacteriostatic but at high concentrations it may exhibit bactericidal activity [4]. ERY does not bind to mammalian ribosomes, it binds specifically to microbial ribosomes [22].

ERY has a broad spectrum of activity which resembles that of penicillins [22]. It is active against gram-positive cocci, particularly against group A β-hemolytic streptococci and *Streptococcus pneumoniae*. The gram-positive bacilli are also susceptible to ERY [4;22]. ERY has also been found to be effective against the anaerobic *Propionibacterium acnes (P. Acnes)*. *P. acnes* is found in the sebaceous glands and follicles in the skin. It produces proteases, hyaluronidases, lipases and chemotactic factors which cause inflammation or produce by-products which cause inflammation [17;18;22].

The activity of ERY *in vivo* is pH dependent. At moderately alkaline pH its activity is increased, up to pH 8.5. This effect is observed particularly for the gram negative species owing to the improved penetration of the ERY into the microbial cell as a result of the neutral charge on the molecule at this pH [4].

1.3.5 Adverse Effects

ERY is generally well-tolerated and serious adverse effects are rare. Adverse effects of the antibiotic are primarily related to irritation of the gastrointestinal tract and possible hepatotoxicity following chronic use, particularly of the estolate derivative. ERY estolate, can produce acute cholestatic hepatitis (fever, jaundice, impaired liver function), probably as a hypersensitivity reaction. Most patients recover from this, but the hepatitis recurs if the drug is re-administered [23].

ERY may cause abdominal discomfort, cramping, vomiting, nausea and diarrhoea following either oral or parenteral administration [4;22]. Hypersensitivity reactions seem to be uncommon and have been reported in about 0.5 % of patients and include pruritis, urticaria and skin rash and the occasional case of anaphylaxis. Most patients receiving intravenous ERY experience burning sensations in the infused vein often with phlebitis necessitating frequent changes of the infusion site [17].

Erythromycin is a mechanism-based inhibitor, also called a suicide inhibitor, of CYP3A4 which means that the inhibition is irreversible. Metabolites of ERY inhibit the hepatic enzymes of cytochrome P450 (CYP450) particularly cytochrome P1A2 (CYP1A2) and cytochrome 3A4 (CYP3A4) by competitive inhibition [4]. This effect results in increased plasma concentrations of

concomitantly administered drugs which are metabolised by CYP450. Examples of such drugs include theophylline, oral anticoagulants, cyclosporine and methylprednisolone.

Recent work by Ray *et al* has shown that the concomitant use of ERY with other drugs known to considerably increase ERY plasma concentrations increases the risk of sudden death due to ventricular tachyarrhythmias. This is due to the fact that ERY in its non-metabolised form prolongs the QT interval by delaying cardiac repolarisation. Drugs such as the azole antifungal agents, certain calcium-channel blockers and some antidepressant drugs are strong inhibitors of the CYP3A4 isoenzyme and elevate the plasma levels of ERY. This increases the risk of the occurrence of ventricular arrhythmias and possibly sudden death. A two-fold increase in the risk of sudden cardiac death has been reported for patients taking ERY alone whereas for those patients taking ERY concurrently with a CYP3A4 inhibitor, the risk of sudden cardiac death was 5.3 fold higher [24].

Hypersensitivity or irritation may occur following topical application of ERY [4]. In addition to these, peeling, redness, dryness, scaling itching and a stinging or burning sensation have been experienced following topical application of either the gel, ointment pledget or solution dosage forms [17].

1.3.6 Contraindications

ERY and its derivatives should be avoided in those known to be hypersensitive to it, or those who have previously developed liver disorders while receiving it. The systemic administration of ERY should be carefully monitored in patients with existing liver disease or hepatic impairment. ERY may interfere with some diagnostic tests including the measurement of urinary catecholamines and should be used with caution in cases where such tests are necessary [4]. Use of ERY in patients with porphyria is considered unsafe [17]. ERY should be used with care in patients with history of arrhythmias or prolonged QT interval [4]. ERY should not be given to pregnant women [25].

Topical use of ERY is to be avoided in patients who are known to be hypersensitive to it [4;17;20].

1.4 PHARMACOKINETICS

ERY is unstable in gastric acid and as a result it is usually administered in film or enteric-coated preparations or one of the more acid-stable salts or esters is employed [4].

Table 1.1 Summary of pharmacokinetic parameters of ERY in healthy volunteers

Route of administration and dose	Concentration achieved (µg/ml)	Half life (h)	T_{max} (h)	% Plasma bound	% Urinary excretion	% Oral bioavailability	References
Oral	-	1.6	-	84	12	35	[26]
Oral (2 g), IV (500 mg)	2 , 10	1.5	-	-	5	-	[23]
Oral, 500 mg	5.4	-	-	-	-	-	[27]
Oral	-	1.5	-	90	5	-	[28]
Oral (500 mg), IV (0.5 – 1 g)	0.1 – 2 µg/ml, 8 – 12 µg/ml	1.5 – 2.0	1 – 4	73 – 81	-	-	[29]
Oral (500 mg)	1.7	-	3.5	-	-	-	[30]
Oral (250 mg, 500 mg)	0.3 – 0.5 µg/ml, 0.3 – 1.9 µg/ml	1.5 – 2.5	1 – 4	70 – 75	2 – 5	-	[4]

1.4.1 Absorption

Orally administered ERY is readily absorbed as the free base, which is the microbiologically active form, mainly in the duodenal part of the small intestine, caecum and large intestine [11]. However, absorption of ERY is variable and unreliable as a result of its instability in gastric acid [4]. ERY undergoes rapid internal cyclic ketal formation leading to an inactive spiroketal. To overcome this problem and as a result improve the absorption of ERY, several salts and esters of the drug have been developed which exhibit improved acid-stability. ERY is also formulated in enteric coated dosage forms to further improve acid stability [31]. Food may reduce the absorption of ERY base or the stearate but this depends on the formulation and the absorption of the esters is little affected by food [4].

The absorption and bioavailability of ERY salt or base is affected by gastric emptying time and the dosage form administered, this is especially true for the unprotected base. Following oral absorption of ERY, peak plasma concentrations generally occur between 1 and 4 hours after administration. The concentrations have been reported to range from 0.3 and 0.5 µg/ml for a 250 mg dose and 0.3 and 1.9 µg/ml for a 500 mg dose. Higher peak plasma concentrations may be achieved on repeated dosing four times daily, if therapeutically required. If a 200 mg dose of the gluceptate or lactobionate salts of ERY are infused intravenously, peak plasma concentrations of 3 to 4 µg/ml can be achieved [4;28;32].

Topical application of the ophthalmic ointment to the eye may result in absorption into the cornea and aqueous humour [11].

1.4.2 Distribution

After absorption, ERY diffuses readily into most body fluids. In the absence of meningeal inflammation, low concentrations are normally achieved in the spinal fluid. ERY does not cross the blood brain barrier well but passage of the drug across the barrier increases in meningitis. Relatively high concentrations are found in the liver and spleen and there is some uptake of the drug into polymorphonuclear lymphocytes and macrophages [4]. Due to the high concentration in phagocytes, ERY is actively transported to the site of infection, where, during active phagocytosis, large concentrations of erythromycin are released [32]. Between 70 – 75 % of ERY base which enters the blood stream following oral or intravenous (I.V.) administration is bound to plasma protein [4].

ERY is excreted in breast milk and crosses the placental barrier but only achieves low concentrations in fetal plasma reported to range from 5 to 20 % of the plasma concentrations in the mother [4]. ERY also distributes into the pulmonary system reaching the bronchial mucosa and also bronchial secretions [33;34].

ERY concentrations in the brain and other tissue persist longer than in serum. ERY concentrates in the bile and liver in patients with normal hepatic function. Levels in semen and prostatic fluid are about 33% higher than in serum. Because of ERY's relatively poor oral absorption, significant concentrations are achieved in the large intestine [32].

1.4.3 Metabolism

Most ERY is metabolised by oxidative demethylation in the liver by CYP3A4 isozymes to give several inactive metabolites [32;35]. The metabolic fate of ERY has not been completely determined [4].

1.4.4 Excretion

ERY is extensively excreted in the bile [22] as unchanged drug and some reabsorption can occur [32]. About 5 – 10 % of a dose taken orally is excreted in the urine unchanged [12]. As much as 12 – 15 % of an I.V. dose may be excreted by the urinary route [4].

In patients with normal renal function, the serum half-life is about 1.5 - 2 hours. Anuric patients may have a serum half-life of around 6 hours [28].

1.4.5 Pharmacokinetics Following Topical Administration

ERY has been shown to be effective in the treatment of *acne vulgaris* [19;36-38] but there are no data to suggest that the drug actually penetrates the skin to reach its' target site, the sebaceous glands and hair follicles which lie in the dermis of the skin [39]. Topical ERY 1.5 % alcoholic solution was compared to oral tetracycline 250 mg twice a day in patients with acne. A 58 % reduction in overall lesion count was observed with ERY topical therapy compared to 38 % with oral tetracycline. Counts of *P. acnes* were reduced by 80 % and 90 % with tetracycline and ERY respectively but the difference was not statistically significant [40].

1.5 PREPARATIONS

There are topical preparations which are available commercially which contain ERY alone and also in combination with other topically used compounds [32]. These formulations are listed in Table 1.2 below and are available on the market in various countries [41]. Topical ERY is available mainly in four different formulations namely ointments, alcoholic solutions, gels and pledgets (See Table 1.2). The innovator company manufacturing ERY topical products is Eli Lilly U. S. Other companies have subsequently developed generics and these include Abbott, Upjohn, Robins and McKesson which are all United States based companies [42]. The topical preparations initially produced by Eli Lilly have been removed from the market citing issues such as resistance, instability issues of ERY in these formulations, and the emergence of more effective treatment regimes for acne as the reasons for the withdrawal [28;41]

It is of interest to note that not all topical formulations of ERY can be found on all markets. Of those which are still available mainly the gels and alcoholic solutions are still in use. The formulations of gels differ in terms of the excipients used thus providing possible sources of variation between such formulations which may affect the release of ERY and consequently impact on the drugs' bioavailability (BA) and the bioequivalence (BE) between products. In addition, there are several multisource topical ERY preparations (See Table 1.2) thereby adding even greater possibility of intra-product variation amongst these formulations.

Table 1.2 Commercially available topical preparations containing ERY used to treat acne

Company	Country	Trade name	Strength	Dosage form	Combination compounds
Abbott Laboratories	U.S.A.	Eryderm topical solution	2 %	Solution	-
Alpharma	U.S.A.	Erythromycin- 2 % topical solution	2 %	Solution	-
Bausch & Lomb Pharmaceuticals	U.S.A.	Erythromycin -2 % topical solution	2 %	Solution	-
Boots Healthcare	Belgium	Aknemycin	-	Emulsion	Ichthammol
Dermapharm	Germany	Inderm	2 %	Gel	-
Dermik Laboratories	U.S.A.	Benzamycin	3 %	Gel	Benzoyl peroxide
Healthpoint	U.S.A.	Akne-Mycin	2 %	Ointment	-
Dura Pharmaceuticals	U.S.A.	Emgel	2 %	Gel	-
Trima Ltd.	U.S.A	Acnetrim	2 %	Solution	-
Fougera	U.S.A.	Erythromycin- 2% topical gel	2 %	Solution/Gel	-
Galderma	Australia	Eryacne	-	Gel	-
Galderma	Canada	Sans-Acne	2 %	Solution	-
Abbott Laboratories	India	Erythrocin-Neomycin	1 %	Ointment	Neomycin sulfate
Olvos	Greece	Acne Hermal	-	Solution	-
Olvos	Greece	Acne Hermal	-	Emulsion	Ichthammol
Hermal	Germany	Aknemycin	-	Ointment/Solution	-
Hermal	Germany	Aknemycin	-	Solution	Tretinoin
Medicis Pharmaceutical	U.S.A.	A/T/S	2 %	Solution/Gel	-
Merz	U.S.A.	Erygel	2 %	Gel	-
Merz	U.S.A.	Erymax	2 %	Solution	-
Morton Grove Pharmaceuticals	U.S.A.	Erythromycin-2 % -topical solution	2 %	Solution	-
Ortho-McNeil Pharmaceutical	U.S.A.	Erycette	2 %	Solution/Pledgets	-
Paddock Labs	U.S.A.	Erythra-Derm	2 %	Solution	-
Paddock Labs	U.S.A.	ETS-2 %	2 %	Solution	-
Pharmafair	U.S.A.	Erythromycin-15 mg/ml topical solution	1.5 % or 2 %	Solution	-
Pliva	U.S.A.	Erythromycin-5 mg/g topical ointment	0.5 %	Ointment	-
Reckitt Benckiser	Germany	Aknemycin plus	4 %	Solution	Tretinoin
Sankyo	Venezuela	Inderm	2 %	Gel	-
Schein Pharmaceutical	U.S.A.	Erythromycin -2 % topical solution	2 %	Solution	-
Stiefel	Germany	Stiemycin	2 %	Solution	-
Stiefel	Canada	Erysol	2 %	Solution	-
Syosset Labs	U.S.A.	E-Solve 2	2%	Solution	-
Westwood-Squibb Pharmaceuticals	U.S.A.	Staticin	1.5 %	Solution/Lotion	-
Westwood-Squibb Pharmaceuticals	Canada	T-stat	2 %	Solution/Pledgets	-
Will-Pharma	Belgium	Inderm	2 %	Gel	-

1.6 ASSESSMENT OF THE SAFETY AND EFFICACY OF GENERIC ERY TOPICAL PRODUCTS

In the early 1980's, several studies were conducted to compare the safety and efficacy of topical ERY formulations with other treatment regimes proven to be effective in the treatment of acne. The studies also looked at topical ERY formulations which contained synergistic compounds to evaluate the added efficacy of such formulations.

In a study conducted in 1980 [43], the effects of a 1.5 % topical solution of ERY were compared with those of its vehicle in a twelve week, double-blind study involving 26 patients. A statistically significant difference between the responses to the two treatments was seen in both lesion counts and overall evaluations. The final reductions in the mean number of papules and pustules in the ERY group were 70.8 and 77.6 % of the initial values, respectively, and the overall evaluations of this group showed that 91.7 % of the patients had achieved good or excellent results. A group of fourteen patients continued therapy with the ERY solution for an additional nine months. Effective control of their acne was maintained, and no serious side effects were observed

In another study, also conducted also in 1980 [44], 253 patients with moderate to severe acne vulgaris were treated with either a 1.5 % topical ERY solution (n = 127) or with the vehicle (n = 126) . The preparations were applied twice daily for 12 weeks. The reduction in the number of inflammatory lesions, papules, and pustules was significantly greater ($p < 0.01$) in the ERY-treated group. No serious adverse effects were encountered.

A further double-blind controlled study conducted in 1980 [45] involved an investigation of the effectiveness of a 4% topical ERY solution with 1.2% zinc acetate and 4% topical ERY gel with 1.2% zinc octoate. The formulations were applied twice daily in comparison with a 250 mg dose of oral tetracycline twice daily and placebo. Analysis of the reduction in the acne severity grade and papule count showed that the ERY/zinc liquid and gel were statistically significantly better than placebo and as effective as the oral tetracycline. Analysis of comedo grades showed that at weeks 8 and 10 the 4% topical ERY/zinc liquid showed a reduction statistically better than placebo.

1.7 SUMMARY

It has already been shown that several different pharmaceutical companies have produced generic topical ERY formulations following the innovator Eli Lilly. This has given rise to multisource topical preparations containing ERY. Subsequent development of these formulations has resulted in

the inclusion of compounds with synergistic effects when combined with ERY base. Although many companies produce the same dosage form, they used different excipients and also different proportions of these excipients. All these factors combined may have provided possible sources of intra-product variability resulting in differences in ERY release from the formulation and subsequently differences in BA and BE.

To date, the only possible method for assessing the BA and BE of such topical formulations is *via c*linical studies. These studies tend to be expensive, lengthy and time consuming. This implies that the development of a suitable surrogate method to use in the assessment of BE of topical ERY products would be valuable.

One of the objectives of the current research work was to explore the use of skin stripping as a possible alternative method for the assessment of the BE of topical ERY gel formulations.

CHAPTER 2

PERCUTANEOUS ABSORPTION AND DRUG DELIVERY

2. INTRODUCTION

The skin acts as a boundary which separates the external environment from the internal organs. The epidermis acts as a barrier between the varied conditions of the external surroundings and the controlled internal environment of the living tissues and body fluids. The skin provides physical protection of internal organs and acts as a sensory organ. It controls body temperature and water loss, and functions as a regulatory barrier which controls the movement of substances into and out of the body [46].

The skin has gained increasing favour as a target site for drug delivery as it avoids problems associated with oral drug administration, namely pH and to some extent, enzyme driven drug degradation and hepatic first-pass metabolism [47]. Cutaneous drug administration is not however without its problems. The drug is subjected to the skin's own first-pass metabolic effect. In addition to this, percutaneous absorption is subject to significant variability owing to differences in age, race, sex, site of administration, species, presence or absence of disease and the skin's reservoir capacity for a specific drug [47].

There are numerous diseases which affect different regions of the skin. Any drug used will be required to reach the site of the disease in order to exert its pharmacological activity. Unless it is for a local effect on the surface only, the drug must either pass through the *stratum corneum* (SC) or go through hair follicles or sweat glands to reach its target site. Once in the skin, a lipid-soluble drug will tend to accumulate in lipophilic regions while more water-soluble drugs will tend to enter the blood capillaries and are removed from the skin [48].

2.1 SKIN STRUCTURE

2.1.1 Epidermis

An important function of the epidermis is the generation of the SC. The epidermis is avascular and is sustained by nutrients it receives by diffusion from the underlying dermal capillaries through the basement membrane [49]. The epidermal layer forms as a result of the death of basal cells via a

specialised differentiative process. The epidermis consists of two layers namely the SC and the viable epidermis [49].

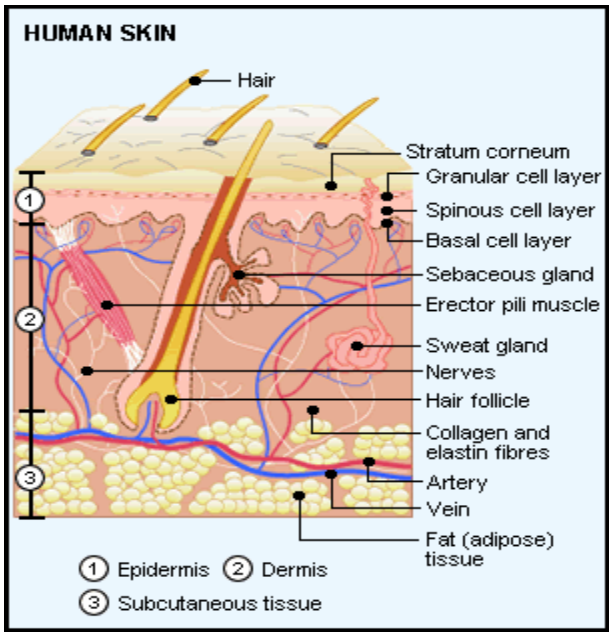


Figure 2.1 Diagrammatic representation of the skin and its structures [50]

2.1.1.1 The Non-viable Epidermis (SC)

This is the outermost layer of the epidermis. It is approximately 10 - 20µm thick and consists of about 15 - 25 layers of flattened, hexagonal and dead cornified cells surrounded by intercellular lipid [48]. The SC thickness varies at different sites of the body, for example, it is thicker in areas such as the palms of the hands and the soles of the feet which are frequently in direct contact with the physical environment. As a result, absorption of compounds through these layers may be slower than in other regions of the body. The SC is a very dense layer of tissue (1.4g/cm^3 in the dry state) [47].

It is believed that most solutes enter the body through the intercellular regions on the SC (0.1 µm wide) which consist of lipids and desmosomes for corneocyte cohesion. The cells of the SC consist mainly of bundled keratins (~70%) and lipid (~20%) [47]. Approximately 15 % of the protein content of the SC consists of enzymes and other non-keratin proteins [51].

The barrier function of the SC is enabled by the continuous desquamation of the non-viable epidermis with a total turnover of the SC occurring once every 2-3 weeks. Highly lipophilic compounds may be less absorbed systemically as they may be sequestered in the horny layer. The

SC prevents loss of internal body components especially water. Disorders of the skin which lead to an increase in the skin turnover, such as psoriasis, will lead to improper SC barrier function [47].

2.1.1.2 The Viable Epidermis

The cells of the SC are generated by the viable epidermis layer of cells. The viable epidermis consists of several cell layers at varying stages of cell differentiation. These layers are the *stratum granulosum*, the *stratum spinosum* and the *stratum basale* or basal layer. The cells of viable epidermis themselves originate from the basal lamina or basement membrane which is located between the dermis and the viable epidermis. Cells located in this layer include melanocytes, Langerhans cells (antigen presenting cells of the skin immune system), Merkel cells (function as sensory receptors on the nervous system) and keratinic cells [47;52].

2.1.2 The Dermis

The dermis is the connective tissue layer which supports the overlying epidermis. It consists mainly of fibrous protein collagen which is secreted by the dermal fibroblasts. The dermis also contains elastin fibres which form a mesh of fine threads, interspaced by collagen, around the hair follicles and sweat glands. In addition to the collagen and elastin tissue, various other substances occur in the dermis. These include mucopolysaccharides and soluble trophocollagen [46]. The dermis contains superficial capillaries, sensory nerves, sebaceous glands, sweat glands and hair follicles [48].

The dermis also houses apocrine and eccrine sweat glands which pass through pores in the epidermis to reach the skin surface, together with hair follicles and sebaceous glands [53].

2.1.3 The Subcutaneous Layer

The subcutaneous tissue is a fatty layer which develops beneath the dermis. It is also called the hypodermis and it is the innermost layer of the skin [53]. Its most important role is to hold the vessels and nerves that supply the skin. It connects to the dermis through the aid of collagen and elastin fibres, and firmly attaches the skin to the underlying muscle tissue [47]. The thickness of this layer is variable and the deeper layers tend to be continuous [54;55].

2.2 PERCUTANEOUS ABSORPTION

After the topical administration of a drug formulation to the skin surface, the drug tends to partition into and diffuse through the lipophilic SC [56]. The drug molecule then moves through the SC into the more aqueous viable epidermis. The drug molecule then moves into the dermis and enters the

systemic circulatory system via the vascular system in the dermis [56]. For a substance to be able to partition into the SC it should be capable of dissolving in the lipophilic SC and have a reasonably balanced affinity for the SC and the aqueous viable epidermal tissue. Generally, ionised chemicals are poor penetrants as they poorly partition into the SC [56].

In order for a drug to be able to penetrate the skin barrier, it must first be released from its formulation into the SC. Due to its barrier function, the SC often is the rate-limiting step in the penetration process [53].

After a drug has been applied to the skin it is possible that there may be a reservoir effect which allows a long-term flux of the drug deep into the dermal tissue [57]. The high concentrations of drug in the SC and low concentrations in deeper dermal layers results in sink conditions.

The purpose of methods utilised to assess skin permeability is to provide information on the quantities of specific substances passing through the SC and entering the underlying tissue. Knowledge of such details concerning percutaneous absorption provides valuable information concerning the bioavailability of drug in the target site [58].

The ideal solute for percutaneous absorption should preferably have a low molecular weight, be non-ionic and have a reasonable degree of lipophilicity with a log *P* value between 1 and 3 as this is thought to be ideal to enhance penetration of the solute through the SC [59-61].

Three pathways of penetration of the SC have been identified and these are transcellular, intercellular and appendageal (primary follicular) these are illustrated in Figure 2.2 below [56].

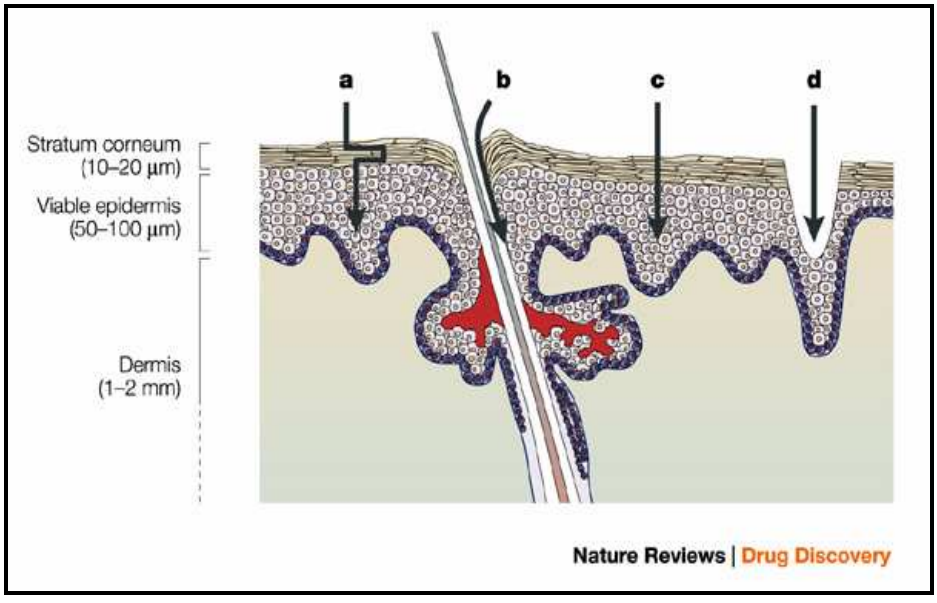


Figure 2.2 The different routes of drug penetration through the skin (a) is the intercellular, (b) and (d) are the appendageal route and (c) is the transcellular route [62].

2.2.1 Appendageal Route

The two main appendages concerned with this type of penetration are the hair follicles and sweat glands. These openings effectively bypass the SC barrier to reach the underlying dermal structures. The role of appendages in transdermal drug penetration has been found to be controversial. For instance, in one particular study, a greater recovery of radio-labelled pesticides in human urine was observed when the pesticide was applied to the scalp as opposed to the forearm (less hairy) [63], while in a different study it was suggested that trans-follicular transport made little contribution to transdermal drug penetration [64]. Early studies using human skin suggested that a follicular or 'shunt' pathway could possibly be important immediately following topical drug application, however, because of its larger surface area, the intercellular pathway becomes dominant after application [65]. As hair follicle density increases the follicular route of drug penetration may become more significant than it has been shown to be in human studies [66]. In humans, the appendageal route is unlikely on account of the limited surface area available [56].

2.2.2 Intercellular

This route of drug absorption appears to predominate for most compounds [56]. It has been suggested that the pharmacological agents applied diffuse through the SC in-between cells following a concentration gradient. That is to say that the drug substances move in the intercellular lipids which exist between keratinocytes in the SC [52]. The rate at which the solute moves through the SC and the skin in this manner is indicated by the diffusion coefficient which considers factors which may hinder the diffusion of the solute such as the tortuosity of the diffusion pathway, the viscosity of the fluids the solute must diffuse through and protein binding of the solute as it moves through the skin [47].

2.2.3 Transcellular

Although the transcellular route makes maximal use of the available surface area for absorption, it is considered to be an unlikely route of administration because of the numerous repetitive partitioning steps of the solute between the several lipophilic and hydrophilic strata in the skin. *In vivo* experiments and morphology studies show that the transcellular route is implausible and that the intercellular path is more likely [56].

2.3 DRUG DELIVERY TO THE SKIN

2.3.1 Physiological Factors Affecting Percutaneous Absorption and Topical Drug Delivery

2.3.1.1 Age

Structural and functional changes occur as the skin ages. It is not clear whether the changes are because of cumulative environmental damage or inherent ageing processes. It has been determined however that the SC seems to be little affected over the normal lifespan of an individual [51]. Studies have shown that the water content of human skin decreases with age [67]. The extent of skin hydration can have a significant impact on the permeation of a drug molecule hence skin ageing could lead to a change in the drug's ability to diffuse through the skin [51].

With age, dermal blood flow tends to decrease thus resulting in a reduction of transdermal drug flux. This factor however is not of major significance for the current research work as it applies more to transdermal drug delivery rather than to topical drug delivery [51]. Ageing was found to not have a significant effect on the permeation of water, oestradiol, caffeine, methyl nicotinate and aspirin and thus it is not expected that the permeation of other similar compounds would be significantly affected by ageing [68-70].

2.3.1.2 Hydration of the Skin

The level of hydration of the SC can have a significant impact on drug permeation. An increase in hydration is known to increase penetration of the drug molecule through the skin for most drugs [51]. This increased penetration of substances is attributed to the softening and swelling of the skin tissue resulting in a 'sponging' effect where pore size is increased hence allowing greater flux of substances through the skin [71]. The use of occlusive dressings and patches or occlusive dosage forms such as ointments have been shown to increase the hydration of the SC and therefore increase percutaneous absorption of drug molecules [51].

The moisture which results in the hydration of the SC originates either from the underlying epidermal tissue of the skin or from perspiration from occluded appendages. The moisture content in normal SC varies between 10 – 25 % of dry tissue weight but this level of moisture content can change in certain disease states thereby affecting the permeation of the skin [48].

2.3.1.3 Race

There are limited literature reports concerning the differences or similarities between races when considering topical drug delivery. Studies which have been conducted on the permeation of benzoic acid, nicotine and aspirin between African, Asian and Europeans skin showed no significant difference between transepidermal water loss amongst these different groups [72;73]. However, there are significant differences in the SC hydration between races suggesting a potential source for variations in inter-racial percutaneous absorption. Due to the limited data available, generalised conclusions cannot be made concerning variations in topical drug delivery between races [51].

2.3.1.4 Body Site

It is obvious that the skin structure varies at different anatomical sites of the body for example, it is much thicker in regions such as that palms of the hands and the soles of the feet than it is at the lips or eyelids [51]. Variations in skin permeation observed at different sites cannot, however, be simply attributed to differences in skin structure at different areas of the body. This point is supported by studies conducted by Wester and Maibach (1989) which showed differences in permeation at different sites of the body which had the same thickness [74].

In addition to differences in skin thickness at different body sites, the density of skin appendages also varies at different areas of the skin and this could further contribute to differences observed in site-to-site permeation [52].

There are some general trends with site-to-site permeability. Generally, the genital tissue is the most permeable and the skin of the head and neck is relatively more permeable than that of the arms and legs [51]. The generalised rank order of site permeability is as follows;

genitalia> head, face and neck > trunk > arm > leg

It is of value to consider variability of permeation at the same site within the same individual. It is estimated that the variation at a body site within the same individual is approximately 30 % while variation at the same site in different individuals is estimated at 40 %. It is clear then that inter-regional variations can exceed these figures [75].

The issue of variation in permeation at different sites is a complex one. Different investigators have reported different rank orders with respect to permeability of the skin at various sites. It has been suggested that skin permeability is a function of the resistance to permeation per unit thickness of the SC at that particular site and the overall skin thickness. For example, the SC

thickness at regions such as the palms may be 400 – 600 µm thick compared to 10 – 20 µm at other body sites. Regardless of the greater thickness of the SC in the palm regions, it has been found that the SC in these areas is less resistant per unit thickness making permeation of a drug lower in such regions but not as low as would be expected when just considering differences in skin thickness [52].

2.3.1.5 Integrity of the Skin

Intact healthy skin forms a barrier difficult to penetrate for many substances as it should be. The occurrence of disease or any form of abrasion or damage which alters the structure of the skin may lead to a modification in the barrier function of the skin. If he skin is inflamed with loss of SC and altered keratinisation then the ability of this organ to prevent absorption of external molecules is compromised and the permeability of the skin is increased. An example of one such disease is psoriasis which results in a defective SC. It has been found that psoriatic skin may absorb as much as twice the amount of 8-methoxypsoralen that is absorbed by uncompromised skin [52;76]. If there is thickening of the skin, for example, through formation of corns, calluses and warts or development of ichthyosis, this leads to a decrease in the permeation of substances through the skin [52].

2.3.1.6 Skin Metabolism

The human skin contains numerous drug-metabolising enzymes. Histochemical and immune-histochemical studies show that most of these enzymes are located in the epidermis, sebaceous glands and hair follicles. Although the enzymes are present at concentrations considerably lower than in the liver, they have been found to exhibit sufficient metabolic activity which may affect the BA of the topically applied medicament. Most of the metabolic reactions which can occur in the skin are oxidation, reduction, hydrolysis, methylation and glucuronidation reactions. The microorganisms which are present in the skin such as *staphylococcus epidermis* may also metabolise topically applied drugs [51]. It has been reported that the skin can metabolise up to 5 % of some of the drugs available for topical treatment, an example being steroidal hormones [52].

2.3.1.7 Other Factors

Since most molecules pass through the skin by diffusion, changes in temperature at the skin surface could result in changes in the skin penetration of the drug. Increase in temperature results in an increase in the diffusion coefficient of the drug molecule ultimately resulting in an increase in the dermal penetration of the drug. The reported temperature of the outer skin surface is approximately

32 ºC and increasing the temperature may induce structural changes within the SC which may increase permeation [51].

In theory, alterations in the peripheral blood flow may affect transdermal absorption of drugs. An increased blood flow could increase the concentration gradient across the skin thereby creating sink conditions which may drive the diffusion process from the skin surface into deeper underlying tissue. In the same way, reducing blood flow to the skin could result in a decrease in the clearance rate of topically applied drugs to the skin. However, the issue of blood flow is more relevant when considering transdermal drug delivery and not topical drug delivery [52].

Generally, rubbing in of the formulation onto the skin will affect the amount of drug absorbed. The longer the period of rubbing and the greater the force used, the greater the absorption of the drug. Another factor that affects percutaneous absorption is the contact period of the formulation. The longer the contact period between the formulation and the skin, the greater the degree of permeation of the drug through the skin. It is important to note that saturation of the skin, changes in hydration of the skin which occur after application of the formulation and changes in the quality/integrity/state of the formulation itself may rule out any significant additional permeation of the drug regardless of how long the formulation remains in contact with the skin [71].

The pH of the skin can have an effect on the ionisation state of a drug molecule. Only unionised molecules can pass through the lipophilic layers of the SC. Thus, the skin pH may alter the ionisation state of weak acids and bases consequently affecting their absorption through the skin [52].

2.3.2 Pharmaceutical Factors Affecting Drug Availability from Topical Formulations

2.3.2.1 Vehicle Composition

Most products applied to the skin for medicinal purposes contain an active drug molecule along with a mixture of inert substances, often called excipients, each with its own unique purpose. Some of these excipients are generally classified as fragrances, co-solvents, preservatives, stabilisers and so on and collectively they are referred to as the vehicle [66].

Absorption of drugs seems to occur best when the drug is in a vehicle which covers the skin easily and mixes readily with sebum to allow the drug to come into direct contact with tissue cells [71].

The pharmacological effect of the drug molecule is determined in part by the ability of the drug molecule to diffuse out of the vehicle and into the skin [66]. The ability of the drug molecule to move out of the formulation is affected by the solubility of the drug in the vehicle and also by the viscosity of the vehicle itself. Highly viscous vehicles will increase the diffusion coefficient of the drug molecule and consequently result in poor penetration of the medicament.

The solubility of the drug in the vehicle will affect the partitioning of the medicament between the vehicle and the targeted skin surface. If the medicament is highly soluble in the vehicle it will tend to partition more favourably into the vehicle resulting in poor penetration into the SC. It is thus important for the vehicle to have a suitable balance in terms of solubility which allows for a formulation with suitable solubility to provide aesthetic appeal while at the same time allowing the drug to partition favourably into the SC lipids in order for the drug to reach its target site [52;77].

Some vehicles have the ability to enhance the hydration of the skin. Oleaginous vehicles occlude the skin surface and thus prevent evaporation of moisture thereby increasing the water content of the SC. This results in an increase in percutaneous absorption of the molecules in the formulation [71].

2.3.2.2 Penetration Enhancers

Penetration enhancers have been reported to work in a number of ways. They may increase the solubility of the drug molecule in the SC intercellular lipids by changing the nature of the SC lipids. Some enhancers alter and denature intercellular keratin in the SC thus causing increased hydration [59;78]. Animal and vegetable oils have been found to facilitate better permeation of drugs than do mineral oils as they penetrate the skin more readily. Organic solvents such as acetone, benzene and propylene glycol (PG) have been found to enhance permeation of molecules dissolved in them due to their ability to penetrate the skin [71].

2.3.2.4 Drug Concentration in the Formulation

According to Fick's First Law of diffusion, the flux of the drug molecule is directly proportional to the concentration gradient of the molecule across the diffusion path (Equation 2.1). It then follows that the amount of drug absorbed per unit area over a specified time interval increases as the drug concentration in the formulation increases [52;71]. Ensuring that the formulation is saturated with the drug will allow for the maximum flux to be achieved in a thermodynamically stable environment [52]. Theoretically, effectively formulated saturated formulations may achieve reproducible percutaneous absorption with drug release kinetics close to zero order kinetics [71].

2.3.3 Physicochemical Properties of the Drug

2.3.3.1 Drug Solubility

Percutaneous absorption is affected to a large extent by the solubility of the medicament in lipophilic media. Generally, the more soluble the drug is in oil the greater the percutaneous absorption. It is, however, important for the drug to possess some degree of solubility in the aqueous phase to allow the drug to penetrate the deeper more hydrophilic layers of the skin which occur beyond the SC. Drugs which have a good balance between lipophilic and hydrophilic solubility tend to achieve higher concentrations in dermal tissue [71].

2.3.3.2 Diffusion Coefficient (D)

The diffusion coefficient (*D*) is used as an indicator of the rate of penetration and degree of resistance to penetration of a molecule through the skin. It encompasses factors such as protein binding of the drug, the tortuosity of the diffusion pathway, some properties of the drug molecule and interactions between the vehicle and the skin. In the skin, the value of *D* decreases as the penetrant reaches deeper more compact layers of the SC [52]. *D* is expressed in Fick's First Law of diffusion:

$$J = -D\frac{dC}{dx} \qquad \text{Equation 2.1}$$

Where;

J = Rate of transfer per unit area of surface (flux)

C = Concentration of diffusing substance

x = Space coordinate measured normal to the section

D = Diffusion coefficient

As indicated by the negative sign on the equation, the flux is in the direction of decreasing concentration. The equation shows that an increase in *D* will result in an increase in the rate of transfer of the drug molecule across the skin and ultimately result in an increase in skin permeation. In biological membranes such as the skin, it is difficult to separate the value of *D* from that of the partition coefficient [52].

2.3.3.3 Partition Coefficient

The partition coefficient (*K*) is a measure of the drug's ability to partition out of the formulation and into the SC. The value of *K* is important in establishing the net movement of drug through the SC. The magnitude of *K* is especially important when the SC is the rate limiting step in the penetration of a drug molecule across the skin. A balanced *K* value is essential in optimising the permeation of substances across the skin. *K* values which are too high are often associated with binding of the drug substances to the structures in the SC and poor penetration of the aqueous layers of the epidermis while low *K* values result in poor partitioning of the drug into the SC.

K is affected by factors such as the drug's solubility in the formulation vehicle, ionisation state of the drug, the drug concentration in the formulation and the drug's own balance between lipophilic and hydrophilic properties [52].

2.3.3.4 Protein Binding

Generally, the more the atoms on a particular molecule, the higher the probability of protein binding through the formation of hydrogen bonds. Thus, the ideal drug molecule should have a small number of atoms to minimise hydrogen bonding. In addition to this, the ideal log P value required for optimal drug penetration through the skin is 2.6. Higher log P values are associated with increased protein binding [60;61].

2.3.3.5 Particle Size and Shape

The ideal molecular weight for optimal drug penetration is considered to be less than or equivalent to 500 Da [60;61]. The molecular weight of a drug molecule is inversely proportional to the flux of the molecule. Smaller drug particles can penetrate the skin faster and with greater ease than larger particles. It is not easy to assess the effect of the particle shape on drug permeation into the skin and thus very little is known about the effect of this parameter on skin penetration [52].

2.4 SUMMARY

The use of percutaneous absorption as a means of delivering a drug molecule either topically or systemically has become increasingly popular. Although it is a route of administration which is riddled with issues of both inter and intra-subject variation, this does not negate the fact that it is a valuable route of administration to use. Amongst its advantages, this route avoids the hepatic first pass metabolism meaning that the BA of the drug substance is improved hence improving the drugs pharmacological efficacy. Being the largest organ in the body, the skin offers a large surface area for use in administering drugs providing formulators a variety of options in creating dosage forms.

Due to the fact that limited concentrations are absorbed systemically for many of the drug molecules applied topically, there is generally little chance of developing toxicity due to overdose.

The degree of variation in terms of percutaneous absorption of topically applied substances does, however, pose a significant challenge in terms of the development of formulations which will ensure sufficient and reproducible cutaneous drug concentrations. There are currently no approved methods available for the assessment of the concentration of non-corticosteroid drug molecules in the skin. Hence, an objective of the current research work was to attempt to develop and optimise a method for assessing the cutaneous BA of ERY in topical gel formulations. Development of such a method would be a valuable step towards ensuring the efficacy, safety and reproducibility of other topical macrolide antibiotics.

CHAPTER 3

FORMULATION OF ERYTHROMYCIN GEL FORMULATIONS

3. INTRODUCTION

The development of an appropriate formulation to deliver any topical therapeutic agent is a complex issue. The composition of the formulation is of particular importance as it may affect the release and consequently the amount of drug which penetrates through the *stratum corneum* into the epidermis/dermis. The formulation can also affect the permeability of the skin and influence the rate of flux of drug across the diffusion path [52].

If the formulation does not alter the structure or function of the skin, the rate of flux of the drug from a saturated formulation will be the same for any vehicle used. However, in an *in vivo* situation, the vehicle usually has some effect on the permeability of the skin, for example, aqueous based formulations usually hydrate the skin and thus affect the permeability of the skin by increasing percutaneous absorption, especially that of hydrophilic compounds [79].

As discussed in Chapter 2, some topical formulations contain additives which are penetration enhancers and through various mechanisms, promote drug penetration through the *stratum corneum*. Aside from these enhancers, some solvents or co-solvent systems incorporated in formulations can themselves act in the same way as a penetration enhancer. An example of one such solvent is propylene glycol. It has been found to increase the solubility of the drug in the skin [80]. Propylene glycol was used in the gel formulations developed for the purposes of the current research work.

ERY has been incorporated in many different vehicle bases in topical formulations (See Table 1.2). Some of the vehicles used are alcoholic solutions, creams, pledgets, ointments and gel formulations. All of these have different advantages and disadvantages which are discussed below.

3.1 TOPICAL FORMULATIONS

3.1.1 Ointments

These are also commonly referred to as oleaginous vehicles. Ointments are considered semi-solid dosage forms and are normally meant to deliver drug products to the skin or to mucosal

membranes. These formulations contain little or no water content and drugs incorporated in them are either in solution or suspension. Ointment based formulations form an occlusive layer on the skin surface which prevents water loss from the skin. This produces the emollient effect observed following topical application of ointments. Ointments are commonly used in the treatment of lesions due to their protective effect [81].

A wide range of medicines have been incorporated in ointment bases and examples of these include anti-acne drugs (sulphur, resorcinol), antibiotics (neomycin, ERY, chlortetracycline), antifungals (benzoic acid, zinc undecenoate), anti-inflammatory agents (betamethasone valerate, triamcinolone actinide), antipruritics (benzocaine, coal tar), antiseptics (zinc oxide), astringents (calamine, zinc oxide), dandruff treatment (salicylic acid), eczema treatment (coal tar, corticosteroids), keratolytics (resorcinol, salicylic acid), protectants (calamine, zinc oxide) and drugs used in the treatment of psoriasis (coal tar, corticosteroids, salicylic acid) [81]. The various different types of ointment bases are classified according to the properties of the base and are described below.

Hydrocarbon bases include all the paraffin bases. These bases are not absorbed by the skin, they are immiscible with water, sticky and almost inert. The main advantage of these bases is that they are readily available and cheap. The disadvantages of using these formulations are that the occlusion of pores and subsequent prevention of water loss may lead to maceration of the skin if the application contact period is prolonged. Also, these formulations result in retention of body heat which may result in an uncomfortable feeling of warmth in patients. These bases do not actively aid absorption of medicaments and this may make them poor candidates when aiming to deliver the medicament to deeper underlying tissues of the skin [81].

Another type of ointment base includes the absorption bases. These are hydrophilic bases which can absorb large amounts of water. They are mainly classified as non-emulsified bases and water-in-oil emulsions, both of which can produce water-in-oil (w/o) emulsions. They are less occlusive than hydrocarbon bases but are also good emollients. The main advantages of these bases are that they facilitate the absorption of oil-soluble medicaments and are ideal for use where the formulation has to mix with biological fluids for example, an eye ointment base. Wool fat and beeswax are examples of absorption bases and are usually combined with other ingredients to produce the ointment formulation. Water-in-oil emulsions can absorb more water than the non-emulsified bases [81].

Further types of ointment bases are the water-soluble bases which are prepared from macrogols. They consist of polyethylene polymeric chains covering a wide range of molecular weights and exist in the form of liquids or solids at room temperature depending on their molecular weights. The advantages of these bases are that they are non-toxic and non-irritating to the skin. They allow for good absorption of medicaments and often lower drug concentrations in the formulation are required to achieve the desired therapeutic effect. They can dissolve a wide range of pharmaceutical products and they are compatible with a wide range of medicaments. The disadvantages include the fact that they are not compatible with some commonly used preservatives such as the phenols, hydorxybenzoates and quaternary ammonium compounds and they are incompatible with certain packaging materials such as polythene and bakelite materials [81].

Generally the preparation of stable, elegant and suitable ointment formulations for topical use is a complex process with several steps and considerations. Furthermore, ointment formulations are not very popular amongst patients for aesthetic reasons and because of the greasiness and discomfort associated with their use.

3.1.2 Alcoholic Solutions

A wide variety of alcoholic solutions of topical ERY are available for use (See Table 1.2). Although these formulations are easy to prepare, the main disadvantage of using them is that some of the alcohols used as the vehicle may irritate and damage the skin surface. Such damage could affect the percutaneous absorption of the medicament. In addition to this, alcohols are rapidly absorbed through the skin and some alcohols have been found to be toxic.

3.1.3 Creams

These are emulsified systems which contain water, that is, most creams are water-in-oil (w/o) emulsions. Emulsions are systems which incorporate two immiscible liquids into a dosage form. The two liquids are usually water and oil-based liquids. The emulsification process results in the dispersion of one of the liquids (the dispersed phase) in the other (the continuous phase). The dispersed phase usually exists as discrete globules or droplets. One of the main disadvantages of a cream is that there is a large surface area of particles dispersed in the continuous phase. This means that there is a lot of interfacial tension in these formulations which may reduce the stability of the formulation [82]. Production of creams is complicated by the need to include several excipients to promote the stability of the formulation. In addition to these disadvantages, owing to the fact that creams contain a significant amount of water, they are susceptible to microbial growth with

subsequent stability implications. Hence, either preservatives must be incorporated or the cream formulation will require to be manufactured under aseptic conditions [48;82].

3.1.4 Gels

Gels are translucent, transparent hydrophilic semi-solid dosage forms. They contain a gelling agent dispersed in water. These gelling agents are usually hydrophilic and swell when they come into contact with water and impart semi-solid properties to such formulations [52]. They are non-greasy and used in topical and other external preparations. They contain a large proportion of water and have a cooling effect. They are easy to apply and the water in the gel evaporates after application to leave a translucent film on the skin. This film usually adheres to the skin quite well and is easily removed by washing with water. Gels are widely used as lubricants and also as topical dosage forms. When preparing a gel formulation, it is important to ensure that the drug is soluble in the gel to ensure even distribution of the active ingredient in the dosage form [48;82].

Gel formulations are stable and although they contain a large proportion of water there is often no need to incorporate preservatives as most gels include alcohol in their formulation. Production of a homogenous stable formulation is a relatively simple process which does not involve a multitude of steps to produce. As illustrated in Table 1.2 (*vide infra* - Chapter 1) some gel formulations of ERY are available. A review of these formulations indicated that the various commercially marketed ERY gels all do not contain the same excipients and gelling agents. For example, aside from ERY and the water, Erygel® contains alcohol (92 %) and hydroxypropyl cellulose only whereas Emgel® contains alcohol (77 %), hydroxypropyl cellulose and propylene glycol [41].

These formulations are both marketed as being effective in the treatment of acne but no information was available on the effect of gel components such as propylene glycol, for example, on the percutaneous absorption of ERY.

Since part of the objectives of this study was to assess the release of ERY from topical dosage forms and in view of the fact that no topical dosage forms of ERY were available on the South African market, gel formulations were chosen to prepare topical dosage forms containing ERY for use in this research project.

As previously discussed, the use of ointments has been found to cause discomfort in patients due to the heat retention properties of these bases. Furthermore, ointments are generally considered to be aesthetically unappealing as they leave a greasy layer on the skin. The use of creams is

complicated by the need to incorporate preservatives during formulation to ensure prevention of microbial growth which may affect the stability of the formulation. Alcoholic solutions have been found to cause damage to the skin surface and may cause itching and irritation. It is for these reasons that gel formulations were chosen instead of the other vehicles mentioned above for the purposes of this study. The use of different percentage content of propylene glycol (PG) in the gels was motivated by formulations for ERY gels which are currently available on the market with little or no PG in them.

3.2 EXCIPIENTS

3.2.1 Gelling Agent

Based on the gel formulations available on various markets around the world, hydroxypropyl cellulose (HPC) was selected as the gelling agent. It is a white to slightly yellow-coloured powder which is odourless and tasteless. Its chemical name is cellulose, 2-hydroxypropyl ether [CAS: 9004-64-2] [83]. It is a non-ionic water-soluble cellulose ether and is a partially substituted polyether of cellulose. It is manufactured by reacting alkali cellulose with propylene oxide at high temperatures and pressures [84].

It is available commercially in many different viscosity grades which are dictated by the intended use for the cellulose. The viscosity grade depends on the molecular weight of the agent used and these vary between 50 000 – 1 250 000 [83]. There are six viscosity grades which are designated by the letters H, M, G, J, L and E in order of decreasing viscosity. The product code combines these letters with the grade designation of the HPC which specifies the intended use of the product.

The melting point range of HPC is 260 – 275 ºC. It is soluble in water (1 in 2 parts), dichloromethane (1 in 10 parts), ethanol (1 in 2.5 parts), methanol (1 in 2 parts) and propylene glycol (1 in 5 parts). HPC has a variety of uses which include its use as a coating agent, thickening agent, viscosity-enhancing agent, encapsulation agent, tablet binder, suspending agent, stabilizing agent emulsifying agent and gelling agent [83;84].

HPC is compatible with substituted phenol derivatives such as methylparaben. Anionic polymers may increase the viscosity of HPC solutions and the stability of the solution may be compromised by high concentrations of inorganic salts in solution [83].

The HPC used was supplied by Klucel ® (Hercules Incorporated, Wilmington, U.S.A.) and two different viscosity grades were investigated, HXF and EXF.

3.2.2 Propylene Glycol

Propylene glycol (PG) is a polyhydric alcohol with a chemical name, (*RS*)-propane-1,2-diol [CAS: 4254-14-2; 4254-15-3]. It has a molecular weight of 76.09, a boiling point range of 184 – 189 ºC and melting point of – 59 ºC. It is a clear, viscous (58.1 cP at 20 ºC), practically odourless, colourless liquid with a sweet taste resembling that of glycerine. PG is miscible with water, ethanol (96 %), acetone, chloroform and glycerine. It is soluble 1 in 6 parts of ether and is immiscible with light mineral oils or fixed oils. PG should be stored in sealed containers, protected from light in a cool dry place [7;83].

PG is incompatible with oxidising agents such as potassium permanganate but is compatible with a number of substances used in topical pharmaceutical and cosmetic products. It is considered non-toxic and this allows for its extensive use in foods, cosmetics and pharmaceuticals. Evidence has shown that PG can penetrate the skin and when applied topically it is regarded as minimally irritant [78]. Some irritation may be experienced when applied to mucous membranes or when used under occlusive conditions [85].

PG can be used for many different purposes, for example, in topical preparations as a humectant, in solutions as a preservative and it is often used as a co-solvent or solvent in aerosol solutions, oral solutions, parenteral solutions and topical solutions [83].

3.2.3 Ethanol

The alcohol used to prepare the gels was ethanol (95 %). Ethanol has a molecular weight of 46.07 and the boiling point is estimated at 78 ºC. It is a clear, volatile, colourless, flammable and hygroscopic liquid. It is miscible with water and methylene chloride. Its relative density ranges between 0.805 – 0812 kg/L [7].

Due to its ability to mix with many organic solvents, ethanol is often used as a co-solvent to promote the solubility of hydrophobic compounds. When incorporated in topical preparations at concentrations higher than 10 %, ethanol can act as a preservative preventing microbial growth and eliminating the need to use additional excipients.

3.4 METHODS

3.4.1 Preparation of Gels

3.4.1.1 Proposed Design

The aim of this experimental work was to prepare two different gel formulations using different percentage contents of PG in order to have different release properties of the included ERY. *In*

vitro microdialysis sampling was used to assess the effect of the different concentrations of PG on the release of ERY from these two gel formulations (See Chapter 6).

3.4.1.2 Preliminary Studies

The initial formula used to produce the gel formulations were sourced from monographs of gels used commercially as well as published data dealing with the preparation of gel formulations using HPC [41;86]. One of the gels, gel 2 (G2), did not contain any PG at all while the other, gel 1 (G1) contained 30 % m/m PG. There was slight crystallisation of ERY which was visible to the naked eye in G2 and a low percentage content of PG was incorporated to enhance the solubility of ERY in the gel. The addition of 10 % PG to G2 resulted in a clear, colourless gel with no crystallisation.

Optimisation of the HPC content was investigated by varying the percentage content of HPC. Two viscosity grades were used initially, HPC EXF and HPC HXF. The EXF grade was a lower viscosity grade and large amounts of the polymer had to be incorporated in order to attain a gel of the desired viscosity. Formulation of the gel using cold water yielded clear, colourless and smooth formulations without lumps. However, addition of such high concentrations (25 %) left a tacky, white film on the skin which as not aesthetically appealing. The HXF viscosity grade resulted in gel formulations which were of suitable viscosity while using very low concentrations of the polymer (3 %). The use of cold water to prepare the gel with HPC HXF resulted in the formation of a lumpy gel due to rapid hydration of the polymer before it was adequately dispersed in the water. To overcome this problem, the water was heated up to 50 ºC before dispersing the HPC to allow the polymer to adequately disperse. Once the gel cooled a clear, colourless, smooth gel was formed with on lumps.

3.4.1.3 Preparation of Gel Formulations

Both gels contained the same excipients and, with the exception of PG and water, the same proportions of these excipients. The water (50 % m/m) was heated to between 45 – 50 ºC and the HPC (3 % m/m) was then gradually dispersed in the water using a magnetic stirrer. Once the dispersion was complete the heat was removed to avoid excessive evaporation of water. While, the water was being heated, the ethanol (15 % m/m) was mixed with the PG using a magnetic stirrer to produce a solvent mixture in which the ERY (2 % m/m) was dissolved. Once the ERY was completely dissolved, the HPC dispersion was added to the ERY solution while still warm and mixed thoroughly until homogenous using a magnetic stirrer. The gel was left at room temperature

(21 ± 3 ºC) to cool and set and to allow the air bubbles produced by the mixing to escape from the gel. Once the gel had set it was stored in the refrigerator at 4 ± 2 ºC and protected from light.

3.4.2 Dosage Form Analysis

3.4.2.1 Drug Content

The drug content tests for both gels were performed over three days using freshly prepared gels on each day. Approximately 7 mg of both gel formulations was dispensed into 50 ml conical flasks using an eppendorf pipette (Eppendorf, Hamburg, Germany) fitted with a 0.2 ml eppendorf combitip (Eppendorf AG, Hamburg, Germany). The 7 mg aliquots of the gels contained approximately 146 µg of ERY according to the manufacturing specifications. Using a pipette, 30 ml of HPLC mobile phase was added to the flask and the mixture was stirred using a magnetic stirrer until the gel dissolved. The flask was sealed with a plug to prevent loss of the solvent *via* evaporation during mixing. The solutions were then transferred to a 2.5 ml HPLC vial and analysed using a validated HPLC analytical method (*vide infra* – Chapter 4).

3.4.2.2 pH

Using about 20 g, the pH of each gel formulation was measured within 24 hours of preparation with a Crison GLP 21pH-meter from Crison Instruments (Lasec, South Africa).

3.4.2.3 Stability Tests

The stability of the ERY gels was assessed by measuring the drug content of the ERY in the two gels each day over a period of one week. For both gels, the stability of the gel when stored on the work bench (~21 ± 3 ºC) and in the refrigerator (~4 ± 2 ºC) was investigated. The samples were protected from light.

3.5 RESULTS AND DISCUSSION

3.5.1 Drug Content

The content of ERY in the gel G1 ranged between 99.4 % and 101.9 % of the expected drug content and in gel G2 it ranged between 99.8 % and 102.3 %. The percentage relative standard deviation (% RSD) 1.12 and 1.80 % for gels G1 and G2 respectively.

3.5.2 pH

The pH of gels G1 and G2 were 6.8 and 6.6, respectively.

3.5.3 Stability Tests

Both gel formulations which were stored in the refrigerator were found to be stable over the duration of the experiment. There was degradation of the ERY in the gels stored on the work bench at room temperature. Figure 3.1 illustrates the decrease in the amount of ERY (N = 4) in the two gels over a period of 7 days.

The rate of degradation was faster in gel G2 than gel G1. The higher degree of degradation in G2 may be due to the lower pH and higher water content in this gel compared to gel G1. ERY is acid labile and it undergoes acid catalysed hydrolytic degradation at low pH values. The lower the pH the greater the degree of degradation [87].

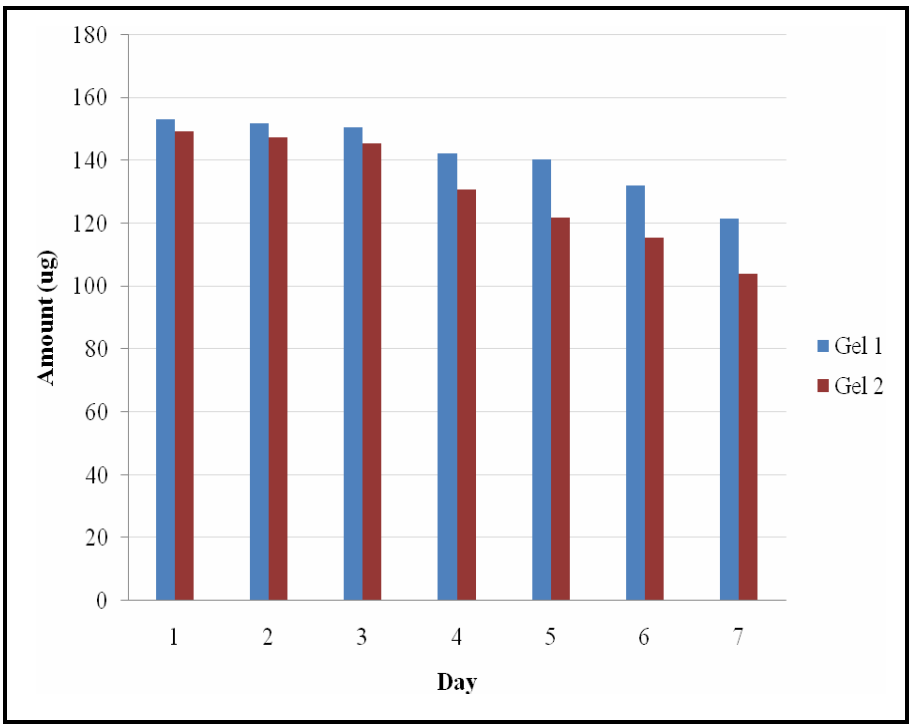


Figure 3.1 ERY gel stability after 1 week of storage at 21 ºC

3.6 CONCLUSIONS

The two different gels were clear, colourless and homogeneous. The latter aspect is an important factor as a lack of homogeneity may produce differences in the percutaneous absorption of ERY from the gels resulting from increased variability of release of ERY. Both gels had the distinct odour of ethanol. When applied to the skin they produced a cooling effect and appeared to somewhat hydrate the skin as evidenced from the soft feel of the skin after application. They left a translucent film on the skin once the water had evaporated and were considered to be aesthetically acceptable.

The stability tests showed that the ERY gels must be stored in a refrigerator at 4 ± 2 ºC and this is an important factor which needed to be considered during the *in vitro* release studies (*vide infra* – Chapter 6) and *in vivo* percutaneous absorption studies (*vide infra* – Chapter 5).

CHAPTER 4

ANALYTICAL METHOD DEVELOPMENT AND VALIDATION

Analysis of Macrolide Antibiotics by High Performance Liquid Chromatography (HPLC)

4. INTRODUCTION

Following successful method development, the analytical procedure must be validated.
The objective of validating an analytical procedure is to demonstrate that it is suitable for its intended purpose [88]. The analytical method which was developed was based on a combination of HPLC methods found in literature. Various methods that have previously been reported were considered and are listed in Table 4.1 below. The various parameters and operating conditions such as mobile phase, type of column and column temperature, method of detection and detection settings, amongst others, were investigated. In view of the fact that ERY does not possess a suitable chromophore to facilitate the optimal use of UV detection of column eluate, an electrochemical method of detection (ECD) was chosen in order to obtain acceptable sensitivity and selectivity for the quantitative analysis of ERY.

4.1 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC has rapidly gained popularity and become the analytical method of choice for quantitative analysis of many compounds. This can be attributed to the fact that HPLC provides rapid high resolution of compounds resulting in an efficient method of analysis which can be completed over relatively short periods of time [89]. In addition to this, coupling HPLC to suitable detection methods often provides increased sensitivity and selectivity to facilitate accurate, precise and reproducible analysis of pharmaceutical products [89]. The use of HPLC often allows greater reproducibility over other methods since operator dependence is reduced and closer control of the parameters affecting HPLC can be ensured [90-92].

In addition to HPLC, various other analytical methods for the analysis of macrolide compounds, such as microbiological assays [99], thin-layer chromatography (TLC) [100;101], gas chromatography(GC) [14], and capillary zone electrophoresis procedures (CZE) [3;102;103].

Table 4.1 Summary of analytical methods used to determine ERY

Column	Matrix	Mobile Phase Composition	Detection	Reference
RSil C_{18} LL, 10 µm (250 x 4.6 mm I.D.)	Solution	acetonitrile/TBA(pH 6.5, 0.2 M)/ phosphate buffer (pH 6.5, 0.2 M)/water (42.5/5/5/47.5)	UV – 215 nm	[16]
Inertsil ODS-2 (50 x 3.0 mm I.D.)	Plasma	water/acetonitrile (50/50) with 2 mM ammonium acetate and 0.1 % acetic acid, 0.7 ml/min.	MSMS/MRM-Positive mode, source temperature 500ºC, cone voltage 60 V, collision energy 12 V	[93]
Symmetry Shield RP8, 5 µm (150 x 3.9 mm I.D.)	Animal stock feeds	Gradient flow with binary mobile phase; Solvent A: phosphate buffer 1 M pH 6.3/water/acetonitrile (12.5/587.5/150) Solvent B: phosphate buffer 1 M pH 6.3/water/acetonitrile (12.5/237.5/600), 0.8 ml/min.	ESA coulometric detector Screening electrode: + 0.8 V Working electrode: + 0.85 V	[94]
Zorbax SB CN,5 µm (150 x 4.6 mm I.D.)	Plasma	50 mM sodium phosphate/acetonitrile/methanol (450/300/50, v/v/v) Apparent pH: 6.6 – 8.0, 1.0 ml/min.	ESA Coulochem II detector Guard cell: +1.0 V Screening electrode: + 0.5 – 0.6 V Working electrode: + 0.8 – 0.9 V	[95]
Xterra RP_{18}, 5 µm (250 x 4.6 mm I.D.)	Gel	0.2 M phosphate buffer, pH7.0/ acetonitrile/water (5/35/60, v/v/v), 1.0 ml/min.	UV- 215 nm	[96]
ODS column, 5 µm (250 x 2.0 mm I.D.)	Plasma	Acetonitrile/Water containing 1.67 mmol/L acetic acid (70/30, v/v), 0.2 ml/min.	MS/SIM –Positive mode, source temperature 250ºC, cone voltage 150 V, collision energy 30 V	[97]
Radial-Pak Resolve, 5 µm (100 x 8 mm I.D.)	Biological fluids	ammonium acetate buffer (pH 7.0;0.25 M) /acetonitrile/methanol (40/50/10; v/v/v), 1 ml/min.	ESA coulometric detector Guard cell: + 0.95 V Screening electrode: + 0.65 V Working electrode: + 0.85 V	[98]

HPLC is based on the separation of components of a mixture by virtue of differences in the equilibrium distribution of the components between a stationary and a mobile phase [92;104]. The compounds partition between the different phases based on their physicochemical properties and affinity for either of the phases and this results in compounds eluting at different times and the consequent separation of the compounds [89;92]. HPLC is ideally suited for the separation of a wide range of pharmaceuticals, food substances, industrial materials, bio-chemicals and biological fluids [104].

The use of HPLC is not without its limitations and some of the concerns associated with the use of this method include the high capital costs of equipment, cost of HPLC columns, need for high purity analytical grade HPLC solvents which are generally expensive. The use of HPLC results in large volumes of organic solvent waste which is not only expensive to dispose of but also has implications for the environment [89]. In many cases however, the advantages of using HPLC over any other method out-weigh the disadvantages [90;104].

Several methods of detection have been used in conjunction with HPLC such as fluorescence [105], UV detection [14;15], ECD [106-108] and liquid chromatography mass spectrometry (LC-MS-MS) [14;109]. As previously mentioned, ERY lacks a good light absorbing chromophore making it a poor candidate for photometric detection [109]. The use of fluorescence with macrolides requires pre-column derivatization with an excitation agent prior to analysis to achieve sensitive assay conditions [105]. This derivatization process is time consuming and is not necessary if other detection methods are chosen. Recent studies have shown that sensitive assays are possible when ECD and LC-MS-MS detection methods are used in conjunction with HPLC [14;93-95;110].

Most analytical methods found in the literature deal with analysis of ERY in biological fluids [14;95;106;109], raw materials [16;109;111] and dosage forms [96;112;113] but no mention has yet been made about the assay of ERY in *in vitro* and *in vivo* microdialysis studies. One of the objectives of this research project was to develop and validate a suitably sensitive and selective analytical method which has the requisite sensitivity, accuracy and precision for the analysis of samples obtained from *in vivo* and *in vitro* microdialysis studies.

4.1.1 Column Efficiency

This is an indicator of the performance of a column and its ability to separate compounds [89;90;114]. Column efficiency is measured by the number of theoretical plates, *N*, or by Height Equivalent to a Theoretic Plate (HETP or H) [7].

The relationship between *N* and H is illustrated in Equation 4.1:

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

Where,

N = Theoretic plate number

L = Length of column packing (cm)

H = Height equivalent of theoretical plate (cm)

For Gaussian peaks, the theoretic plate number can also be defined by either of the following equations [5]:

$$N = 16 \left(\frac{Rt}{W} \right)^2 \qquad \text{Equation 4.2}$$

$$N = 5.54 \left(\frac{Rt}{W_{h/2}} \right) \qquad \text{Equation 4.3}$$

Where,

W = Width of the peak at its base

$W_{h/2}$ = Width at half of the peak height

Rt = Retention time of the substance

The theoretical plate model implies that the HPLC column is subdivide into a number of theoretical plates or discs [7]. In each plate, equilibrium of the movement of analyte between the mobile phase and stationary phase exists. The theoretic plate model assumes that the substance being analysed moves down the column from one plate to the next [7;17]. It then follows that the greater the number of plates the more efficient the column [89].

The use of the theoretical plate model is however limited by the fact that the model is based on gaussian peak shape and not all chromatographic peaks display this form. This means the model does not consider variables which cause peak tailing and shouldering and hence result in the formation of non-gaussian peaks. This suggests that the calculated value for *N* may not be accurate. It is for this reason that the use of *N* is limited to describing column efficacy and not to describe the behaviour of chromatographic peaks. The rate or kinetic theory is used for the later instead. Ideally *N* should be greater than 2000 [114].

4.1.2 Capacity Factor

The capacity factor, $k\alpha$, can be used to describe the migration factor of an analyte through a column [7]. It gives a measure of the location of the peak of interest with respect to the void volume [114]. Migration of component molecules of a mixture will occur only when the molecules are in the mobile phase. The $k\alpha$ is a measure of the degree with which a compound partitions into

the stationary phase from the mobile phase [89;104]. The greater the $k\alpha$ the greater the retention time [89]. The $k\alpha$ can be described by the following equation:

$$k\alpha = \frac{(Rt-t_O)}{t_O} \qquad \text{Equation 4.4}$$

Where,

$k\alpha$ = Capacity factor

Rt = Retention time of the analyte

t_O = Elution time of the void volume (dead time)

Substances with $k\alpha < 1$ elute too soon [91]. The recommendation is that the peak for the analyte of choice should be well resolved from other peaks and the void volume and a value for the capacity factor which is above 2 is considered acceptable [114].

4.1.3 Selectivity

The selectivity factor, α, is a measure of the efficiency of a column to separate peaks [89]. The selectivity of the analytical method is shown by proving that extraneous peaks do not interfere with the analyte peak [17]. All components in the mixture must be well resolved at the baseline with or without stress testing [7]. The selectivity factor can be defined as:

$$\alpha = k'_B / k'_A \qquad \text{Equation 4.5}$$

Where,

α = Selectivity factor

$k\alpha_B$ = capacity factor for the strongly retained species

$k\square_A$ = capacity factor for the more rapidly eluting solute

An $\alpha > 1$ is the fundamental requirement for separation in chromatographic techniques [91] and it is necessary to provide chromatograms to demonstrate selectivity especially in the presence of co-eluting peaks. Determination of selectivity is not essential as long as the resolution falls within the acceptable range.

4.1.4 Resolution

Resolution, *R*, is a measure of the extent of separation of two peaks [7]. Well-separated peaks are essential for reliable and accurate quantitation [114]. Resolution is defined as:

$$R = \frac{(Rt_2 - Rt_1)}{(1/2)(t_{w1} + t_{w2})} \qquad \text{Equation 4.6}$$

Where,

R = Resolution

Rt_1 = Retention time of first eluting peak

Rt_2 = Retention time of second eluting peak

t_{w1} = Width of first eluting peak at the base

t_{w2} = Width of second eluting peak at the base

Values above 2 for resolution between two peaks are desirable [114]. Values falling below 1.5 indicate incomplete separation of peaks [17;114].

4.2 METHODS

4.2.1 Method Development

4.2.1.1 Reagents and Chemicals

ERY and clarithromycin (CLM) were obtained from USP Reference Standards (Rockville, USA). HPLC grade acetonitrile (UV 200) and methanol (UV 215) ROMIL - SpS™ Super Purity Solvents were obtained from Romil Ltd (Waterbeach, Cambridge, UK). Potassium dihydrogen-orthophosphate AR was obtained from Associated Chemical Enterprises (Pty) Ltd (Theta, Johannesburg, South Africa). Tetrabutylammonium hydrogen sulphate (TBA) was obtained from the Sigma® Chemical Company (St Loius, USA) and potassium hydroxide pellets C.P. were obtained from Holpro Chemical Corp (Pty) Ltd (Port Elizabeth, South Africa). The water used for chromatography was initially purified by reverse osmosis followed by filtration through a Milli-Q system (Millipore, Bedford, MA, USA). The water purification system consisted of a Milli-Q® Academic A10 with a Quantum™ EX Ultrapure Organex Cartridge equipped with Q-Gard® 1 Progard pre-treatment packs.

4.2.1.2 Instrumentation and Chromatographic conditions

The experiments were performed on an analytical system which comprised of a solvent degasser (Model ERC-3710, ERMA Optical Works Ltd, Tokyo, Japan), a precision isocratic pump (Model SP8810-020, Spectra-Physics, San Jose, California, U.S.A.) connected to a fixed loop autosampler (Model AS1000 Spectra Series, SP Thermo Separation Products, San Jose, California, U. S. A.), a column heater (Model TC931, HPLC Technology, Macclesfield, U.K.), an ESA Coulochem® electrochemical detector (Model 5100A, ESA Inc, Massachusetts, U.S.A.) connected to a guard cell (Coulochem Model ESA 5020) and a dual electrode analytical cell (Coulochem Model ESA 5010). The detector output was recorded on a chart recorder (Model 561, Perkin Elmer, Hitachi Ltd, Tokyo, Japan). The column temperature was maintained at 35 $\pm$ 2 ºC and the injection volume was 20 µl. Samples were placed in glass HPLC vials (Waters Cooperation, Milford, Massachusetts, USA).

4.2.1.3 Preparation of Standard Solutions

The stock solutions of ERY and CLM (0.5 mg/ml each) were prepared by dissolving 5 mg of powder accurately weighed on an analytical balance (Mettler Toledo Model MX5, Uznach, Switzerland) in 10 ml of mobile phase (acetonitrile/methanol/phosphate buffer – see below) in a volumetric flask. The stock solution was then diluted to prepare calibration standards over the range 1 – 10 µg/ml. Quality control (QC) samples were similarly prepared from a separate stock solution over the range 3 – 9 µg/ml. The stock solution, calibration standards and QC samples were vortexed for 30 s on a Lab Dancer Vario vortexer (Yellowline, Staufen, Germany). These solutions were prepared on three different days for use in linearity, precision and accuracy experiments and also to determine the limits of detection and quantitation.

4.2.1.4 Preparation of Mobile Phase

HPLC-grade solvents, including HPLC-grade water, were used to prepare the mobile phase. Analytical grade potassium dihydrogen-orthophospate was used to prepare a 0.02 M phosphate buffer adjusted to pH 7.7 using potassium hydroxide pellets. The mobile phase was prepared by adding the organic modifiers, acetonitrile and methanol to the phosphate buffer (48:10:42). Tetrabutylammonium hydrogen sulphate (TBA) was then added to the mobile phase to yield a concentration of 1.8 mM. The mobile phase was then degassed using an Eyela Aspirator A- 2S (Tokyo Rikakikai Co. Ltd, Tokyo, Japan) and filtered through a 0.45 µm HVLP filter paper (Millipore, Bedford, MA, USA).

4.2.1.5 Column Selection

The selection of a suitable column for a HPLC system forms is of primary importance for the development of an appropriate analytical method [115]. The physicochemical properties of the analyte such as the solubility, pKa, lipophilicity and ionic structure, amongst others, influence the choice of column [92].

Reverse phase (RP) chromatography makes use of a non-polar stationary phase coupled with a polar mobile phase as the basis for separating compounds. The stationary phase commonly used comprises of a silica backbone which is covalently bonded to alkyl chains [116]. The alkyl chains retain non-polar hydrophobic compounds while polar hydrophilic solutes are eluted with minimal retention [90;92].

ERY is a slightly water soluble lipophilic compound (Log P 3.1) [12] with a macrocyclic fused ring system which largely contributes to the hydrophobic nature of the analyte. These factors promote the preferential partitioning of ERY from the mobile phase onto the stationary phase favouring the non-polar nature of the alkyl bonded silica backbone to the predominantly hydrophilic mobile phase.

A reversed phase Luna C_{18} (2) (Phenomenex, 411 Madrid Ave., Torrence, C.A., U.S.A.) column was used for the HPLC analysis of ERY. The column has an internal diameter of 4.6 mm and a length of 150 mm and contains 5 μm particles with a pore diameter of 100 Å.

4.2.1.6 Mobile Phase Selection

The selection of a suitable mobile phase for RP columns involves using a system which combines an aqueous phase, usually a buffer, along with an organic modifier, usually acetonitrile and or methanol [14]. Previous studies have shown that ERY and its esters are unstable in methanol [87;117]. For this reason, acetonitrile was chosen as the main organic modifier. Several analytical methods have been described which utilise acetonitrile as the organic modifier resulting in an accurate and precise analytical method (See Table 4.1). The addition of a small proportion of methanol as an additional organic modifier resulted in sharper peaks.

The selection of a suitable buffer depends on the buffering capacity, pKa and the corrosive effect of the buffer salt on the HPLC system [115]. When selecting a buffer, the pKa of the buffer should be near the desired working pH. The potassium dihydrogen phosphate buffer salt was chosen as it has

a pKa of 7.2 and a buffering range between 6.2 and 8.2 [118]. This makes it suitable to use at the chosen working pH of 7.7.

4.2.1.6.1 Selection of Buffer Molarity

The molarity of the buffer can have a profound effect on the retention time (Rt) and peak shape of an analyte especially when the pH of the mobile phase is greater than 5 [90;92]. Above pH 5 the silanol groups of the stationary phase backbone exist predominately in their ionised state [119]. These exposed ionised groups can interact with basic ionic groups via an ion exchange mechanism and the result is drastic peak tailing [120;121]. One way of reducing this effect is to increase the buffer molarity resulting in a reduction of the interaction of cationic analytes with the ionised silanol groups due to increased competition between the cationic analyte and the buffer cations for interaction with the exposed silanol groups [119;121].

Research has shown that when using a phosphate buffer for analysis of macrolide antibiotics, increasing the molarity of the buffer will result in a reduction in retention time [115]. However, this is also coupled by an alteration in the shape of the peak resulting in broader peaks [11;115]. In addition to this, use of higher buffer molarities may result in re-crystallisation problems of the salt which ultimately will adversely affect the analytical system.

Based on the published methods listed in table 4.1 the buffer molarity which was chosen for this work was 20 mM of the potassium dihydrogen orthophosphate salt.

4.2.1.6.2 Selection of Organic Modifier Content

Selection of the appropriate content for the organic modifier depends heavily on the desired run time for the specific analytical procedure. An increase in the content of the organic modifier results in a decrease in Rt [92;119]. Generally a short run time, under 10 minutes, is preferred as it is associated with reduction in cost in terms of maintenance of equipment and chemicals [90].

A mobile phase composition of acetonitrile/buffer (60/40) resulted in a lack of resolution between ERY and CLM with retention times of 4.9 and 5.3 minutes for ERY and CLM respectively (R = 0.9). Reduction of the acetonitrile content to 50 % resulted in improved separation however the peaks were still considerably close with retention times of 7.2 minutes for ERY and 7.8 minutes for CLM (*R* = 1.4). An acetonitrile/ buffer composition of 40/70 resulted in baseline resolution (R =

2.4), however, the retention times were too long, 9.1 and 10.2 minutes for ERY and CLM respectively. In addition, to this the peaks obtained were quite broad.

The addition of methanol (10 %) to the 48/42 v/v (acetonitrile/buffer) mobile phase resulted in an improvement in the peak shape without significantly altering the Rt. Further attempts to improve resolution were carried out by adjusting the pH of the buffer and addition of TBA.

4.2.1.6.3 Selection of Mobile Phase pH

In HPLC, pH can have a profound effect on the Rt and resolution of analytes depending on their physicochemical properties [91]. Increasing the pH for weak acids will result in a reduction of Rt while for weak bases it will result in an increased Rt [92;119]. The increased Rt for weak bases is explained by the fact that at high pH values ionisable compounds exist in their unionised state and as a result partition preferentially into the stationary phase [90]. The use of pH values above 7 enables better separation of erythromycins compared with acidic pH values where ERY is unstable in acidic media [15].

Increasing pH in the mobile phase has also been found to adversely affect the shape of the macrolide peaks and also results in broader peaks [115].

At pH 7 the Rt for ERY was 7.2 minutes and 7.8 minutes for CLM. At this pH, the peaks were just barely resolved at the baseline (R = 1.4). Increasing the pH to 7.4 yielded a Rt of 7.7 and 8.8 minutes for ERY and CLM respectively. The baseline resolution was still not satisfactory (R = 1.48) and the pH was further increased to 7.7 which yielded Rt of 8.2 and 9.7 minutes for ERY and CLM respectively. At this pH the peaks were well resolved (R = 2.1) however the increase in pH resulted in slightly broader peaks. An organic salt, TBA, was used to improve peak.

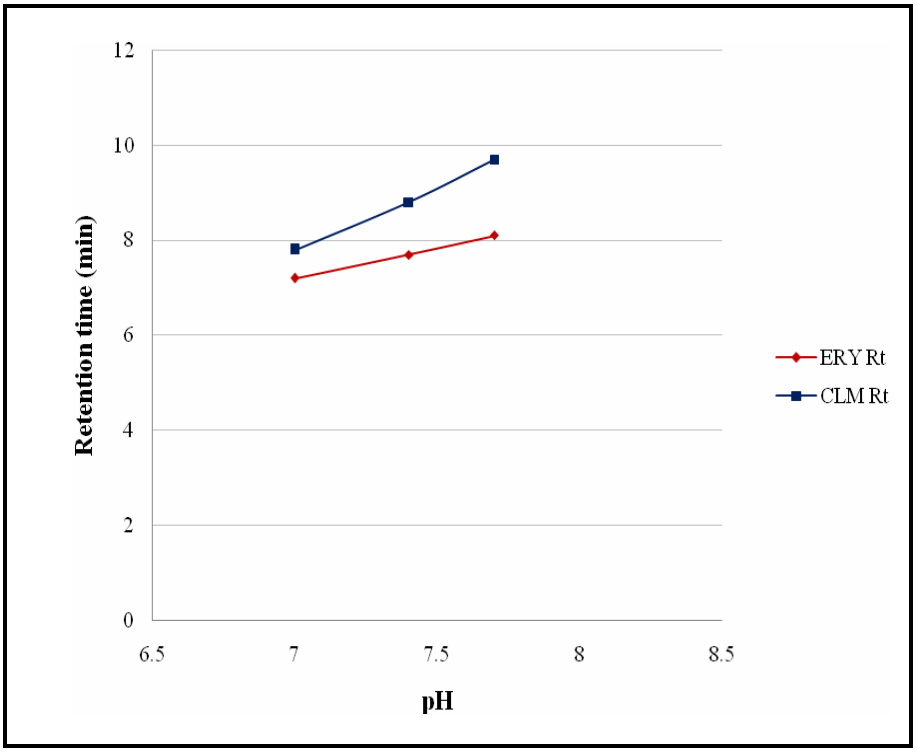


Figure 4.1: pH dependent retention time profile for ERY

4.2.1.6.4 Addition of TBA

Quaternary ammonium compounds such as TBA have been used successfully to improve peak shape and to reduce the Rt of basic compounds [15;16;122]. These compounds act as silanol-blocking agents and weaken interactions between basic groups and the silanol groups on the stationary phase backbone. The result of these reduced interactions is improved peak symmetry [122].

In pH values close to neutral, TBA exists as positively charged ions which can form a layer on the stationary phase causing repulsion of the basic ERY molecules and hence reduce the Rt [16;122].

The addition of TBA resulted in reduced Rt and improved peak shape. Addition of 10.5 mM [15] TBA resulted in the peaks being eluted in the solvent front. Reduction of the amount of TBA to 0.55 mM resulted in Rt's of 5.4 minutes for ERY and 10.6 minutes for CLM. The TBA content was increased to 2.1 mM to bring the retention time of CLM under 10 minutes. The resultant retention times were 4.3 and 7.2 minutes for ERY and CLM respectively where the ERY peak ran too close to the solvent front. Reduction of the concentration of TBA to 1.8 mM resulted in the Rt's of 4.8 minutes for ERY and 8.2 minutes for CLM. These respective peaks were sharp and well-resolved.

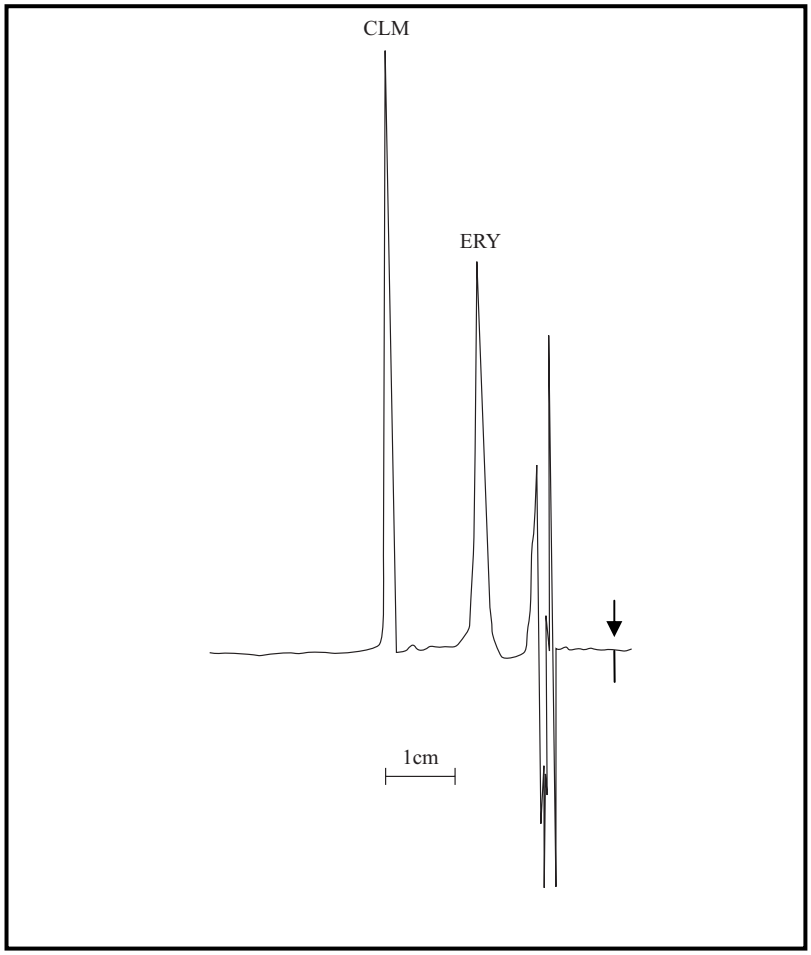


Figure 4.2: Typical chromatogram showing ERY and CLM peaks.

4.2.1.7 Detection Method

An ESA Coulochem® Model 5100A detector connected to an ESA Model 5020 guard cell and an ESA Model 5010 dual electrode cell was used. The potentials of the analytical cell electrodes were set at +0.4 V and +0.9 V for the screening and working electrodes respectively. The gain on the working electrode was set at 10 x 40. The guard cell potential was set at +1.00 V.

The optimal conditions for the applied potentials for both the electrodes of the analytical cell were determined by means of a cyclic voltammogram (Figure 4.3).

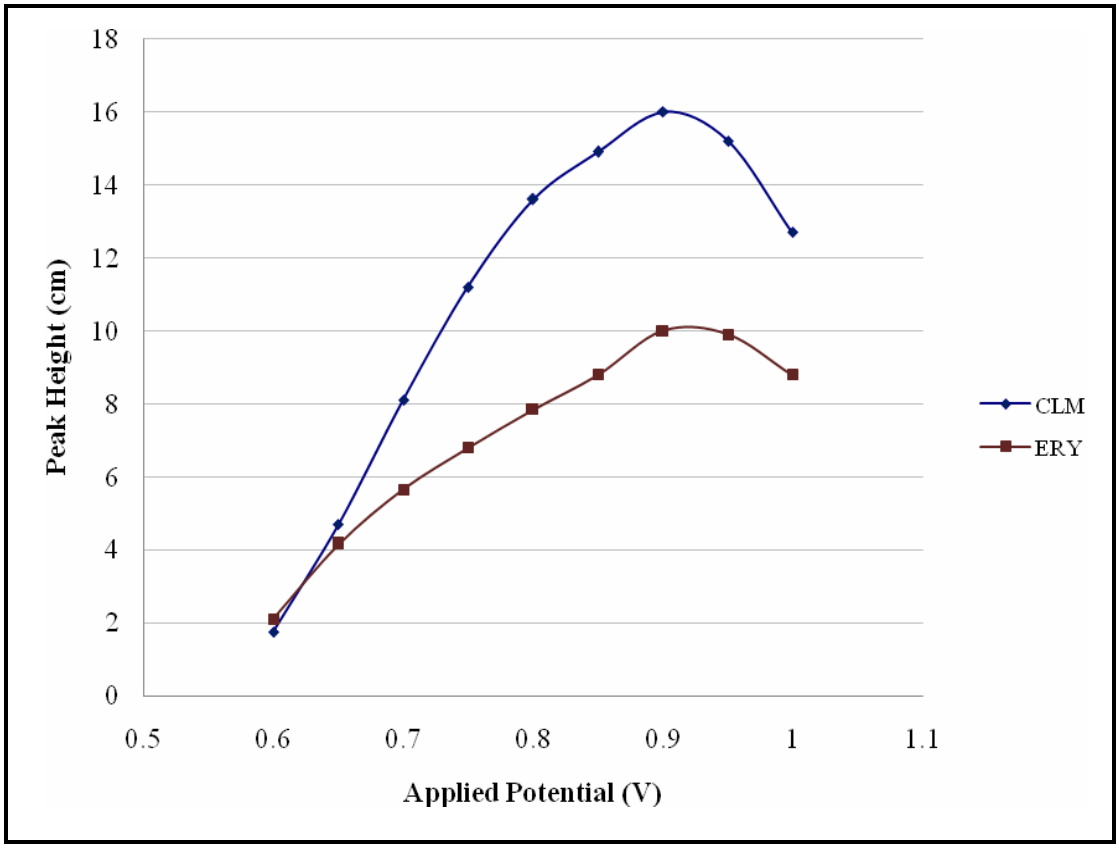


Figure 4.3 Cyclic voltammograms for ERY and CLM.

4.2.1.8 Chromatographic Conditions

Table 4.2 Chromatographic conditions

Column	Phenomenex ® Luna C_{18} (2) column Length 150 mm Internal diameter 4.6 mm Particle size 5 µm Pore diameter 100 Å
Detector	ESA Coulochem® Model 5100A detector
Upstream electrode applied potential	+0.40 V
Working electrode applied potential	+0.90 V
Pump	Spectra Series P100 isocratic pump, TSP Thermo
Injector	Separation Products Spectra Systems AS1000 autosampler
Recorder	Linear Instruments chart recorder
Flow rate	1.0 ml/min
Injection volume	5 µl
Temperature	35 ± 2 °C
Mobile phase	20 mM potassium dihydrogen phosphate (pH 7.7)/ acetonitrile/methanol (42/48/10) with 1.8 mM TBA
ERY retention time	ca 4.8 minutes
CLM retention time	ca 8.2 minutes
Column pressure	1100 psi

4.2.2 Stability Studies

The stability of ERY (n = 3) in normal saline (7 µg/ml) was assessed after one week of storage on the work bench (~21 ± 3 ºC), in the autosampler (~21 ± 3 ºC) and in the refrigerator (~4 ± 2 ºC). The samples were protected from light.

4.3 RESULTS AND DISCUSSION

4.3.1 Assay Validation

4.3.1.1 Accuracy and Bias

Accuracy is a measure of the closeness of agreement between the experimentally determined value and the true value. Accuracy should be established across the specified range of the analytical procedure [114]. According to the recommendations made by the Tripartite International Conference of Harmonisation (ICH) no less than 9 determinations over a minimum of 3 concentration levels covering the specified range should be achieved by means of inferring accuracy [88].

Accuracy should be reported as percentage recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals [88]. Accuracy can also be reported as the % bias which is calculated as below;

$$\text{Percentage bias} = \frac{\text{true value} - \text{measured value}}{\text{true value}} \times 100 \qquad \text{Equation 4.7}$$

According to the Centre for Drug Evaluation and Research (CDER) the acceptance values for % bias should be less than ±5 % [114].

Table 4.3: Accuracy data for the analysis of ERY

Theoretical Concentration (µg/ml)	Calculated Mean Concentration Determined (n=6)	%RSD	% Bias
3	3.03 ± 0.049	1.65	- 1.16
7	7.09 ± 0.035	1.89	- 1.45
9	9.17 ± 0.132	1.44	- 2.48

The bias was found to be less than ± 5% which met the requirements for the maximum allowable deviation and thus complied with the validation requirements for accuracy.

4.3.1.2 Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions [114]. There are three levels to precision; repeatability, intermediate precision and reproducibility [88]. Precision is usually expressed as the variance, standard deviation or percentage coefficient of variation (CV % or % RSD). According to the CDER [114], % RSD values ≤ 1 are ideal, however values less than 10 % are acceptable. The analytical system was assessed before and during use to ensure that it remained functional over the period of the analysis.

4.3.1.3 Repeatability

Repeatability, also known as intra-day precision, expresses the precision under the same operating conditions over a short period of time [88].

Table 4.4: Intra-day precision data for the analysis of ERY

Theoretical Concentration (µg/ml)	Actual Concentration Determined (µg/ml) (n = 6)	Standard deviation	% RSD
3	2.97	0.032	1.08
7	6.99	0.114	1.63
9	8.93	0.164	1.83

4.3.3.2 Intermediate Precision

Intermediate precision expresses with-in laboratories variations: different days, different analysts, different equipment and so on [114]. Intermediate precision is also termed inter-day precision [88]. Intermediate precision is used to evaluate the effect of random events on the precision of an analytical procedure [114].

Table 4.5: Inter-day precision data for the analysis of ERY

	Theoretical Concentration (µg/ml)	Actual concentration determined (µg/ml) ± SD (n = 6)	% RSD
Day 1	3	2.92 ± 0.047	1.52
	7	6.99 ± 0.166	2.37
	9	8.95 ± 0.238	2.66
Day 2	3	3.06 ± 0.056	1.83
	7	6.98 ± 0.167	2.38
	9	8.93 ± 0.238	2.67
Day 3	3	3.08 ± 0.049	1.61
	7	7.02 ± 0.148	2.10
	9	9.04 ± 0.219	2.42

4.3.3.3 Reproducibility

Reproducibility expresses the precision between laboratories as a means of standardising methodology. If intermediate precision is shown it is not necessary to show reproducibility[88].

4.3.4 Linearity

The linearity of a method is a measure of how well a calibration plot of response against concentration approximates a straight line, or how well the data fit to the linear equation [92];

$$y = mx + c \qquad \text{Equation 4.7}$$

Where;

y = Response

m = Slope

x = Concentration

c = y-intercept of a regression line fit to the data

The linear range of detectability that obeys Beer's Law is dependent on the compound being analysed and detector used [114]. The working sample concentration and samples tested for accuracy should be in the linear range [114]. Ideally a linear relationship (c = 0) is preferred as it is more precise and can be defined with fewer standards [92]. The results of linearity should be evaluated using the calculation of the regression line by the method of least squares [88]. The correlation coefficient (R^2), the y-intercept and the slope of the regression line should be reported. The ideal R^2 value should be more than or equal to 0.998 [88].

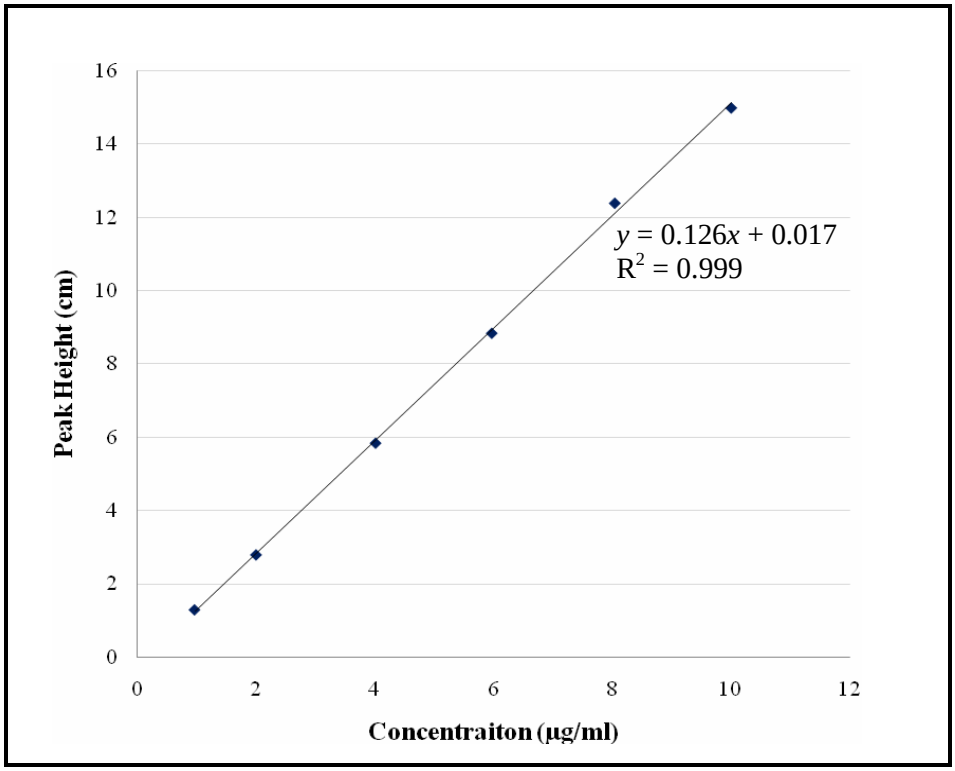


Figure 4.4: Calibration line of the plot of peak height versus ERY concentration

4.3.5 Limit of Quantification (LOQ) and Limit of Detection (LOD)

The LOD is the minimum amount of analyte that can be reliably detected but not necessarily quantitated under the stated experimental conditions [92;114]. The LOQ is the lowest amount of analyte which can be quantitatively determined with a suitable degree of precision and accuracy [88]. The LOQ need should be validated using an analyte sample with a concentration at or near the LOQ [88].

There are several approaches to determining the LOD and LOQ depending whether the procedure is non-instrumental or instrumental [88].

4.3.5.1 Visual Evaluation

Visual evaluation may be used for non-instrumental or instrumental methods [88]. The LOD or LOQ may be determined by the analysis of samples with known concentrations of analyte and establishing the minimum level at which the analyte can be reliably detected or quantitated with acceptable accuracy and precision [123;124].

4.3.5.2 Signal-to-Noise ratio

This approach is recommended for application only to analytical procedures that exhibit baseline noise. Determination of signal-to-noise (S/N) may be performed by comparing measure signals from samples with known low concentrations of analyte with those of blank samples, and establishing the minimum concentration at which the analyte can be reliably detected or quantitated [88]. A S/N ratio of between 3 or 2:1 is generally recommended for establishing the detection limit and a S/N ratio of 10:1 is recommended for establishing the quantitation limit [114].

4.3.5.3 Standard Deviation of the Response and the Slope

It is recommended that the LOD and LOQ be expressed as [124];

$$LOD = 3.3\,\delta / S \qquad \text{Equation 4.8}$$

$$LOQ = 10\,\delta / S \qquad \text{Equation 4.9}$$

Where;

δ = Standard deviation of responses

S = Slope of the calibration curve

The slope (S) may be estimated from the calibration curve of the analyte [124;125]. The estimate of δ may be carried out in a variety of ways [124;126]. One way is based on the standard deviation of the blank which measures the magnitude of analytical background noise of a number of samples and calculates the standard deviation of these responses [126]. The other method is to use calibration curve samples in the range of the LOD and LOQ and use the residual standard deviation of the regression line or use the standard deviation of the y-intercepts of regression lines as the standard deviation [126].

The LOD and LOQ quoted in this research were determined using the Tripartite ICH guidelines on method validation based on the use of the S/N ratio. The LOD = 0.05 µg/ml (S/N = 3.1) and the LOQ = 1.0 µg/ml (S/N = 10.4).

4.3.6 Stability Studies

ERY was found to be stable on the workbench (21 ºC), in the autosampler (21 ºC) and in the refrigerator (4 ºC) over a period of one week and the assay values were greater than 99 % in all cases.

4.4 CONCLUSIONS

The analytical method was validated in accordance with the recommendations of the US Food and Drug Administration (F.D.A.) and the International Conference on Harmonisation (I.C.H.) and complied with those acceptance criteria and specifications.

The described HPLC method is rapid, accurate, precise and reproducible and it provides an effective method for use in the quantitative determination of ERY. Baseline separation was achieved within ten minutes and the use of ECD allowed for high specificity and good resolution between the ERY peak and that of the internal standard, CLM.

CHAPTER 5

MICRODIALYSIS

5. INTRODUCTION

The use of microdialysis (MD) in assessing dermal drug delivery has been receiving increasing attention over the years. MD is a sampling technique which allows quantitative and qualitative analysis of endogenous and exogenous substances found in extracellular fluids [127]. MD was originally used in neuroscience to measure the release of neurotransmitters in the animal brain [128;129] but its use as an analytical technique has spread to various different fields such as DPK.

MD is a probe-based sampling technique which allows for continuous sampling of unbound analyte at its target site. For this reason, MD was acknowledged by the United States FDA advisory committee as an attractive technique to use in studying tissue concentrations of substances in the extracellular fluid (ECF) [130;131].
A diagrammatic illustration of the *in vivo* cutaneous MD sampling technique is shown in Figure 5.1.

MD is based on the principle of passive diffusion of an analyte down a concentration gradient across a semi-permeable membrane. The probe is placed in ECF) and a liquid referred to as the perfusate is pumped through it. The unbound substances which are in the ECF can cross the semi-permeable membrane and diffuse into the liquid, now referred to as the dialysate. The dialysate is collected and analysed using a suitable analytical technique [127;132]. When the technique is applied specifically to measure drug concentrations in the skin it is referred to as cutaneous MD [133].

Due to the fact that there is constant flow of perfusate in the probe, the concentration of the dialysate never reaches equilibrium. The actual concentration of drug in the ECF (higher) will thus be different from the concentration of the dialysate (lower). For this reason the concentration of the analyte in the dialysate is described in terms of the relative recovery which is a ratio between the concentration of drug in the dialysate and the fluid surrounding the probe also called the periprobe fluid (See Equation 5.1) [127].

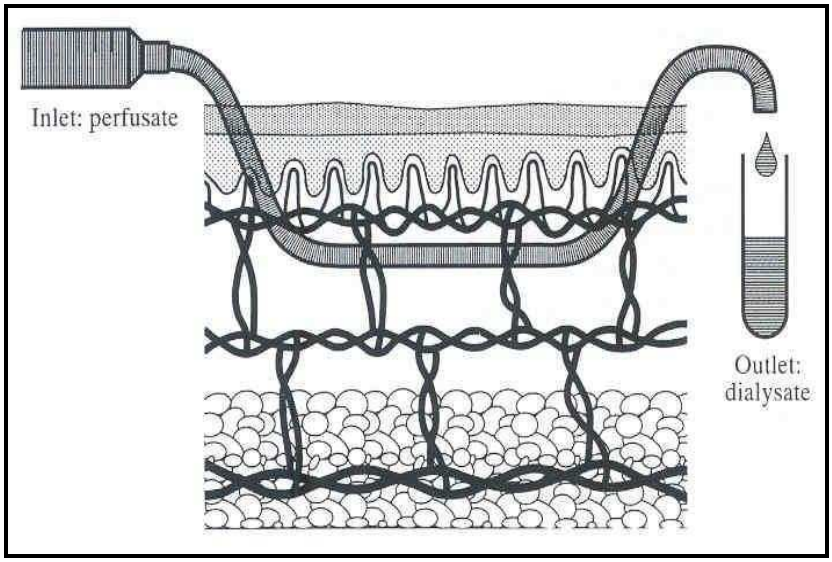


Figure 5.1 Schematic representation of *in vivo* cutaneous MD [134]

The relative recovery is affected by factors such as the perfusate flow rate, the probe material used for the membrane, the length of the membrane, blood flow, temperature, metabolism, depth of the probe in the dermis and factors pertaining to the analyte itself such as molecular weight, lipophilicity, electrical charge and protein binding [127;132].

$$\text{Relative recovery} = \frac{C_d}{C_m} \qquad \text{Equation 5.1}$$

Where;

C_d = Concentration of analyte in the dialysate

C_m = Concentration of analyte in medium surrounding the probe

The main focus of cutaneous MD has been to determine tissue concentrations of substances which are administered either topically or systemically [133;135-139]. The cutaneous MD sampling technique is considered to be semi-invasive and involves insertion of a needle under the skin to allow for the insertion of the probe. The procedure is considered to be minimally painful and many subjects in reported studies do not complain of discomfort [131].

Most recently, MD has been used *in vitro* to assess the release of an active ingredient from topical dosage forms [140]. It proved to be an extremely useful tool for the *in vitro* assessment of the release of mometasone furoate (MF) from topical preparations. Application of a MD technique was successfully used to discriminate differences between various topical ointment and cream

formulations containing MF. Hence, based on the MF release studies, one of the objectives of the current research was to apply an MD sampling technique and develop an appropriate *in vitro* MD method which could be used to assess the release of ERY from topical gel formulations which were produced extemporaneously (*vide infra* - Chapter 3).

5.1 COMPONENTS OF THE MICRODIALYSIS SYSTEM

The MD probes used in the current work were linear probes which were manufactured in-house. Linear probes are used in cutaneous MD to maximise the recovery of substances. The probes were composed of a hollow section of membrane fibre which was made of cellulose and obtained from dialyser cartridges. Two types of dialysis membrane were used, a Haemophan fibre dialysis cartridges, Al wall GFS plus 12 and GFS plus 20 (Gambro, Leuven, Belgium). These membranes are normally used during the haemodialysis of patients suffering from kidney failure. The membranes both had internal diameter of 210 μm and molecular weight cut-off of 5 and 10 kDa, respectively. The cut-off value, supplied by the manufacturer, is the molecular weight in Daltons beyond which 80 % of molecules cannot pass through the membrane [141].

A length of stainless steel guide wire with o.d. 100 μm (Metalann, Meslin-I-'Eveque, Belgium) was inserted into each fibre to provide mechanical support to the membrane.

Portex® tubing (Scientific Laboratory Suppliers Ltd, Nottingham, UK) which consists of nylon with an internal diameter (i.d.) of 0.50 mm and an outer diameter (o.d.) of 0.63 mm was attached on either side of the membrane fibre using a cyanoacrylate glue (Bostik® Ltd, Swindon, England) to produce a linear microdialysis probe.

Other components of the MD system include MD glass syringes (CMA/ Microdialysis, Solna, Sweden), tubing adapters (Ref 340 9500, CMA/ Microdialysis, Solna, Sweden), a CMA 400 Syringe Microinjection precision pump (CMA/Microdialysis AB, Solna, Sweden), 300 μl glass micro-insert vials (Waters Cooperation, Milford, Massachusetts, USA) and a custom built MD chamber placed on a shaker platform. An image of the chamber is shown in Figure 5.2 below.

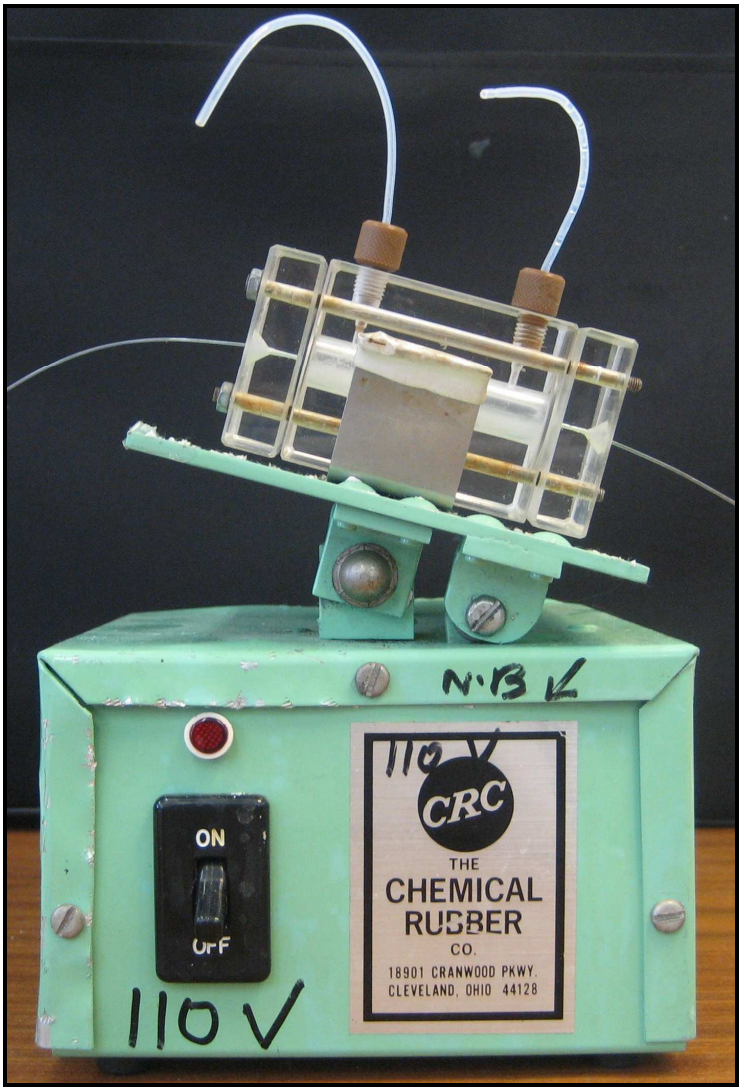


Figure 5.2 The MD chamber used for in vitro retrodialysis and MD experiments

Figure 5.3 is a diagrammatic representation of the probes which were used in the MD studies.

5.2 PREPARATION OF MICRODIALYSIS PROBES

The lengths of dialysis membrane fibre which were removed from the dialysis cartridge were cut into 50 mm in length before insertion of the guide wire. A 70 mm length of guide wire was inserted into each membrane fibre before gluing the Portex ® onto either side of the membrane fibre.

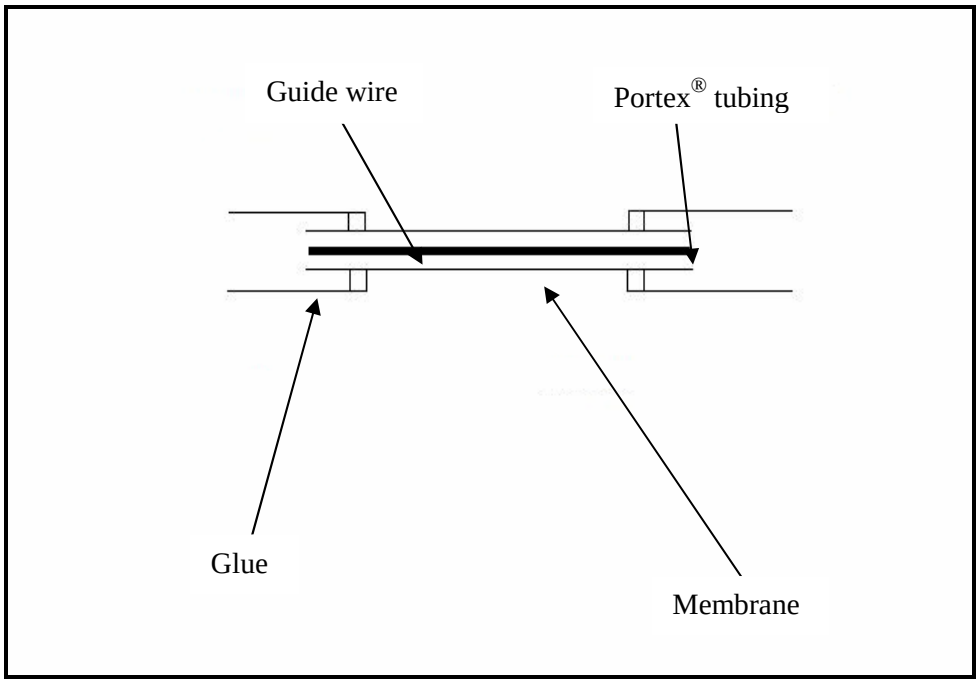


Figure 5.3 Diagrammatic scheme of a linear microdialysis probe

Two different lengths of Portex ® tubing were used, one being 20 cm in length and the other 25 cm. The differences in length where to allow for flexibility when working with a custom-built MD chamber, made specifically for the *in vitro* studies. The extra 5 cm was necessary to facilitate the connection of the probes to the chamber whilst mounted on a shaker platform. The Portex® tubing was glued to the fibre membrane 0.5 cm from the edge of the membrane on both sides leaving 4 cm of exposed membrane which is the actual surface area over which diffusion could occur. The probe inlet was attached to a precision glass syringe which was connected to a MD syringe pump.

5.3 *IN VITRO* MICRODIALYSIS TECHNIQUES

5.3.1 Advantages Of Microdialysis

MD allows for efficient and hassle free real-time sampling and the fact that the MD membrane does not allow macromolecules such as proteins to enter the dialysate eliminates the need for an extraction step and means that the dialysate can be analysed immediately after collection. Analysis of samples can be done online allowing rapid and efficient analysis of samples [131;132].

The use of MD requires only small volumes of liquid making it a relatively low cost analytical technique. Use of MD to assess *in vitro* release properties of different topical formulations has

proven to be effective in showing differences or similarities between formulations opening it up for use in *in vitro* release studies [140].

5.3.1 Limitations of Microdialysis

Since very slow flow rates are used, samples collected are usually very small volumes, in the micro-litre range, generally requiring highly sensitive analytical methods [127;132]. The manufacture of the linear microdialysis probes requires adeptness and skill in order to produce probes with the necessary properties and integrity to ensure reproducible results. Since not all analyte molecules may be able to diffuse across the range of currently available membranes depending of the cut-off values, the use of the technique may be limited to certain molecular weight ranges of analytes [127;141].

Since most perfusates used in MD experiments are hydrophilic, application of the technique to analysis of lipophilic substances is limited as issues concerning the solubility of the lipophilic analyte and the potential of the analyte to stick to the MD membrane must be addressed [142].

The perfusate used depends on the solubility properties of the analyte since the analyte must be soluble in the chosen perfusate. Clearly, perfusates used must be aqueous-based and preferably compatible and safe for use *in vivo* should the need to perform MD *in vivo* at a subsequent time be envisaged. This is highly desirable since it would provide extremely useful information to attempt to establish *in vitro*-*in vivo* correlations [131].

5.3.2 Calibration of Microdialysis Probes

In vitro MD experiments are aimed at ensuring that the relative recovery of the analyte is not affected by the membrane or the concentration of the analyte in the periprobe fluid or perfusate. This parameter is tested by assessing both the percent loss (% loss) and percent gain (% gain). Using the concentration of perfusate spiked with analyte and the concentration of analyte in the dialysate, it is possible to calculate % loss (Equation 5.2). In the same way, using the known concentration of the analyte in the periprobe fluid and concentration appearing in the dialysate, the % gain can also be determined (Equation 5.3). Under these conditions, the % loss and % gain should be equal [143]. Several different concentrations of the analyte should be assessed in both ways to ensure that concentration does not change the values of % loss or % recovery.

Calculation of % loss is shown in Equation 6.2 and Equation 6.3 shows the calculation for % gain [127].

$$\% \text{ loss} = [(C_p - C_d)/C_p] \times 100 \qquad \text{Equation 5.2}$$

$$\% \text{ gain} = (C_d/C_m) \times 100 \qquad \text{Equation 5.3}$$

Where;

C_d = Concentration of substance in the dialysate

C_p = Concentration of substance in the perfusate

C_m = Concentration of substance in medium surrounding the probe

5.3 METHODS

In vitro microdialysis was applied to topical formulations for the assessment of the release of ERY as a measure of pharmaceutical availability. Several experimental considerations were necessary before such evaluations could be carried out.

5.3.1 Preliminary Investigations and Optimisation Of Experimental Conditions

5.3.1.1 Perfusate Selection

The main concern when considering a suitable perfusate is the solubility of the substance of interest in the perfusate. It is essential to ensure that the analyte is soluble in the intended perfusate to avoid poor recoveries caused by solubility issues. Generally, the solution in which ERY is optimally soluble is the most appropriate one to ensure that sink conditions are maintained during the experiment. An important consideration which should be addressed when selecting a suitable perfusate for MD experiments is the compatibility between the intended perfusate and future *in vivo* applications when MD may need to be considered to monitor analyte in ECF. The perfusate should be as similar to the tissue or fluid surrounding the probe as possible in terms of tonicity and solute content. The most obvious choice of perfusate for cutaneous MD experiments is Ringer's solution or normal saline (NS) with or without glucose [127]. To increase the recovery of lipophilic compounds, the perfusate may be modified by including albumin and cyclodextrins or by using intralipid solution [144].

Loss of perfusate is known to occur during MD studies but it has been reported that when working with dextran 70 solutions (40 g/l of dextran 70), there is no net loss of perfusate even when the perfusate flow rate is as low as 0.075 µl/min [145;146].

The solubility of ERY in various solvents which were considered for use as possible perfusates was investigated. The solvents which were investigated were normal saline with 5 % ethanol (NS), a solvent system containg propylene glycol (PG) and water (PG/H_2O) in a 30/70 ratio respectively and a solvent system (SOLV) consisting of 4 % solution of dextran 70 in NS with 5 % ethanol (i.e. = SOLV). Two milligrams of ERY was dissolved in 10 ml of each of the solvents and also dissolved in methanol, a solution in which ERY is known to be soluble [7] which was used to compare the solubility of ERY in the other solvents. The 12 ml Kimax tubes containing these solutions were shaken on a Junior Orbit Shaker for 24 hours and all experiments were performed in duplicate. The above method was used instead of the solvent saturation method due to the limited availability of ERY. The amount of ERY which dissolved in the solvents should yield concentrations of ERY (200 µg /ml) well above the maximum concentrations used in the current experimental work (50 µg/ml) in order to eliminate the possibility of solubility related problems with the relative recovery. Stability of ERY in these solutions on the workbench and in the refrigerator was also assessed over a 48 hour period. Samples were analysed using a validated HPLC method.

5.3.1.2 Membrane Structure and Selection

Two different Hemophan® fibres with different cut-off values were investigated for use as previously described. The polymer for Hemophan® originates from cellulose, in which a well-defined portion of hydrophilic hydroxyl (-OH-) groups are substituted by hydrophobic diethylaminoethyl groups. The cellulose backbone provides optimum aqueous solute clearance by its hydrogel structure. Large numbers of water molecules are incorporated between the fibrils of the polymer inside the membrane wall. Thus solutes, especially hydrophilic solutes, can easily diffuse through the membrane. The substitution of some of the hydroxyl-groups achieves the desired balance between hydrophilic and hydrophobic domains. This balance mimics human cell membranes and thus delivers the best biocompatibility comparable to that of synthetic membranes [147].

One of the membranes (M1) had a cut-off of 5 kDa (Alwall GFS plus 12) while the other, membrane (M2) had a cut-off of 10 kDa (GFS plus 20). Both membranes were assessed using the relative recovery of ERY in NS to determine their suitability for *in vitro* use. Four probes were used for each membrane, the periprobe fluid was a 54.2 µg/ml solution of ERY in NS and the perfusate was blank normal saline. The microdialysis flow rate was 3 µl/min. The experiment was run over five hours with hourly sampling intervals. An equilibration time of one hour was allowed before commencing the experiment and samples were analysed using the validated HPLC analytical method (*vide infra* – Chapter 4).

5.3.2 Retrodialysis in Air in the Assessment of Adsorption

Depending on the lipophilicity of a molecule, it is possible for the analyte to bind to the glass syringes, plastic connectors, tubing or membrane of a probe especially in the absence of an organic solvent. If this occurs it presents a challenge as it affects the relative recovery of the analyte [140]. Two separate solutions of known concentration of ERY were prepared one in the PG/H_2O solvent (52.1 µg/ml) and the other in SOLV (50.9 µg/ml) in 100 ml volumetric flasks. The solutions were perfused (3 µl/min) through the MD probes (n = 4) which were suspended in air in a covered container to prevent loss of dialysate volume by evaporation. The dialysate was collected and weighed every hour for five hours and the samples were analysed by HPLC.

5.3.3 Assessment of *In Vitro* Retrodialysis and Microdialysis of ERY

To assess the *in vitro* retrodialysis, MD probes (n = 4) were perfused (3 µl/min) with a solution of ERY in SOLV or PG/H_2O and the probes were immersed in SOLV or PG/H_2O respectively. To assess the *in vitro* microdialysis of ERY, SOLV or PG/H_2O was perfused through MD probes (n = 4) that were submerged in the periprobe fluid which was a solution of ERY dissolved in SOLV or PG/H2O respectively.

For both the MD and retrodialysis experiments four different solutions were used over the concentration range of 5, 10, 20 and 50 µg/ml. This was done to ensure that relative recovery is not influenced by the concentration of the solution. Ideally, the values for percentage relative recovery for both experiments should remain the same over the entire concentration range.

All MD and retrodialysis experiments were conducted in a custom-made microdialysis chamber which had a self contained stirrer and held a volume of periprobe fluid of approximately 4 ml. Figure 5.2 shows the chamber fitted with a probe inside it.

5.3.5 *In Vitro* Release Assessment Of ERY Gels

The release of ERY from the topical gel formulations was assessed using *in vitro* MD as a sampling technique. The perfusates used were SOLV and PG/H_2O and they were both run at a flow rate to 3 µl/min. The probes (n = 4) were immersed in approximately 4 g of each gel and the experiment was run over five hours. Samples were collected every hour and were analysed using HPLC as previously described.

5.4 DATA ANALYSIS - ASSESSMENT OF PHARMACEUTICAL EQUIVALENCE

Comparing the release data obtained from the *in vitro* MD release tests for the two gel formulations provided information which could be used to determine the pharmaceutical equivalence of the two gel formulations. Statistical methods and mathematical models have been used to study drug release kinetics.

5.4.1 Mathematical Treatment of *In Vitro* Release Data

5.4.1.1 ANOVA Analysis

The different perfusates that were used for the assessment of ERY gels were evaluated for potential discriminatory behaviour using ANOVA analysis. Two-way ANOVA analysis was performed using GraphPad Prism software Version 4.00 for Windows (GraphPad Software, San Diego California USA) to assess whether differences between each time point of the *in vitro* release profiles existed for each of the formulations and perfusates tested.

ANOVA assesses each time point in the drug release profile between the test and the reference products and takes into account the variability in data at the single time point. Any differences in the profiles at any given time point are shown thus the release mechanism of the formulation can be inferred from ANOVA studies [148].

The disadvantage of ANOVA testing is that each time point is treated independently from the rest of the profile. A consequence of multiple comparisons is that the overall risk of incorrectly concluding that products are pharmaceutically not equivalent is greater than the nominal 5%. Multiple comparisons may be statistically significant at some points in the profile, but not all, making it difficult to conclude whether differences truly exist. The use of ANOVA is not recommended for release profiles other than immediate release [148].

5.4.1.2 f_1 and f_2 difference and similarity factors

This approach uses mathematical indices to describe differences (f_1) and similarity (f_2) factors for comparison of the release profiles. The equations for the f1 and f_2 factors are described below;

$$f_1 = \{[\sum_{t=1}^{n}|R_t - T_t|] / [\sum_{t=1}^{n}.R_t]\}.100 \qquad \text{Equation 5.4}$$

$$f_2 = 50 \log \{[1 + (1/n).\sum_{t=1}^{n}.W_t.(R_t - T_t)^2]^{-0.5}.100\} \qquad \text{Equation 5.5}$$

Where;

n = Number of time points

W_t = Optional weight factor

R_t = Dissolution value of the reference method at time t

T_t = Dissolution value of the test method at time t

The f_1 value indicates the percentage difference between two profiles at each time point and is a measurement of the relative error between them. The f_2 value is a measure of the similarity between the dissolution profiles. Generally, to ensure sameness between profiles, f_1 should be in the range of 0 – 15, and f_2 in the range of 50 – 100. To calculate the fit factors, the mean dissolution values from both profiles at each time interval are used, including only one sampling point at greater than 85% level of drug release in order to avoid bias in the similarity assessment [149;150]. At least three dissolution time points are needed to calculate the f_2 and the two profiles are identical if $f_2 = 100$. An average difference of 10% at all measured time points results in an f_2 value of 50. The FDA has set a public standard of f_2 value between 50 - 100 to indicate similarity between two dissolution profiles [149].

The main advantages of the use of the f_1 and f_2 difference and similarity factors are that they are easy to compute and each gives a single number that indicates the degree of closeness between the two *in vitro* dissolution profiles being compared [151]. However, limitations in the use of the similarity factor include failure to take into account the shape of the curve and unequal spacing between time points on *in vitro* release profiles. The similarity factor is often too liberal in concluding similarity between two dissolution profiles [152]. The use of these fit factors is further

limited by the fact that values of f_1 and f_2 are sensitive to the number of sampling points used after 85 % of the drug present in the dosage form has dissolved [148].

The release profiles of the two ERY gels using the two different perfusates were investigated using these two fit factors which were determined using an excel spreadsheet.

5.4.2 Model-Dependent Methods

Model-dependent methods make assumptions about the shape of the curve, fitting data using equations in which parameters defining the shape of the curve are optimised [153]. Zero order, First order and Higuchi models are examples of commonly used model-dependent methods that have been used [152]. These models can be used to establish the mechanism of drug release from delivery systems and are useful in understanding factors pertaining to the formulation that alter the *in vitro* release of an analyte from a dosage form. Such knowledge can be used for optimising properties of a formulation during the development process [154].

5.4.2.1 Zero Order Kinetics

The zero order model can be used to model drug release from dosage forms that do not disaggregate and release the same amount of drug per unit time to obtain an expected and continuous release of a medicinal agent [152]. The zero order model can be expressed using Equation 5.5.

$$Q_t = Q_0 + K_0 t \qquad \text{Equation 5.5}$$

Where,

Q_t = amount of drug released at time t

Q_0 = initial amount of drug present in solution

K_0 = zero order release constant

t = time

When drug release follows a zero order model, the amount and rate of drug released is independent of time and change in the concentration of the reactants does not speed up or slow down the rate of reaction.

5.4.2.2 First Order Kinetics

The application of a first order model to describe the *in vitro* release/dissolution kinetics of a pharmaceutical ingredient was reported by Gibaldi and Feldman [155]. The first order release model may be expressed mathematically using Equation 5.6.

$$\ln Q_t = \ln Q_0 + K_1 t \qquad \text{Equation 5.6}$$

Where,

Q_t = amount of drug release in time t

Q_0 = initial amount of drug present in solution

K_1 = first order release constant

t = time

Water-soluble drugs that are formulated into porous matrices are released according to first order kinetic principles [152] and that the rate of release is proportional to the amount of drug remaining to be released from a matrix. The rate of release therefore declines with time as the amount of drug remaining in the dosage form diminishes over time. That is to say in these systems the rate of release is dependent on the concentration of the analyte in the dosage form.

5.4.2.3 Higuchi Model

The models for drug release developed by Higuchi [156] describe the mechanism of release of water-soluble and water-insoluble substances that were homogeneously incorporated into non-eroding semi-solid and/or solid matrices. The model applies to systems where the drug is saturated in the vehicle. A simplified version of the Higuchi model is depicted in Equation 5.7.

$$Q_t = K_H . t^{1/2} \qquad \text{Equation 5.7}$$

Where,

Q_t = Amount of drug released at time t

K_H = Higuchi dissolution constant

t = Time

The model was derived for a system which fulfilled the following three requirements:

The drug was in a semi-solid dosage form in a finely divided state such that the particles were smaller than the thickness of the layer of formulation placed on the surface being assessed. In

addition to this, the amount of drug per unit volume of the formulation was substantially greater than the solubility of the drug per unit volume of the vehicle. Lastly, the surface to which the drug formulation was applied was immiscible with the formulation and thus constituted perfect sink conditions for the released drug [156].

The Higuchi model describes drug release based on Fick's First Law of diffusion and release is considered to be diffusion controlled if the release of drug is square root time dependent [152].

5.4.3 Method of Data Analysis

The ERY release profiles were analysed using the various models described above. Differences and similarities in the release profiles were evaluated using two-way ANOVA and an Excel® spread sheet to calculate f_1 and f_2 fit factors.

5.5 RESULTS AND DISCUSSION

5.5.1 Microdialysis Conditions Selected For Experimental Work

5.5.1.1 Perfusate Selection

The results of the solubility investigations showed similar results for ERY solubility in SOLV, NS (5 % ethanol) and PG/H_2O solutions. For the purposes of this investigation, the results were compared in terms of the peak heights obtained when samples of the respective ERY solutions were analysed by HPLC. The average peak heights for the SOLV, NS (5% ethanol) and PG/ H_2O were, 7.89 $\pm$ 1.2 cm, 7.81 $\pm$ 1.1 cm for NS (5 % ethanol), 7.9 $\pm$ 0.9 cm, respectively compared to the average peak height of 8.07 $\pm$ 0.7 cm for the methanolic solution. ERY was found to be stable on the workbench (21 ºC) and in the refrigerator (4 ºC) over a period 48 hours in all the solvents.

In initial experiments involving perfusion of the two ERY gel formulations, the chosen perfusate was NS with 5 % ethanol. The gravimetric checks which were performed at each sampling interval to ensure no net loss of fluid in the dialysate indicated that up to 60 % of the dialysate was not being recovered. At a flow rate of 3 µl/min, a volume of dialysate close to 180 µl should have been collected but only 60 µl resulted. This significant difference was thought to be due to the loss of water in the perfusate to the gel. This was attributed to the much higher osmotic potential of the gels due to the high concentration of solutes.

To overcome the issue of volume loss due to osmotic differences, the perfusate selected was the PG/H_2O solvent system. When this solvent was used, an average loss of only a 5 % of the dialysate

occurred, thus justifying the use of this solvent mixture for *in vitro* work. The ratio of 30/70 PG/H_2O was chosen as it gave a composition similar to that in G1 not the same as G2 (10 % PG). This was because the use of a perfusate ratio of 10/90 PG/H_2O would still lead to volume loss when perfusing G1 which had a higher concentration of PG. Thus, using the 30/70 PG/H_2O would circumvent the problem of volume loss when perfusing both gels.

In view of the fact that it would be useful to consider the application of *in vivo* microdialysis of topical ERY formulations at some further point in time, an alternative perfusate which would be physiologically compatible, safe and appropriate for use *in vivo*, was also investigated. As a result, 40 g/l of dextran 70 was added to a normal saline solution (i.e. SOLV) in order to avoid the risk of a reduction in the collection volume of dialysate due to osmotic pressure differences and resultant water loss as experienced when NS only was used and as previously described.

5.5.1.2 Membrane Selection

Of the two membranes selected the one with the higher cut-off (M2) resulted in a higher rate of recovery of ERY than the one with the lower cut-off (M1). A higher extent of recovery was also obtained using M2 compared to M1. The approach to equilibrium was faster for M2 than it was for M1. Figure 5.4 shows the percentage relative recoveries for the two membranes over the five hour experimental run time.

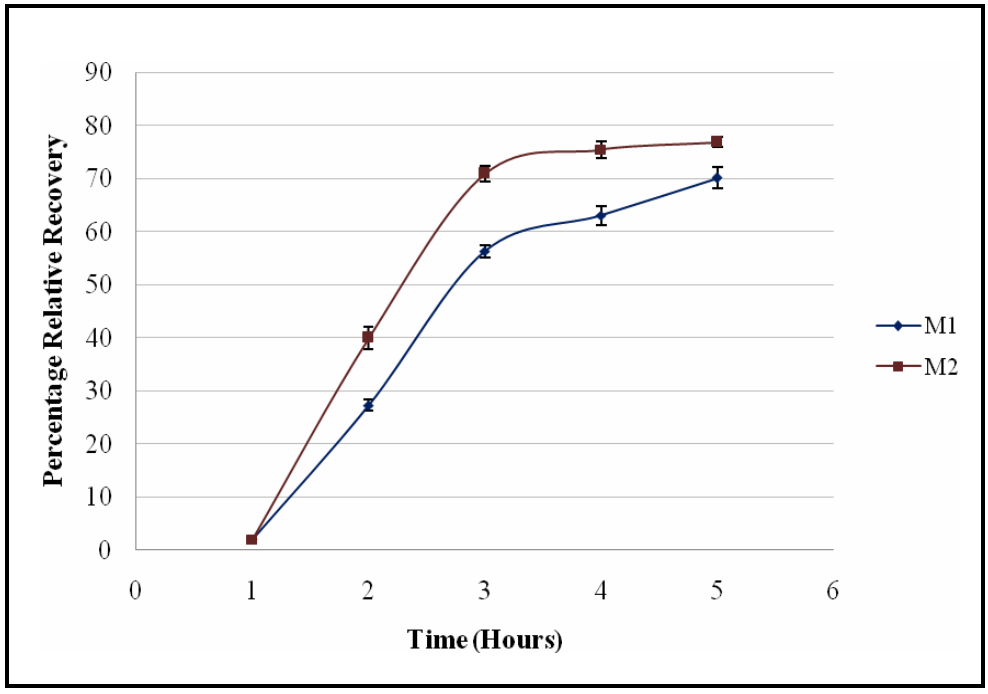


Figure 5.4 Differences in percentage relative recovery between M1 and M2

The final conditions selected for the *in vitro* microdialysis experiments are summarized in Table 5.1.

Table 5.1 Microdialysis conditions selected

Perfusates	a) PG/H2O (30/70) b) Normal saline with 4 % dextran 70 and 5 % ethanol (SOLV)
Flow rate	3 µl/min
Membrane	Gambro GFS Plus 20
Molecular weight cut-off	10 kDa
Membrane length	4 cm
Tubing	Portex® tubing, 20 cm and 25 cm
Temperature	Ambient

5.5.2 Retrodialysis in Air to Assess Adsorption

The average relative recovery of ERY in the dialysate was 99.3 % (n = 4) suggesting that there was no significant binding of the ERY to any aspect of the MD apparatus including membrane.

5.5.3 Retrodialysis and MD of ERY

Both the retrodialysis and MD results obtained over the five hour sampling time for the two perfusates are shown below. The retrodialysis and MD profiles for SOLV are shown in Figure 5.5 and Figure 5.6 shows the profiles for PG/H_2O.

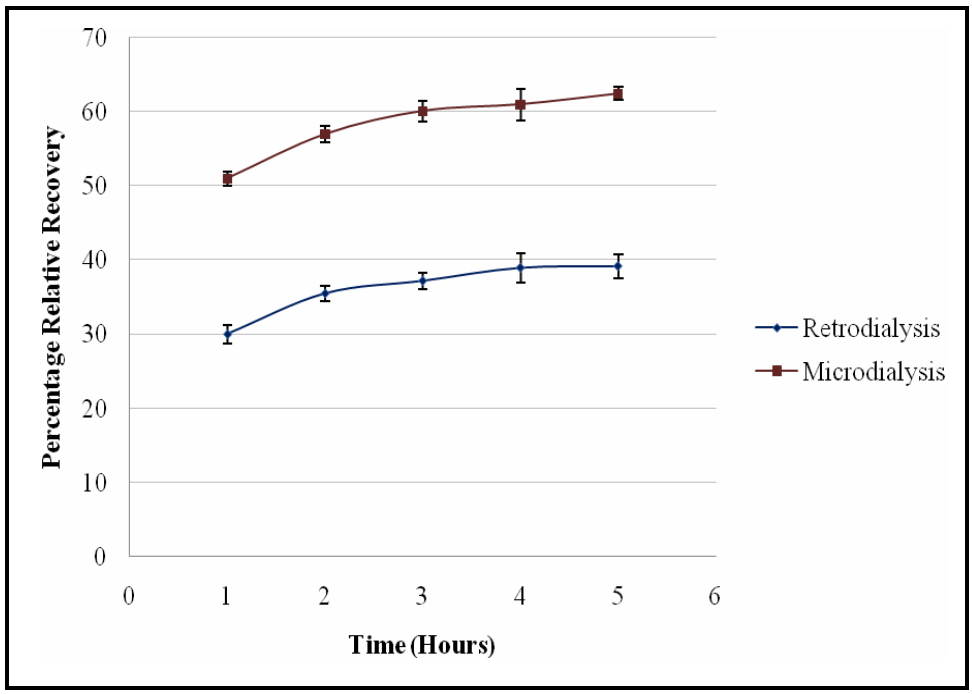


Figure 5.5 Retrodialysis and MD of ERY solution using SOLV

For SOLV, the amount of ERY recovered by MD averaged 62.4 % whereas the average loss of ERY (from retrodialysis) was 39.5 % and for PG/H_2O 64.2 % was recovered as opposed to 36.1 % loss using the respective methods. The rate of recovery using either retrodialysis or MD processes slowed down 3 hours into the experiment. Reconciliation of the % loss in retrodialysis and the % recovered in MD, which should give a total of 100%, resulted in acceptable values of 101.9 % and 100.3 % for SOLV and PG/H_2O, respectively.

Whilst both Figures 5.5 and 5.6 represents the highest calibration concentrations used for each solvent, the profiles for the other concentrations showed similar results with comparable averages for the % loss and % gain.

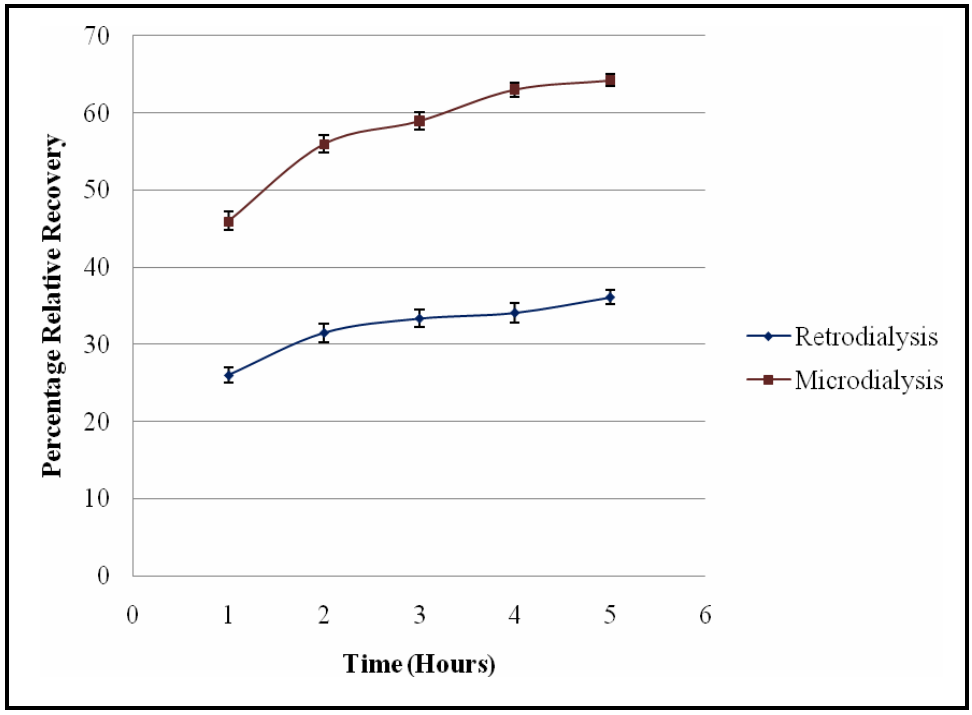


Figure 5.6 Retrodialysis and MD of ERY solution using PG/H_2O

5.5.6 *In Vitro* Release Of Erythromycin Gels

The results shown in Figure 6.6 illustrate the differences in both the rate of recovery and extent of recovery. Generally, the cumulative amount released and the rate of release was greater for G2 than for G1 for both perfusates. When SOLV was used, G1 released 1.56 mg/cm^2 and G2 released 1.74 mg/cm^2 at the end of the sampling period. When PG/H_2O was used, G1 released 3.94 mg/cm^2 and G2 released 7.80 mg/cm^2 over the sampling period. The apparent rate of release of G1 with perfusate SOLV was 0.303 mg/cm^2/hr and 0.337 mg/cm^2/hr for G2. For the PG/H_2O solvent, the apparent rate of release for G1 and G2 was 0.751 mg/cm^2/hr and 1.486 mg/cm^2/hr respectively.

The difference observed in the order of release (G2 > G1) was due to the different PG content in the two gels. The presence of PG in a higher concentration in G1 (30 %) results in a decreased tendency of the ERY to diffuse out of the gel and into the dialysate. The ERY partitions more into G1 than the dialysate when compared to G2. This is true for both perfusate fluids which were used.

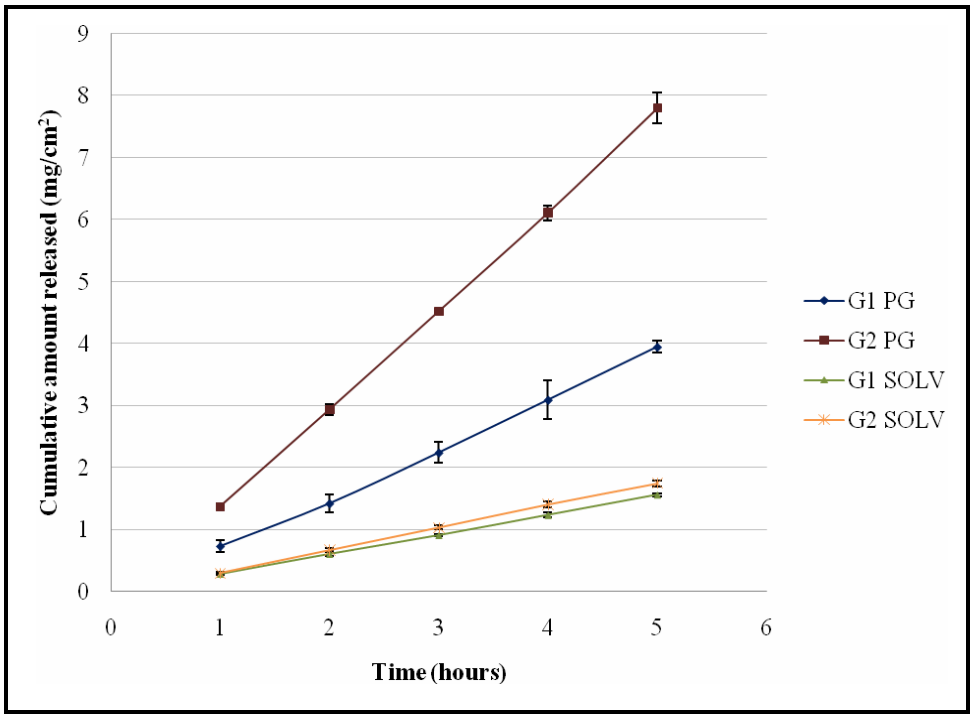


Figure 5.6 Drug release profiles from 2 % ERY gel formulations using two perfusates

Comparing the R^2 values from the linear regression lines for the Higuchi, zero and first order plots suggests that the release of ERY from all formulations follows zero order kinetics. However, the high regression correlation factors for the Higuchi plots suggest that the diffusion process has some degree of impact on the release of ERY from the gels. The lower R^2 values for the First order plots suggest that the release of ERY from the gels is least likely to follow these kinetics. In summary, the R^2 values suggest that the release of ERY from the gels is not affected by the concentration of ERY in the gels and that diffusion has some part to play in ERY release from the gel formulations to a lesser extent. The results for the R^2 values obtained when the release data was fitted to the Higuchi, Zero and First order models are shown in Table 5.2 below.

Table 5.2 Comparison of ERY release kinetics from gel formulations using different perfusates

Formulation and perfusate	Higuchi R^2	Zero Order R^2	First Order R^2
G1 SOLV	0.976	0.999	0.943
G2 SOLV	0.989	0.999	0.934
G1 PG/H_2O	0.982	0.999	0.952
G2 PG/H_20	0.984	0.998	0.944

Visual assessment of the *y*-error bars on the two SLOV profiles suggests that at sample times of one and two hours there is no significant difference between the release profiles for the two gels but after the second hour the release profiles begin to show greater differences. The *y*-error bars on the PG/H_2O profiles show distinct differences in between the two profiles at all time points.

Two-way ANOVA was one of the methods used to test for significant differences or similarities between profiles when using different perfusates (Table 5.3). The means at each time point were compared to determine if there were differences and the results are shown in Table 5.3 below.

Table 5.3 ANOVA values for Pharmaceutical Equivalence

Comparison between release profiles	Sample Time (Hours)	Mean difference	P-value	95 % Confidence interval		Summary
				Lower limit	Upper limit	
G1 PG/H_2O vs G2 PG/H_2O	1	0.6346	P < 0.001	0.3665	0.9027	Significant
	2	1.512	P < 0.001	1.244	1.780	Significant
	3	2.279	P < 0.001	2.011	2.547	Significant
	4	3.019	P < 0.001	2.751	3.287	Significant
	5	3.854	P < 0.001	3.586	4.122	Significant
G1 SOLV vs G2 SOLV	1	0.01841	P > 0.05	-0.2497	0.2865	Not significant
	2	0.06296	P > 0.05	-0.2051	0.3310	Not significant
	3	0.1256	P > 0.05	-0.1425	0.3936	Not significant
	4	0.1692	P > 0.05	-0.09889	0.4373	Not significant
	5	0.1824	P > 0.05	-0.08564	0.4505	Not significant

The results clearly indicate that there is a significant difference between the profiles for G1 and G2 when using PG/H_2O as the perfusate. However, the results also indicate that there is no significant difference between the G1 and G2 profiles when using SOLV as the perfusate.

Table 5.4 Fit factor values for Pharmaceutical Equivalence

Comparison between release profiles	f_1 value	f_2 value
G1 PG/H_2O vs G2 PG/H_2O	34.2	46.7
G1 SOLV vs G2 SOLV	10.8	97.9

The fit factors were also used to determine differences and similarities between profiles generated using different perfusates and the values obtained were contradictory (Table 5.4).

When comparing G1 and G2 profiles generated using SOLV as the perfusate, the f_1 factor was10.8 and the f_2 factor was 97.9. Both the f_1 and f_2 factor suggest that the profiles are similar as both values fall within the similarity limits previously mentioned. The profiles generated using PG/H_2O as the solvent gave f_1 of 34.2 and f_2 46.7. The f_2 factor falls just outside the limits for similarity (50 – 100) but the f_1 factor falls well outside the range indicative of similarity between profiles. This suggests that the use of fit factors for assessing the similarities and differences between profiles in the current work does show differences and similarities and may be a suitably mathematical tool to use to discriminate difference between both sets of data.

The differences in the extent and rate of drug release from the gel formulations with both perfusates can be attributed to the difference in the PG content in the gels. Being a hydrophobic drug, ERY is more soluble in PG than it is in water. This means that ERY was more soluble in G1 than it was in G2 as gel one had a higher percentage content of PG. As a result, the tendency for ERY to remain in the gel and not enter the perfusate was higher in G1 than it was for G2 thus explaining the order of release.

When PG/H_2O was used as the perfusate the ANOVA and fit factor results showed significant differences between G1 and G2 release profiles. This suggests that from a quality control point of view the PG/H_2O solvent is a better solvent to use when trying to show differences in pharmaceutical availability *in vitro* as it allows for greater sensitivity when testing for discrepancies between the gel formulations than SOLV. The use of SOLV for inferring pharmaceutical availability is not possible as the solvent did not discriminate between the two gel formulations *in vitro*. The lower degree of release of ERY from the gel formulations using SOLV as the perfusate can be attributed to the poor solubility of ERY in aqueous media which affects the tendency of the analyte to move out of the gel where it is more soluble into the predominantly aqueous perfusate. Thus, the degree of solubility of the ERY in the perfusate affected the relative recovery of the analyte in both gels to the same extent leading to profiles which were different only upon visual assessment.

However, SOLV was originally investigated as a solvent to use possibly in future *in vivo* MD studies and whether or not SOLV can discriminate differences between the gels *in vivo* still needs to be determined.

5.6 CONCLUSIONS

The linear MD probes which were made in-house gave reproducible results (% RSD of < XXX) and can be reliably used for these types of MD experiments. When conducting MD experiments it is important to ensure that there is no binding between the substance of interest and the MD apparatus. Under the specified conditions, it has been shown that there is no binding of ERY to the membrane, probe tubing, glass syringes and tubing connectors.

More ERY was released from G2 per unit time compared to G1 regardless of the perfusate used. These results show that MD can be used to determine release rates of ERY from topical formulations. However, the results showed that the extent of the differences shown is dependent on the nature of the perfusate. Using SOLV as the perfusate showed no difference in the release profiles of the two gels however using the PG/H_2O solvent showed marked differences in the release of ERY from the two gels. Hence, it can be concluded that *in vitro* MD can be used to establish differences in pharmaceutical availability however careful consideration must go into the choice of perfusate as this will have a considerable effect on the release profiles of the formulations being compared.

There is great potential for use of MD to assess the pharmaceutical availability of topical macrolide preparations. However, further investigations and modifications may be required before MD can be used in quantitative and qualitative assessments *in vivo*.

CHAPTER 6

DERMATOPHARMACOKINETICS

Skin Stripping

6. INTRODUCTION

According to the Food and Drug Administration (FDA) guidelines [157], pharmacokinetic (PK) measurements in blood, plasma and/or urine of topical dermatological drug products are not feasible to document bioequivalence (BE) since the active ingredient(s) in topical formulations is/are not intended to be absorbed into the systemic circulation and in addition, concentrations in extracutaneous biological tissues would generally not be measurable. This limits determination of bioavailability and assessment of BE of such products to pharmacodynamic measurements, clinical trials and dermatopharmacokinetic (DPK) measurements such as tape stripping (TS) and MD [157].

One method which has received a great deal of attention is TS, also sometimes referred to as a DPK method. It has been widely investigated as a possible method for use in BE and BA studies of topical drug products [157]. Research has shown that it is possible to generate data on area under the curve (AUC) and maximum concentration using DPK but use of this method has been hampered by variable and contradictory results [39]. In light of such discrepancies, the FDA concluded that the methodology was not adequately developed to satisfy the regulatory requirements for BA or BE assessment and the proposal was subsequently withdrawn [157].

TS involves removal of successive layers of the SC using adhesive tape placed on the skin. Each strip removes approximately 0.5 – 1 µm of SC and the procedure is relatively pain free [158]. TS is considered to be a non-invasive sampling technique and although it causes minor distortion of the skin structure, the skin has the ability to rapidly regenerate and restore barrier function [158]. The successive removal of the SC has been shown to result in an increase in water loss through the skin as the barrier function of the skin is altered. The measurement of this water loss during TS studies has lead to the development of methods to assess the thickness of the skin in relation to transepidermal water loss (TEWL) [159;160].

The use of TS is limited by the fact that currently there are no standardised and universally acceptable TS methods to determine the SC concentration of a compound following topical

administration. Standardization of such methods are essential for routine use of TS for BA determination and BE assessment of topical dosage forms. Another challenge is that once TS data have been collected it has to be processed in a manner which takes into account the variability of data due to inter-individual differences in the thickness of the SC. Whilst it is essential to quantitatively determine the amount of SC removed by each strip, consensus has not yet been reached on a simple and rapid method to do so [158].

A correlation has been found to exist between TEWL measurements and SC thickness. Using sequential stripping processes followed by gravimetric determination of the SC thickness, it was found that TEWL is inversely proportional to SC thickness and it is possible to use TEWL readings to determine SC thickness [161].

TS has found use in a variety of applications such as:

- assessment of pH of the SC environment [162]
- estimation of the amount of SC removed by TS [160]
- assessment of the BA of topically applied substances [163-168]
- correlation of TEWL results obtained from TS to correlate skin barrier function and percutaneous absorption [169;170]
- determination of diffusion characteristics at different anatomical sites of the body [171]
- investigation of the effect of certain skin pathologies such as inflammation, neoplastic disorders or xerotic conditions [172], amongst other uses.

In DPK, TS is used to determine the cutaneous concentrations of substances applied topically. The technique is particularly useful when considering substances whose intended site of action is the SC such as antifungals, UVA/UVB filters and antiseptics [158]. Thus the use of TS presents a potential technique for use in assessing topical BA and BE of topically applied formulations targeting the SC and underlying tissues of the skin.

6.1 FACTORS AFFECTING THE SKIN STRIPPING TECHNIQUE

6.1.1 Experimental Considerations

6.1.1.1 Selection of adhesive tape

Different types of adhesive tapes tend to remove different amounts of SC and do so in a variable fashion [173]. The choice of tape for skin stripping is affected by a number of factors. The tape

should ideally be capable of removing the SC using a reasonable number of strips (~ 10 – 15 strips). In addition to this, extraction of the active ingredient from the tape should be simple, rapid, efficient and reproducible. Finally, depending on the chosen analytical method, the tape chosen should not contain substances which could interfere with the determination of the analyte following extraction [158].

For the purposes of the current work two types of tape were investigated, Transpore™ 1527 tapes (Kleefpleister, Isando, South Africa) and Scotch® Magic™ tape (3M, St Paul, Minnesota, USA). The former was chosen for the current work as it produced cleaner samples following extraction without any interference from any of the components of the tape compared to Scotch® magic tape which resulted in the presence of three additional components in the HPLC chromatogram, two of which interfered with the quantitative analysis of ERY.

6.1.1.2 Application of Tapes Prior to Stripping

Application of the tape on the demarcated application site without applying any pressure may result in minimal contact of the tape with the extreme corners of the area intended for stripping. The use of pressure on the tape after it has been placed on the application site ensures that all areas of the skin on the application site come into proper contact with the tape prior to stripping. Increasing the applied pressure on the skin from 165 g/cm^2 to 330 g/cm^2 has been found to result in more rapid increases in TEWL. In addition to this, increasing the length of time that force is applied from 2 seconds to 10 seconds also results in a faster rate of increase of TEWL [174]. A correlation exists between the thickness of the SC removed and TEWL [160;161]. Thus, as the force applied at the application site affects TEWL, it follows that the pressure used to apply the tape strips affects the thickness of the SC removed by TS.

6.1.1.3 Method of Stripping

The speed, force and direction of stripping has been found to affect the amount of skin removed during the TS process. The speed of removal of the tape strip affects the stripping/peeling force applied to remove the tape strip [158;173]. There is a scarcity of published information pertaining to the effect of speed, force and direction of stripping and the effect these factors have on inter-subject and inter-investigator variation.

6.1.1.4 Anatomical Site

The amount of skin removed during the TS process is affected by the anatomical site of the body where the skin is removed. As discussed in Chapter 2, the thickness of the SC at different anatomical sites varies and depends upon the extent of physical contact which the skin has with its physical surroundings. In other words, increased physical contact of a particular area of the body with the external environment results in thickening of the skin in that area in order to prevent damage caused by physical trauma. These differences may affect the amount of SC removed relative to the entire thickness of the SC during the stripping process. Correlation of TEWL from the skin of the back, abdomen, forearm and thigh with SC thickness have shown that the SC thickness at the extremities is thicker than that of the abdomen [171].

6.1.1.5 Removal of Excess Formulation

It is considered to be of importance to clean the treated area of the SC before beginning the stripping process. The method chosen must be capable of efficiently removing excess formulation from the skin without inadvertently forcing the drug into deeper layers of the skin [158]. During the current research, the effect of removing excess formulation from the application site was investigated using different solvents.

6.1.2 Inter-Individual Factors

6.1.2.1 Cohesion of stratum corneum cells

The cohesive strength between the SC cells affects the amount of skin removed by TS. Previous studies have reported that progressive stripping of the SC resulted in decreased removal of skin in the deeper layers. As a fresh strip of tape was used for each stripping, the adhesive properties of the tape remained the same during the TS process. Hence, the decrease in the amount of SC removed is probably due to an increase in the cohesiveness of the deeper layers of the SC [173].

6.1.2.2 Race

Comparative structural studies of black and white skins suggest that cell cohesion of SC cells as well as desquamation are increased in black people [175;176]. As previously stated, the cohesiveness of the SC affects removal of SC cells during the tape stripping process. It goes without saying then that such differences in skin structure between different races will have an effect on the amount of skin removed during the TS process.

6.2 OBJECTIVES

The objectives of the study were to:

- develop and validate a TS method which could be used to assess cutaneous levels of ERY in the skin following application of topical gel formulations.
- develop and validate an extraction procedure which could be used to efficiently and reproducibly extract ERY from tape strips.
- determine the effect of PG content of the two gel formulations on the percutaneous absorption of ERY from the topical dosage forms.
- assess the BE of topical gel formulations containing ERY using TS.

6.3 METHODS AND PROCEDURES

6.3.1 Selection Criteria

6.3.1.1 Inclusion Criteria

Subjects had to meet the following criteria to qualify for inclusion into the study:

- healthy and normal in terms of physical and dermatological examination at the pre-study screening and who were available for the duration of the study.
- willing to adhere to the protocol requirements and follow study restrictions.
- complete an informed consent form (in relation to age and mental well-being).
- aged between 18 and 50 years.

6.3.1.2 Exclusion Criteria

The following exclusion criteria were applied to subjects who:

- were breast feeding.
- were contemplating becoming pregnant in the time immediately following the study.
- were pregnant.
- had a known allergy/hypersensitivity to ERY or any macrolide antibiotics.
- had any history of drug or alcohol abuse.
- had any mental deficiency or handicap.
- had hairy ventral forearm surfaces and/or abrasions on the underside of their forearms.
- exhibited signs of sun-tanning.
- participated in another tape stripping study within 2 months of the study date.
- used any macrolide antibiotics within three months of the start of the TS study

- suffered from any allergic conditions (hay fever, allergic rashes, asthma or childhood eczema).
- suffered from any relevant skin disorder such as psoriasis or eczema.
- were on chronic medication or who used any creams within the week preceding the study (excluding contraceptive pills).
- had a history of any neurological, kidney or liver disorders.

6.3.1.3 General Study Restrictions

- Use of prescription drugs a week prior to the beginning of the study and during the course of the study was not allowed.
- Eligible subjects not permitted to use over the counter medicines, vitamins, homeopathic medicines, herbs and natural products one week prior to commencement of the study and during the study.
- Consumption of alcohol 24 hours before the study and during the study was prohibited.
- Participation in strenuous physical exercise or physical exertion 12 hours before the beginning of the study was not allowed.
- Smoking was not permitted 24 hours before and during the study.
- Subjects were asked not to apply any moisturiser or any other topical product to the skin of the forearms 24 hours before the study commenced.

6.3.1.4 Criteria for Removal from the Study

Removal of any subject from the study at anytime would occur under the following conditions:

- voluntary withdrawal by the subject for any reason.
- illness or injury if regarded as clinically significant.
- development of an adverse event(s) or toxicity if regarded as clinically significant.
- failure of the subject to comply with, or who is uncooperative towards any study requirements or restrictions if regarded as clinically significant by the study investigator.

Examples of the informed consent forms and information brochures given to volunteers prior to commencement of the study are included in the appendices.

6.3.2 Study Products

The study products were two gel formulations which were developed specifically for the purposes of this research work (*vide infra* – Chapter 3). The products were protected from direct light and were refrigerated at 4 $\pm$ 2 °C for the duration of use to ensure the stability of the gel formulations.

6.3.3 Study Populations

The volunteers considered for this study were aged between 18 and 50, and were in general good health. In the pilot study, four black African volunteers (2 females, 2 males) with no history of dermatologic disease were chosen to participate. The data generated in the pilot study was used to calculate the power and number of volunteers required to show BE. The pivotal study included 14 black African volunteers (8 females, 6 males).

6.3.4 Product Application

Three 1 x 1 cm^2 application sites were demarcated on the volar aspect of the forearm using Micropore™ 1530 surgical tape (Kleefpleister, Isando, South Africa) with three sites cut out of the tape. The appropriate gel formulation was dispensed from a 0.5 µl Eppendorf® combitip (Eppendorf Ag, Hamburg, Germany) mounted on an Eppendorf® pipette (Eppendorf Ag, Hamburg, Germany) set to dispense approximately 7.3 mg of gel.

The gels were spread over the two application sites using a stainless steel spatula, care being taken to avoid application of the gels to the edges of the tape demarcating the application site. The spatula used to spread the gels was weighed before and after application of the gel to determine the exact amount of gel applied to each site. The third site was used to measure baseline TEWL from the untreated site and used to determine SC thickness. A vaporimeter was used to measure the TEWL following stripping. The blank site from which the TEWL readings were taken was stripped using the same method for the treated sites. Care was taken to ensure that the aperture of the vaporimeter was placed over the stripped site immediately after the removal of the tape strip to avoid bias in the TEWL readings.

Protective covers made of polyvinyl material were used to prevent inadvertent contamination of the application sites and accidental smearing or removal of the gel from the treated sites. Utmost care was taken to avoid unintentional contact of the protective cover with the formulation. The protective covers allowed free flow of air circulation and thus did not occlude the sites.

6.3.5 Validated Tape Strip Extraction Procedure

A suitable extraction procedure to ensure efficient and reproducible removal of ERY from the tape strips was developed. Strips of Transpore™ 1527 tape measuring 1.2 x 1.2 cm^2 were spiked with methanolic solutions of ERY using a Hamilton syringe (Supelco, Buchs, Switzerland). Four solutions were used, the LOQ (1 µg/ml), (3 µg/ml), (7 µg/ml) and (9 µg/ml) and were spiked onto the adhesive side of the pre-cut tape strips in triplicate. The samples were extracted on three consecutive days and samples were analysed using a validated HPLC method. Investigation of the extraction efficiency at concentrations at or close to the LOQ is important since any loss of analyte at these low concentrations could have a profound impact on the results. The LOQ was established as 1 µg/ml of ERY.

Care was taken to prevent seepage of the spiked solution through the porous tape whilst the tape strips were drying in air. Once the tape strip had dried it was placed in a 1.5 ml Eppendorf microcentrifuge tube (Eppendorf Ag, Hamburg, Germany) and 1.2 ml of methanol (HPLC grade, Romil Ltd, Waterbeach, Cambridge, UK) was added in the tube using a micropipette. The tubes were capped, vortexed for 1 minute at high speed on a Lab Dancer Vario vortexer (Yellowline, Staufen, Germany), sonicated for 5 minutes in a sonicator (Ultrasonic Manufacturing Company, Krugersdorp, South Africa) and centrifuged for 5 minutes at 12 000 rpm using an Eppendorf 5414 centrifuge (Eppendorf Ag, Hamburg, Germany). The extracted solution was placed in a glass HPLC vial (Waters Cooperation, Milford, Massachusetts, USA) and analysed.

Samples covering the range of the calibration curve were also spiked onto blank tape strips and treated as described above and used to generate a calibration curve based on extracted samples.

6.3.6 Removal of Excess Formulation

Before commencing the TS studies a protocol was developed to determine the best method for removing excess formulation left on the skin at the end of the dose duration which was previously determined by the pilot study (Section 6.3.6). The use of a cotton swab treated with different solvents to remove excess formulation was investigated. The solvents which were considered were ethanol, isopropyl alcohol (IPA), soapy water and deionised water. A 1 % soapy solution was prepared using Medisan liquid soap (Designer Group, Germiston, South Africa). Application sites were treated with 7.3 g of formulation G1 only as it was assumed if there were any solvent effects at the application sites they would be the same with formulation G2. Ten sites on the forearm were treated with gel and the removal test was performed in duplicate for each solvent. The excess formulation on the remaining two sites was not removed to determine if removal made a significant

difference. The gels were applied one after the other with lag times to make sure that the gels were not left on the skin for longer than one hour. Cotton swabs were used to gently remove the excess formulation and these swabs were assayed to determine the amount of ERY in the dose which did not penetrate through the SC. Five Transpore™ 1527 tape strips were removed sequentially from each of the ten sites. The strips were extracted individually as described and analysed using a validated HPLC method.

6.3.7 Pilot Study

A pilot study was undertaken to determine the dose duration - response relationship of the macrolide under specified study conditions. The aim of conducting the pilot study was to investigate the optimal application time for the gel formulations which would allow for differences between the gel formulations to be accurately and reproducibly assessed. The pilot study was also used to determine the number of subjects required to assess bioequivalence of the ERY topical formulations.

Strips of pre-cut Transpore™ 1527 tape strips (1.2 x 1.2 cm^2) were pre-weighed using an analytical balance (Mettler Toledo Model MX5, Uznach, Switzerland) and placed face down on non-adhesive paper to prevent desiccation of the tape strips. Each gel was applied to three sites on the skin as previously described, for dose durations of 0.5, 1 and 2 hours after which the gels were removed using the developed gel removal protocol. A 15 minute drying period was allowed before stripping to allow TEWL to normalise and hence avoid the possible introduction of bias to the results. Baseline TEWL readings were taken from a site adjacent to the treated sites. The tape strips were pressed firmly onto the skin with two back-and-forth motions using a pair of stainless steel tweezers. Fifteen sequential tape strips were removed, in alternating directions, from each site. Each tape strip was immediately re-weighed after the TS process, placed in a micro-centrifuge tube and extracted at the end of the study. Samples were analysed as previously described using a validated HPLC method.

6.3.8 Pivotal Study

The purpose of the pivotal study was to assess the BE of two different ERY gel formulations. The pivotal study was conducted using the same parameters as those used during the pilot study. The optimal dose duration determined by the pilot study was 2 hours thus the pivotal study was conducted using the same dose duration. All subjects were informed about the nature and purpose of the study prior to its commencement both verbally and using an information brochure and were

requested to complete and submit informed consent forms before agreeing to participate in the study.

6.3.9 Data Analysis

The tape stripping data obtained were analysed using both the one-way ANOVA (GraphPad Prism software Version 4.00 for Windows, GraphPad Software, San Diego California USA) and Locke's Method which is used to obtain a Fieller-type confidence set [180] for the ratio of test product/reference product, the latter being used to determine BE between two formulations in a standard two-period crossover study using untransformed data [181,182].

6.4 RESULTS AND DISCUSSION

6.4.1 Validated Tape Strip Extraction Procedure

The extracted calibration curve resulted in a correlation coefficient (R^2) value of 0.999 and the percentage recovery (% recovery) results are recorded in Table 6.1.

Table 6.1: Percentage Recovery of ERY from spiked tape strips

Actual Concentration (µg/ml)	Calculated Mean Extract Concentration (n=3)	% Recovery	%RSD
1.02	0.97 ± 0.016	94.6	1.61
3.05	2.89 ± 0.058	94.7	2.02
7.03	6.63 ± 0.183	94.4	2.76
8.99	8.53 ± 0.265	94.9	3.11

The results obtained showed that the extraction procedure was efficient and reproducible and hence was suitable for use in the current research work.

6.4.2 Removal of Excess Formulation

The first two tape strips taken from the sites which were not treated with any solvent were analysed but the values obtained were not included in the calculation of the average cumulative amount of ERY on the tape strips as they were assumed to contain ERY which did not penetrate through the SC. Of the four solvents investigated, ethanol and IPA were found to enhance penetration of ERY into deeper SC layers since more ERY was recovered in the tape strips removed from sites treated with these solvents compared to the two aqueous based solvents. The penetration enhancing effect of ethanol was much more profound resulting in approximately twice as much ERY on later tape

strips than with the aqueous based solvents. The deionised water and soapy water gave similar results in terms of the depth and amount of ERY which penetrated the SC.

Figures 6.1 below shows the average cumulative amount of ERY recovered from the five tape strips removed from the relevant skin sites.

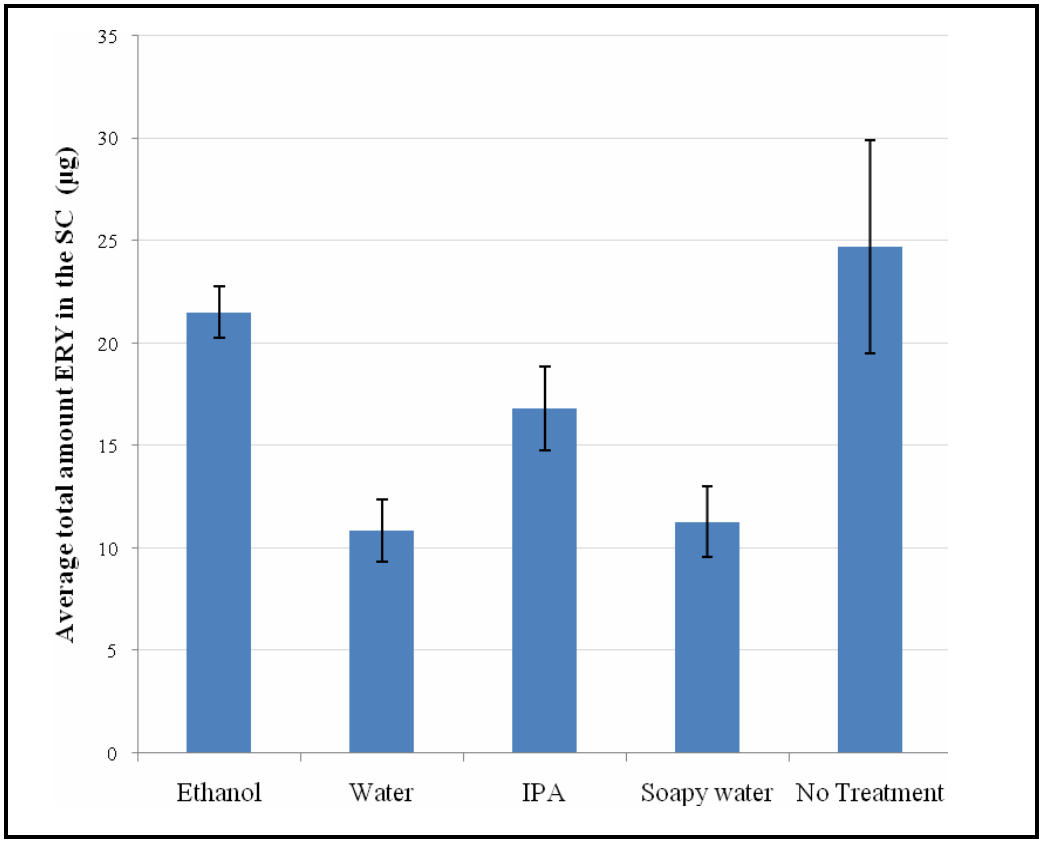


Figure 6.1 Average cumulative amount of ERY recovered from tape strips removed from sites treated with different solvents

A distinct difference was observed between the solvents used when comparing the amount of ERY on the second tape strip to that on the corresponding tape strip on the sites where excess formulation was not removed. The amount of ERY on the second tape strip of the later sites was considerably higher than that on the sites treated with any solvent. In addition to this, the amount of ERY recovered from the sites which were not treated with a solvent was greater than from all the other sites. This may have been due to the presence of furrows [177] in the skin which still contained ERY as there was no physical removal of excess formulation from the skin surface at these sites. These findings confirm the importance of removing excess formulation from the application sites before beginning the stripping process.

The solvent used for the removal of excess gel was deionised water due to the fact that it did not cause any apparent increased penetration of ERY into the SC. It was also chosen because the gel polymer (HPC) is highly soluble in water thus using water would also ensure removal of the cellulose polymer during the removal process. This is an added advantage as it should decrease the possibility of introducing bias when determining the weighed amount of SC removed. Soapy water, although aqueous based, was not selected to avoid possible interaction or interference in the analysis by the various excipients found in the soap.

6.4.3 Pilot Study

All volunteers completed both the pilot and pivotal studies. No adverse events occurred during the treatment period or after the end of the experiments. The average cumulative amounts of ERY were determined and used to compare differences in dose duration-response for the two gels over the three application times. The results obtained are shown in Figure 6.2.

In the female volunteers, the ERY appeared to have penetrated into deeper layers and also to a greater extent than it did for the male volunteers. As a result, the inter-subject variation was much higher when using data from both sexes than when considering a single sex**.** The results showed different absorption trends for the two gels. ERY in formulation G1 appeared to continue to penetrate through the skin 2 hours after topical application while formulation G2 showed a maximum penetration at 30 minutes followed by decreasing amounts of ERY thereafter in both males and females (Figure 6.2).

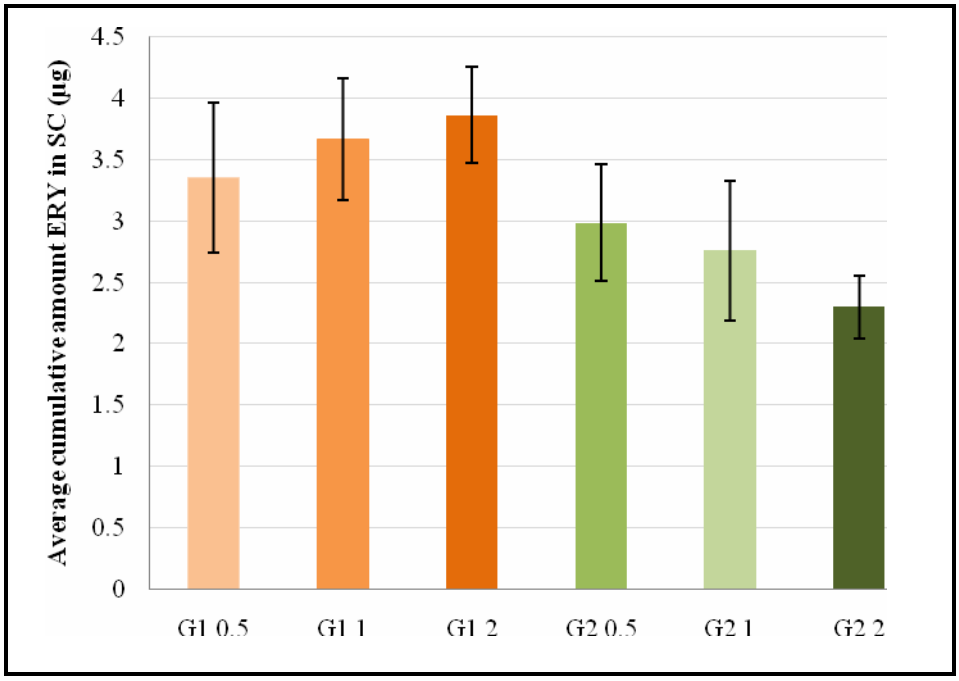


Figure 6.2 Comparison between formulations G1 and G2: Average cumulative amounts of ERY collected after different dose durations in male and female volunteers.

The two gels exhibited the greatest difference in the average cumulative amount of ERY after a dose duration of 2 hours. For each volunteer, the ratio of the cumulative amount of ERY recovered for formulation G1 and formulation G2 was determined (i.e. G1/G2). The CV % (16.2 %) obtained by comparing the mean of this ratio from data obtained from four volunteers was used to calculate of the power of the BE study using a modified version of the Hauschke *et al* formula [183]. The minimum number of volunteers required to infer BE in the pivotal study determined using this method was 14.

6.4.4 Pivotal Study

The average extent of absorption of ERY was different between the two gel formulations. More ERY was recovered from the extracted tape strips for formulation G1 than G2 (Figure 6.3). The extent of absorption for ERY was also greater in the female volunteers than in the male volunteers, as observed in the pilot study (Figure 6.4). The one-way ANOVA analysis of the mean total amounts ERY absorbed from formulation G1 compared to G2 showed that there was a significant difference ($P < 0.0001$) between the means.

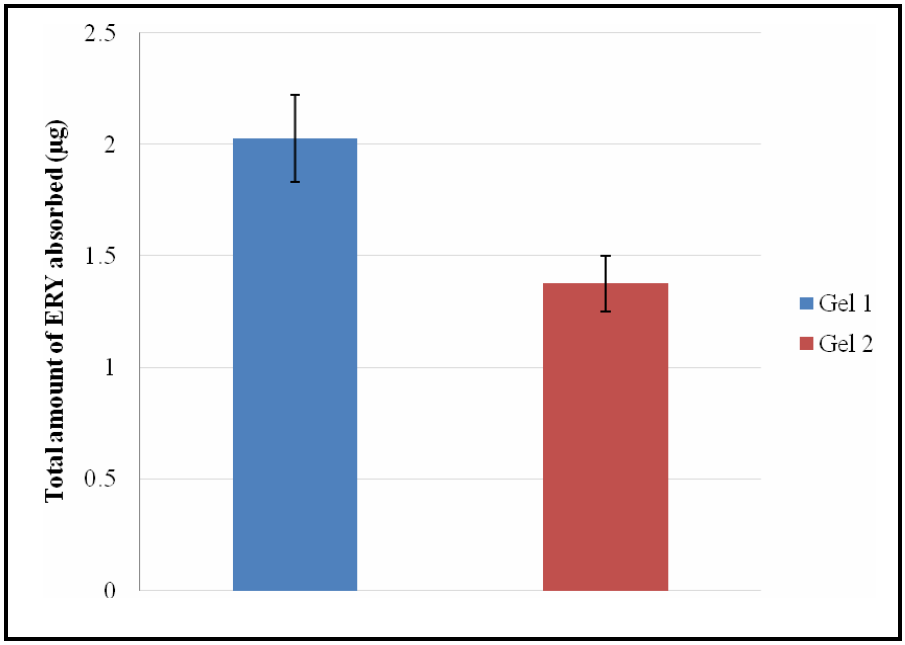


Figure 6.3 Average total amount of ERY absorbed for 14 subjects

The baseline TEWL data and weight of SC removed were used to calculate the apparent thickness of the SC (L) in each volunteer using a spreadsheet gratefully provided by Dr. Richard H. Guy. The thickness of the SC removed with each tape strip (*x*) was calculated using the recovered weight of the SC, the known area of the treatment site and the density of the SC (1 g/cm^3) [178]. (Appendix III).

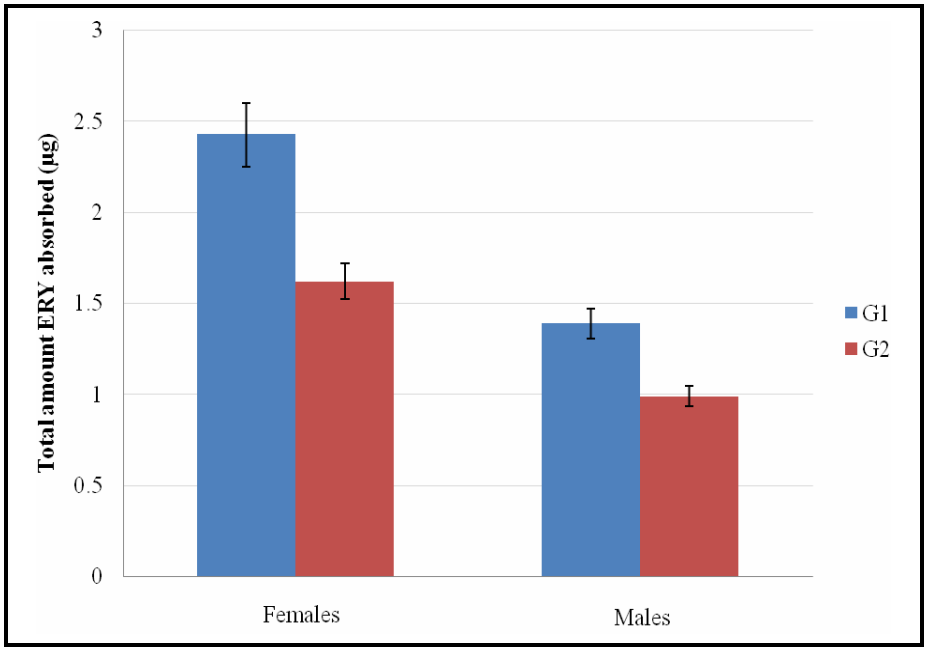


Figure 6.4 Comparison between males and females of total amount absorbed

The values of L and *x* were used to generate drug concentration profiles which were expressed as a normalised function of the relative thickness of the SC (*x*/L) making it possible to compare data obtained from different volunteers. The cumulative drug concentration profiles obtained in this manner are shown in Figure 6.5 below.

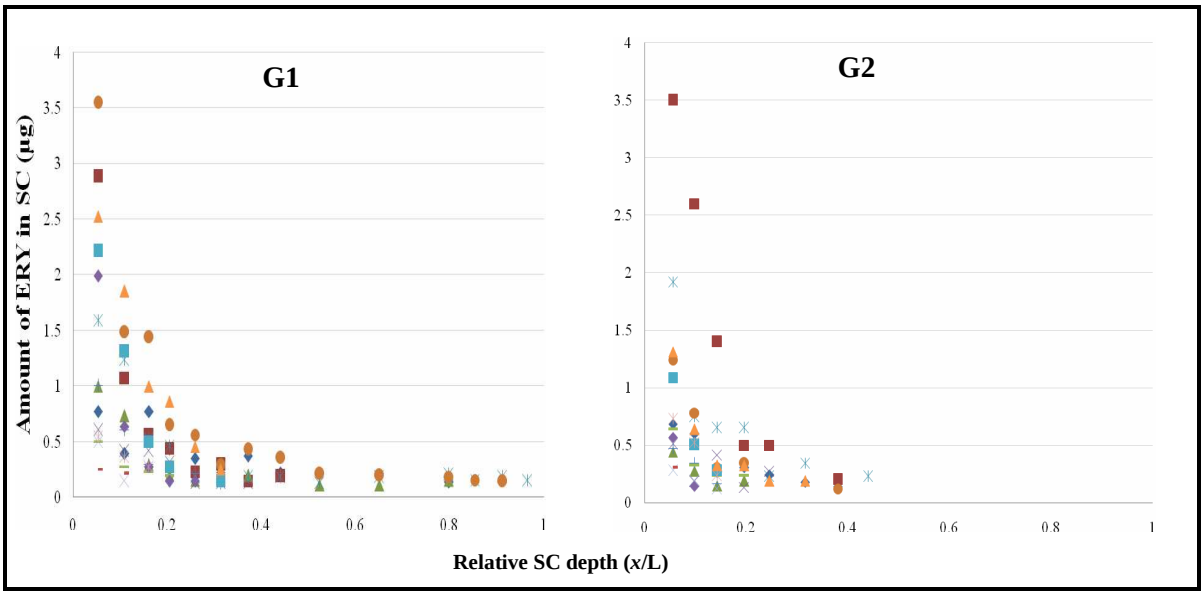


Figure 6.5 Comparative cumulative amount ERY profiles of 14 volunteers for the two gel formulations

The results obtained show that ERY from formulation G1 penetrated deeper into the SC (*x*/L ~ 1) when compared to formulation G2 (*x*/L ~ 0.5). The diagram also shows that for formulation G1 greater amounts of drug were recovered at lower levels of the SC than from formulation G2. These differences can be attributed to the different percentage content of PG in the two gel formulations. As discussed in Chapter 2, PG has been found to be a penetration enhancer and it acts by increasing the solubility of the penetrating substance in the SC. Hence, using higher concentrations of PG should result in a larger increase in the amount of ERY which is able to penetrate the skin.

Figure 6.6 shows the mean penetration profiles of ERY into SC after the 2 hour dose duration following application of the gel formulations to the skin of the volunteers. The results clearly show that ERY penetrated into deeper levels when formulation G1 was applied compared to formulation G2. The results also show that more ERY penetrated into the SC from formulation G1 compared to formulation G2. This clearly indicates that different percentage content of PG can affect the extent and depth of penetration of ERY following topical application.

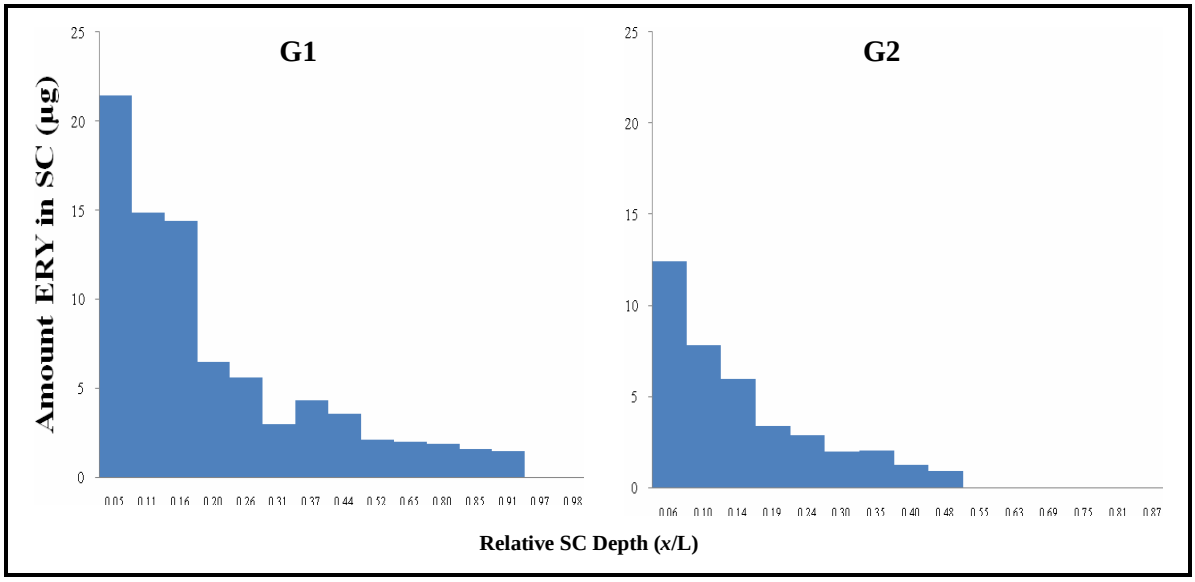


Figure 6.6 Typical penetration profiles of ERY in the same volunteer

6.4.4.1 Bioequivalence assessment

For the purposes of the current research work formulation G1 was considered the reference product (R) and formulation G2, the test product (T). Declaration of BE requires that the test product meets the acceptance criteria stipulated by the FDA and most other regulatory authorities/agencies. Generally, this is achieved when the means of the AUC ratios (T/R) fall within the bioequivalence limits of 80 to 125 % using a 90 % confidence interval [179]. The average T/R ratios were obtained from data of the cumulative amount ERY absorbed through the SC.

Locke's method was used to assess BE between formulations G1 and G2 using an Excel® spread sheet to generate the observed results. The mean ratio of T/R expressed as a percentage was 118.4 % and the calculated limits associated with this mean were 98.7 and 142.6 %. The use of Locke's method results in the generation of a value, G [179], which is calculated inorder to predict whether or not the data being used generates a proper confidence interval which satisfies *in vivo* BE requirements. A value of $G < 1$ is required to infer a proper confidence interval and to concluded that the formulations being assessed meet *in vivo* BE requirements. If $G \geq 1$, the study does not meet the *in vivo* BE requirements. The value of G was found to be 1.25 which indicated that the BE criteria were not met. In addition to this, the data obtained for the two gels, computed from Locke's method fell outside the BE limits of 80 – 125 %.

The results obtained suggested that the amount of ERY released from the test product, formulation G1, was significantly higher than that from formulation G2. The calculated interval of 98.8 – 142.6

% fell outside the BE limits and indicated, unsurprisingly that the two gel formulations were not equivalent. The results indicate that the PG content included in the various formulations had a significant effect on the BA of ERY from the respective formulations. Inclusion of a higher percentage content of PG in formulation G1 resulted in higher percutaneous absorption of ERY compared to formulation G2. These findings were in accord with work published by C. Herkenne *et al* who investigated the effect of PG on the *in vivo* absorption of ibuprofen in human skin. The findings concluded that the greater the amount of PG found in the vehicle the greater the amount of drug taken into the skin as was the case in this particular investigation [178].

6.5 CONCLUSIONS

The high % recovery and low % RSD values obtained during the validation of the TS extraction procedure (Table 6.1) showed that the method used was efficient and reproducible and suitable for use in the current research work. The use of a solvent to remove excess formulation from the application site proved to be an essential step in ensuring the accuracy and reliability of the TS data obtained. It also proved to be an essential step in reducing the variability of the TS data.

From the pilot study, it was estimated that fourteen volunteers were needed for a pivotal study to produce results which would be statistically significant and reliable to assess BE between the two gels under the specified experimental conditions.

The relatively low variation observed with the results from the pivotal TS procedure suggested that the TS method which was developed was efficient and reproducible. The results obtained indicate the utility of TS as a potential tool to determine BE of topical ERY formulations and its possible application to other topical products.

The use of different concentrations of PG in the gel formulations was shown to affect the amount of ERY taken up into the SC. Gel G1 which contained a higher proportion of PG resulted in greater penetration of ERY through the skin compared to gel G2. This sequence of events was the exact opposite of the order of release obtained during the *in vitro* MD studies (*vide infra* – Chapter 5). This observed result may have been a result of the inability of the *in vitro* MD method to predict the penetration enhancing effect of the PG *in vivo*. Studies investigating the effect of PG on skin penetration have shown that PG has the ability to enhance the percutaneous absorption of topically administered medicines (*vide infra* – Chapter 2). In addition, the use of an *in vitro* MD model conducted under predominantly aqueous conditions (30/70; PG/H_20) to attempt to correlate the diffusion characteristics of ERY in the human skin, a predominantly lipophilic medium, was not achievable and hence it was an *in vitro-in vivo* correlation was not established. It was however

possible to conclude that PG does enhance the percutaneous penetration of ERY in the same way as it has been found to enhance the percutaneous penetration of other molecules.

CONCLUDING REMARKS

There is a definite need for an *in vivo* sampling technique which can be used to determine the bioavailability of the active component(s) and bioequivalence of topical formulations. Whereas TS has been extensively used in comparative BA studies, the use of this method has been hampered by issues concerning reproducibility and inter-subject variation, amongst others.

A TS method was developed and validated for use to determine the bioavailability of ERY from two different gel formulations. Factors such as the tape strip application force, removal of excess formulation and the type of tape used were investigated and optimised. Consequently, a reproducible and efficient *in vivo* sampling technique was developed and used to determine the BA/BE of ERY following topical application of two different gel formulations.

In vitro MD showed good potential to determine the pharmaceutical availability by monitoring the release of ERY from topical dosage forms. The *in vitro* MD studies using PG/H_2O as the perfusate lead to the conclusion that the two gels had significantly different pharmaceutical availabilities, and this fact was backed up by the *in vivo* TS studies. The use of SOLV as a perfusate showed that this was not a suitable solvent to use when trying to determine pharmaceutical availability *in vitro*. However, this solvent was developed for possible use as a perfusate for future *in vivo* MD studies and it still remains to be determined whether the use of SOLV as the perfusate in *in vivo* MD will have the necessary discriminatory power to determine similarities and/or significant differences in the BA of topical formulations for successful application for BE assessment of topical dosage forms.

The statistical power of the pivotal study proved to be sufficient to conclude that the two gel formulations were not BE. The differences were attributed to the different percentage content of PG in the gels. The *in vitro* MD studies provided a valuable tool for the assessment of the pharmaceutical availability of ERY from the topical formulations and showed that in the case of formulation G1, less ERY diffused out of the gel into the surrounding medium than formulation G2. However, this order was reversed in the *in vivo* TS studies. The reason for this was that a predominantly aqueous medium was used for the *in vitro* studies and since ERY is a hydrophobic drug, the tendency for the drug to diffuse out of formulation G1 which had more PG (hence was more lipophilic) was less than the tendency for ERY to diffuse out of formulation G2 into the aqueous perfusate. In addition to this, in the *in vivo* studies, the PG in the formulations enhanced

the penetration of ERY into the SC and this effect increased as the percentage content of PG in the formulation increased which accounted for the higher release of ERY from formulation G1 compared to formulation G2.

An HPLC method was successfully developed and validated and was suitable for use to analyse samples generated by both the *in vitro* MD and *in vivo* TS studies.

Two gel formulations which differed in the percentage content of PG were formulated and produced. The gels were clear, transparent and homogenous and had the characteristic odour of ethanol. They were stored in a refrigerator at 4 $\pm$ 2 ºC and remained stable over the period of use.

In vitro MD and TS techniques were successfully applied to determine the pharmaceutical availability and BA/BE of ERY from topical gel formulations, respectively. The data indicate that *in vitro* microdialysis may be useful as a tool for the assessment of the pharmaceutical availability of topical formulations and that the BA/BE of ERY from topical gel formulations can be determined using TS.

Establishment of *in vitro-in vivo* correlations provide valuable data to assist in the design of dosage forms as well as providing useful insight on BE. During in vitro experiments, choice of conditions and in particular the perfusate was extremely challenging and attempts to identify the 'ideal' perfusate were constrained by attempts to simulate both the properties of human skin and as well as considering physiological compatibility. A physiologically compatible perfusate consisting of 4 % dextran 70 in normal saline was chosen to prevent volume loss during the *in vitro* MD experiments due to osmolarity differences between the gel formulations and normal saline perfusate.

The use of SOLV as the perfusate showed no differences in the release properties of the two gel formulations whereas marked difference were shown when using PG/H_2O as the perfusate. The issue of whether or not these differences were real is debatable and in future studies emphasis should be placed on backing up *in vitro* MD release studies with suitable *in vivo* experimental work in an attempt to confirm any differences or similarities observed and to establish an *in vitro-in vivo* correlation. Although the use of SOLV as a perfusate did not show any differences in the release properties of the gels *in vitro*, further application of this system to an *in vivo* setting would confirm that any differences observed following topical application of the gels to the skin are real and are

not influenced by the perfusate or other factors associated with the experiment, but are a result of true differences in the diffusion characteristics of ERY from the gels into the skin.

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APPENDIX I

INFORMATION FOR VOLUNTEERS BROCHURE

INFORMATION FOR VOLUNTEERS BROCHURE

APPLICATION OF TAPE STRIPPING FOR THE ASSESSMENT OF THE BIOAVAILABILITY OF ERYTHROMYCIN IN HEALTHY VOLUNTEERS

STUDY NUMBER: NM_01_2007

PROTOCOL VERSION 1

BROCHURE VERSION 1 JULY 2007

1 Study objective

The study involves research to assess the rate and extent to which erythromycin diffuse into the skin from a gel product using tape stripping as a measure of efficacy. This study will provide valuable insight for use in the assessment of sameness of two erythromycin gel formulations.

The study will involve 14 subjects.

2 Ethical considerations and standards of practice

Several different topical erythromycin formulations have been used in the past in treating *acne vulgaris*. To date, there are no approved methods for use to assess the bioequivalence of topical dermatological products such as these, with the exception of topical corticosteroids. There are many different vehicles used in the production of topical ERY formulations but so far, no studies have been carried out to assess the bioequivalence of these formulations. Medicine control bodies worldwide require that studies should be conducted to provide evidence that such products are indeed equivalent in their therapeutic efficacy. This is an indirect way of demonstrating that the products will both be effective clinically and is demonstrated by what are known as ‘comparative bioavailability or bioequivalence studies’.

2.1 Written informed consent

Preceding the study, the nature, purpose and risk of participating in the study will be explained to you. Should you wish, you will be given time overnight to consider the information and any questions that you might have will be answered. The nature of the insurance cover will also be explained. If you decide to participate in the study you will sign a consent form in the presence of a witness. You are encouraged to consult your parents or personal medical doctor for approval in this study.

3 Voluntary nature of participation

Your participation in this study is entirely voluntary and you may withdraw from the study at any time, without prejudice. Should you decide to participate, we ask that you try to be committed to completing the study if at all possible. Should you encounter any problems along the way, please speak to me so that every effort can be made to assist you.

4 Dates and duration of the study phases

The study consists of one phase only which will run over a day. The study will be conducted from 0900 hours until 1300 hours.

5 Place of study

The study will be conducted in the clinic (Room T17) in the Faculty of Pharmacy building at Rhodes University. Room T17 is on the top floor of the Pharmaceutical and Chemical Sciences building in Artillery Road on the Rhodes University campus. The principal investigator, supervisor and the assistant investigator will be suitably qualified, trained and experienced to perform the study procedures.

6 Background information

Erythromycin is used in the management and treatment of patients with *acne vulgaris*. The gel is usually applied to the affected areas twice daily.

Tape stripping is a technique employed to study the penetration of drugs into the skin. Tape stripping involves the sequential removing of microscopic layers (typically 0.5 -1 µm) of the stratum corneum (outer dead layers of skin) by placing an adhesive tape strip onto the skin surface with uniform pressure which is then subsequently removed. Tape stripping is simple and has been described as a minimal invasive technique. Although tape-stripping is considered to be essentially non-invasive, stripped sites in certain dark-skinned individuals may remain darker the usual for several months after healing.

7 Study design

Templates for use with the tape stripping study will be affixed on your right arm with pieces of Micropore™ 1530 tapes. These will be used to demarcate (1 cm x 1 cm) the stratum corneum sampling site. The templates will be designed to have three sampling sites, two sites for the application of the formulations and the other for use as a control. The test and reference formulations will be applied to the two application sites and after a 2 hour exposure period the

excess drug will be removed by wiping with a wet cotton swab. Adhesive tapes (Transpore™ 1527 tapes) will be applied concurrently to both application sites with uniform pressure and then subsequently removed. 15 successive strips will be removed from each site. The amount of moisture loss measurements will be recorded from the control site using a small instrument which is placed on the skin. This is entirely pain free. This procedure will take approximately 1 hour. The entire study will conclude at 1300 hours.

8 Adverse effects

For topical preparations, delayed hypersensitivity dermatitis may occur at the site of the application, but this is uncommon. Cases of reddening of the skin and inflammation of the skin have also been reported. It is advised that erythromycin gel preparations should not be used on open wounds or lesions on the skin or near the eye. The skin reactions are however reversible on discontinuation of therapy. Erythromycin must not be administered to healthy or ill individuals who have an allergy to this compound or to any of the ingredients in the formulation. In this study, a single application to a limited area is unlikely to invoke any adverse effects but procedures will be in place to address any discomfort noted during and after the study.

The tape stripping technique is safe and no adverse reactions such as irritation or allergic reactions to the tape have been reported so far. Although tape-stripping is considered to be essentially non-invasive, stripped sites in certain dark-skinned individuals may remain hyperpigmented for several weeks after healing.

9 Conditions of participation in the study

To participate in this study you must:

i. Undergo a skin test to assess any possible allergy or sensitivity to the adhesive in the medical tape that will be used.
ii. Fulfil certain inclusion and exclusion criteria which have been set out in the protocol.
iii. Agree to be fully committed to the study and conscientiously abide by the restrictions required of you as listed in section 11.

NB: It is extremely important that you divulge any past medical history and abide by the rules for participation in this study. This is to protect you from unnecessary risks and to help ensure the reliability of the data gathered.

10 Volunteer inclusion/exclusion criteria

You will be considered for this study if you are aged between 18 and 50, in general good health, available for the entire study period and if female be on reliable contraception or abstaining from sex.

Please do not consider participating in this study if you

i. are breast feeding.
ii. are contemplating becoming pregnant in the time immediately following the study.
iii. are pregnant.
iv. have a known allergy/hypersensitivity to erythromycin or any macrolide antibiotics.
v. have any history of drug or alcohol abuse.
vi. have any mental deficiency or handicap.
vii. have hairy ventral forearm surfaces and/or abrasions on the underside of their forearms.
viii. have engaged in any sun-tanning or taken any sunny vacations within the last month.
ix. have participated in another tape stripping study within 2 months of the study date.
x. have used any macrolide antibiotics within the last three months.
xi. suffer from any allergic conditions (hayfever, allergic rashes, asthma or childhood eczema).
xii. suffer from any skin disorder such as psoriasis, eczema or other relevant skin disorder.
xiii. take regular medicine or tablets or used any creams within the last week (contraceptive pills excluded).
xiv. have a history of any neurological, kidney or liver disorders.

11 Study restrictions

Restricted item	Duration of restriction	Examples of restriction	Comments
Prescription drugs	From 1 week before the start of the study, until the end of the study.	-all medication obtained on prescription -antibiotics, vaccinations -anti-inflammatory medicines -anti-asthmatic drugs -anti-acne drugs etc.	This includes all long term medication
Over-the-counter (OTC) drugs	From 1 week before the start of the study, until the end of the study.	-anti-flu drugs, sports supplements, antacids, paracetamol -vitamins, minerals -homeopathic medicines	This includes herbs, natural products & all medications that can be bought without a prescription.
Alcohol	From 24 hours before the start of the study and during the study. **No alcohol for a total of just over 2 days**	All alcoholic drinks and alcohol containing foods.	**It is important that this requirement is taken seriously and observed, as alcohol can significantly affect the liver.**
Strenuous Physical Exercise	From 12 hours before the start of the study.	Rugby, Squash, Rowing, Gym, Tennis etc.	Light exercise such as walking is permitted.
Smoking	No smoking will be permitted during the study.	Cigarettes	No cigars or pipe smoking.
Moisturising creams	You must refrain from applying any type of skin conditioning creams to their forearms from 24 hours before the study until the end of the study	All skin creams, e.g. moisturizers, Vaseline, medicated creams, aqueous cream and tanning lotions.	This could interfere with the absorption of the study gel.

NB: Random checks will be done to ascertain whether you have managed to adhere to the above restrictions. If you have not been able to adhere to the study restrictions please inform us immediately or else you may not be able to participate with a reduced remuneration as detailed in section 15.

12 Procedures and duration of the study

The study consists of a single phase. The procedures and duration of the study are detailed below.

On the morning of the study you must check into the clinic (Room T17) in the Faculty of Pharmacy building at 08:45 hours. At check-in you will be:

i. Questioned and undergo a brief medical examination to establish whether you still fulfil all the inclusion and exclusion criteria since your pre-study medical examination and that you have complied with the study restrictions.
ii. Prepared for product application by having medical tape applied to your forearms.
iii. The study will commence at 0900 hours.
iv. You are not restricted with regard to food and drink during the study period.
v. Should any new and significant information about the study medication become available during the course of the study, this will be communicated to you.
vi. A tube of 0.1% m/m hydrocortisone cream will be given to you to self medicate your arms twice daily for one week.

13 Financial compensation

You will receive a gratuity of **R 50.00** for full participation in the study. Payment following withdrawal from the study will be calculated on a pro-rated basis from the start of the study to the end of the study.

14 Adverse medical events

You will be monitored during the study for any adverse events whether or not they are thought to be related to the investigational products or procedures. If any adverse events are reported, a decision will be made as to whether or not to withdraw you from the study and what treatment is appropriate. All adverse events will be monitored and treated appropriately until a satisfactory outcome is attained.

15 Confidentiality

Your medical history and physical examination records and any other information or data generated during this study will be kept confidential. However, you must agree that all the above mentioned documentation and data can be released for any lawful purpose and released for publication in scientific journals and/or presentation in a thesis submitted to Rhodes University in fulfilment of the requirement for the degree of Master of Science (Pharmacy) after the completion of the study.

In such cases your name will be removed from all documentation to ensure your anonymity. In signing the consent form for the study, you agree to the granting of access to your medical data. Your medical data will be provided to you upon request and you will be informed of significant abnormalities identified before or after the study.

16 Amendments/Changes

Should there be any changes made to the trial protocol these will be communicated to you verbally and in writing in time to enable you to reconsider your decision to participate in the study.

17 Withdrawal

You may withdraw from the study at any time due to the following:

i. Voluntary withdrawal by yourself due to any reason.
ii. Illness or injury during the study if regarded as clinically significant by the principal investigator and the supervisor.
iii. Any adverse event or signs of toxicity if regarded as clinically significant by the principal investigator and the supervisor.

18 Termination of the study

The BRG reserves the right to terminate the study prematurely in the interests of your welfare.

19 Emergency contacts

You are obliged to notify the principal investigator as soon as possible if you are unable to follow the procedures or if you suffer any adverse event we have not told you about. In particular, you should make every effort to contact me if you suffer a Serious Adverse Events (SAEs) or need to take additional medication of any kind. This applies to out of hours, as to normal work time.

In such cases of medical emergencies during the study, or if you have any urgent questions relating to adverse effects or unrelated illness, please feel free to telephone the principle investigator.

Work 046 603 8412 Cell 072 721 1357

Contacts for additional information The following individuals are responsible for conducting this study and as such may be approached for more information:

Principal Investigator **Nyaradzo Mandimika** *BPharm, MSc Candidate (Rhodes)*

Supervisor **Isadore Kanfer** *BSc (Pharm), BSc (Hons), PhD (Rhodes)*

If you have questions about this study which have not been answered adequately by the principal investigator you should first discuss them with your doctor.

APPENDIX II

STUDY PARTICIPATION INFORMED CONSENT FORM

STUDY PARTICIPATION INFORMED CONSENT FORM

APPLICATION OF TAPE STRIPPING FOR THE ASSESSMENT OF THE BIOAVAILABILITY OF ERYTHROMYCIN IN HEALTHY VOLUNTEERS

STUDY NUMBER: NM_01_2007

I ..... Born on.....
Present address .....
..... hereby give permission that the necessary tape stripping procedure conducted as described to me by the principal investigator and that the formulations stated below be applied to my skin during the course of this study.

Test product: Erythromycin G1 2 % m/m gel.

Reference product: Erythromycin G2 2 % m/m gel.

My consent is given freely and I realise that it may be withdrawn at any time, without penalty to me. Furthermore, I understand that I do not give up any of my legal rights by signing this consent form.

I have been fully informed by ..... regarding the possible adverse effects of the medication, procedures to be used in this study and the risks thereof, as detailed in the "Information for Volunteers Brochure". I will receive a copy of the information brochure and signed consent form for my records.

I undertake to comply with all the relevant conditions contained in the Information to Volunteers Brochure and confirm that I understand that it is important not to withhold or misrepresent any information asked of me.

I undertake to inform the principal investigator immediately of any symptoms -expected or unexpected -which I might experience.

I agree to my medical records being reviewed in the event of an audit, enquiry, monitoring and/or inspection on the understanding that my anonymity will be maintained.

I have been informed that if I do not adhere to the protocol, it may result in my exclusion from the study and forfeiture of the agreed upon remuneration. I acknowledge that instructions relating to my participation in this study have been communicated to me both verbally and in writing, and that I understand them.

I also declare that I have made the necessary arrangements regarding the attendance of lectures and

other academic activities.

I understand that a policy to cover volunteers in clinical studies against death or disablement arising as a direct result of participation in such clinical studies has been taken out by the Biopharmaceutics Research Group. I accept the conditions of the policy as set out in the insurance policies.

I acknowledge that I will receive **R 50.00** for full participation in this study and that I will receive a pro-rated amount if I withdraw from the study before it has been completed.

Signed at .....this .....day of ..... 20.....

..... **.....**
Volunteer (Print name) **Volunteer (Signature)**

..... **.....**
Witness (Print name) **Witness (Signature)**

.....
Nyaradzo Mandimika
Principal Investigator
BPharm, MSc Candidate (Rhodes)

APPENDIX III

TEWL READINGS FOR EACH INDIVIDUAL VOLUNTEER

Individual Volunteer TEWL values (g/m^2h)														
Site Number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Blank	2.6	3.5	1.9	4.1	2.9	4.8	11.7	3.4	3.2	3.4	4.9	3.6	3.8	1.6
1	4.3	3	2.3	1.5	1	3.7	9.4	3.5	2	2.5	3.9	3.7	2.6	1.6
2	3.5	2.3	2.2	1.9	1.7	4.8	5.6	2.7	2.2	2	4.1	4.8	2.8	1.4
3	3.4	3.4	3	1.3	1.4	3.7	5.7	3.3	3.2	3.1	4.6	3.5	1.5	1.2
4	3.2	3.7	2.2	2.7	3.2	4.3	5.5	4	3.6	3.1	4.8	4	2.2	2.5
5	3.8	4.9	3.6	2.9	3.2	4.3	7.9	3.6	4.3	2.7	5.6	5.2	3.6	2.7
6	3.9	6.9	3.5	3.6	3.4	5.2	6.1	5.3	4.7	4.1	5.5	5	4.1	2.5
7	3.8	5.2	3.7	12.7	3.2	6.5	7.2	5.5	6	6.4	6	6.2	2.3	3.5
8	5.2	6.1	4	11.6	3.1	6.3	7.1	5.7	7.4	5.4	6.8	7.7	5.2	3.6
9	5	6.4	4	11.5	5.2	5.6	7	7.6	7.7	5.2	6.9	7	7.2	3.2
10	6.5	6.1	4.4	12.9	6.3	6.2	8.2	5.6	8.8	7.5	10.3	7.5	7.7	6.7
11	5.5	7.3	4.7	15.3	6.8	7.7	10.3	4.3	11.2	7.3	10.1	8.2	8.4	5.3
12	5.5	7.3	4.8	15.2	7.2	5.6	10.8	5.6	12.9	8.3	10.2	8.6	10.2	5.6
13	7.9	9.4	4.7	18.8	9.2	9	12	10	12.4	10.1	9.3	10.7	7.4	7.3
14	7.6	9.3	5.8	19.5	8.3	11.9	11.1	8.2	12.1	9.8	10.2	9	11.7	9.5
15	8.9	10.4	8.1	18.8	8.2	9.2	12.6	11	11.9	10.1	11.6	11.7	11.1	10.1
SC Thickness (µm)	12.43	3.37	5.45	3.72	2.77	5.34	9.15	5.49	4.53	2.78	4.56	3.63	4.99	4.34