A STUDY OF THE TRANSDERMAL DRUG DIFFUSION PROPERTIES OF ROOPEROL TETRA-ACETATE

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ABSTRACT

The rapidly growing interest in the potential use of topical drug delivery formulations has resulted in increased use of the skin as a vital port for drug delivery. Extensive research has been conducted in designing vehicles capable of delivering a desired amount of drug to a specific site, to produce the desired pharmacological response. Rooperol tetra-acetate is a lipophilic, cytotoxic drug with the potential for use in the treatment of solar keratosis. For effective pharmacological action, delivery of the drug to the epidermal/dermal junction of the skin is required. A study of the topical penetration properties of rooperol tetra-acetate from different topical bases, each possessing different physico-chemical properties, was performed. The assessment involved a comparison of the diffusion properties under occlusive and non occlusive conditions when the drug was formulated into a gel, Cetomacrogol Cream B.P. (oil-in-water), Simple Ointment B.P. and an extemporaneously prepared water-in-oil topical cream. The *in vitro* experiments were conducted using polydimethylsiloxane and rat membrane mounted in a Franz diffusion cell. The topical permeation kinetics of rooperol tetra-acetate were determined by exploring the release characteristics of the active ingredient from the vehicles formulated and the permeability properties of the drug through the membranes employed. Further studies involved investigating the utilization of supersaturated systems intended to increase the thermodynamic activity of the drug when formulated into a propylene glycol/water vehicle (with and without polymer).

To measure the release of rooperol tetra-acetate into the skin from a topical base it was necessary to, firstly, develop a suitable quantitative method for the analysis of the active drug in the aqueous receptor phase of *in vitro* diffusion cells. The second stage of product development was the design of an effective delivery system to facilitate the release of the diffusant from its base. A high performance liquid chromatographic method was utilized for the identification and quantification of the active drug. As validation is an important aspect in the development and subsequent utilization of an analytical procedure, the developed HPLC technique was validated by determining the precision, accuracy, range, limit of quantitation and sensitivity of the

system. Lastly, the stability of rooperol tetra-acetate at elevated temperatures was assessed and a stability profile of the drug was generated for the three-month period of analysis.

The results obtained following chromatographic analysis of the receptor phase sampled during the diffusion experiments indicate that the gel and oil-in-water formulations most effectively promoted the diffusion of rooperol tetra-acetate across polydimethylsiloxane membrane. The water-in-oil system exhibited lower flux rates and the ointment showed the least drug release. Occlusion of the topical vehicle increased the diffusitivity of the permeant from all formulations analysed. The permeation assessment results of the supersaturated systems showed enhanced diffusion of rooperol tetra-acetate across polydimethylsiloxane and rat membrane. The high thermodynamic activity existing in supersaturated systems most effectively increased the driving force for drug diffusion resulting in enhanced percutaneous penetration of rooperol tetra-acetate beyond the release and transport limitations of saturated solutions. These results provide the basis on which an effective topical drug delivery vehicle may be designed for this new drug entity.

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

1.1. IDENTIFICATION OF THE PROBLEM

1.1.1. SOLAR KERATOSIS

Solar keratosis mostly arises as a result of chronic skin exposure to ultraviolet A radiation. The first indications of recurrent sun damage of the skin are small hyperkeratotic lesions (solar keratoses) which, if untreated, may develop into more aggressive forms of skin cancer such as squamous cell carcinoma. The keratotic lesions are easily recognised as pinkish macules or papules with a rough adherent scale and they usually appear on body parts most frequently exposed to the sun, especially, the face, neck, hands and forearms [FIG 1: see arrows].

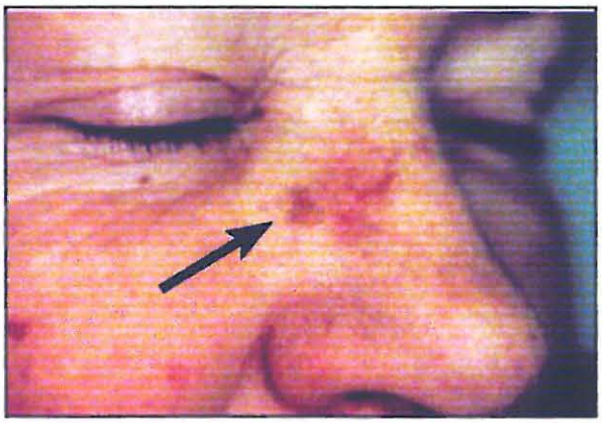


FIGURE 1a: Keratotic lesion on the facial region

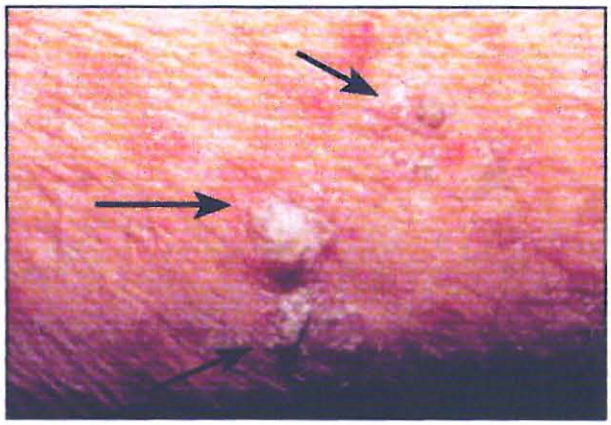


FIGURE 1b: Close-up of keratotic lesions

Typical histological changes which occur within keratotic skin are illustrated in Figures 2a and 2b. Abnormally high keratin production is particularly evident in Figure 2b while Figure 2a clearly shows characteristic overgrowth and thickening of the epidermis accompanied by degeneration of the elastic fibres of the connective tissue in the stratum corneum [A and B in FIG 2]. The incidence of photo carcinogenesis is related to the amount and type of melanin synthesized in the skin. Light-skinned persons produce less melanin and are therefore more susceptible to solar keratosis. The most common treatment for skin cancers is surgical excision. Alternatively, intensive x-ray

therapy can be used, however, this procedure is less successful than surgery. Most cytotoxic drugs currently in use fail to distinguish between cancer and normal healthy cells thus resulting in considerable damage to the normal cells. Newer treatment options involve the use of selectively delivered cytotoxics. Rooperol tetra-acetate is a novel drug designed to retard the metabolic and proliferative activity of malignant cells through direct targeting of the tumour. Such selective therapy is desirable since potential side effects are minimized as a result of limited damage to normal cells.

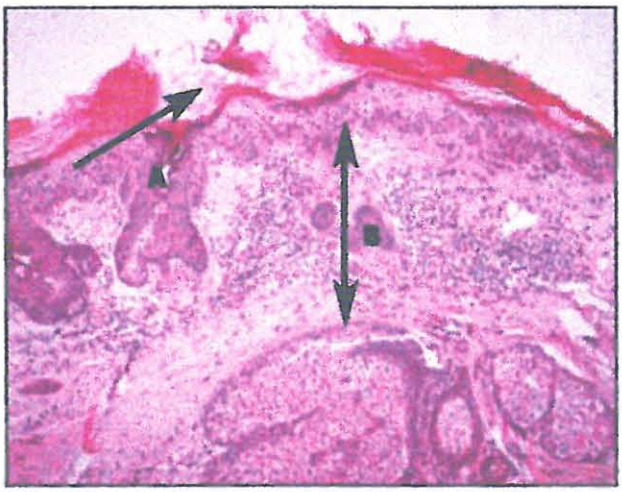


Figure 2a: A histological illustration of keratotic skin. (A) shows degeneration of the stratum corneum and (B), overgrowth of the epidermis.

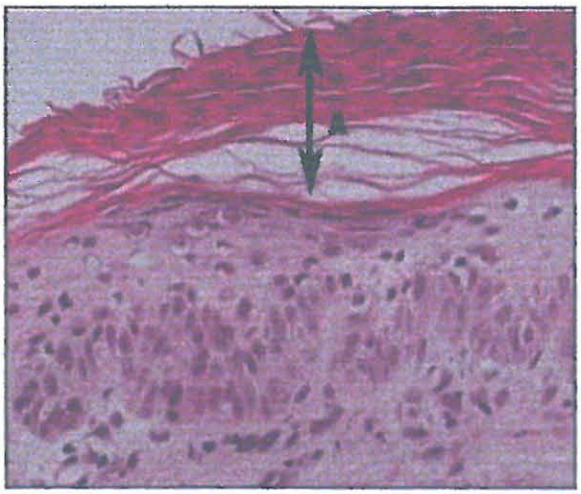


Figure 2b: A histological illustration of keratotic skin showing hyperkeratosis in the stratum corneum.

Most cytotoxics are administered orally, hence, they are rapidly eliminated from the body by hepatic first pass metabolism. In contrast, transdermally administered compounds bypass hepatic metabolism resulting in constant and therapeutically effective drug levels in the vascularized dermal layer. Additionally, simple and rapid termination of drug input in problematic situations allows for better control over topically applied dosage forms [1,2,3]. Skin application of cancer growth inhibitors can also mean reduced treatment stress for the patient due to the design of a non invasive, painless and less aggressive way of treating cancer.

1.2. THE ACTIVE INGREDIENT: ROOPEROL TETRA-ACETATE

Rooperol, when exposed to air, is highly susceptible to oxidation. Acetylation of rooperol results in the formation of rooperol tetra-acetate, a chemically more stable derivative of the parent compound [FIG 3]. Various clinical studies on the aglycone of the hypoxoside, rooperol, have successfully demonstrated the usefulness of the chemical as a cytotoxic substance with the potential for use in the treatment of solar keratosis [4-6].

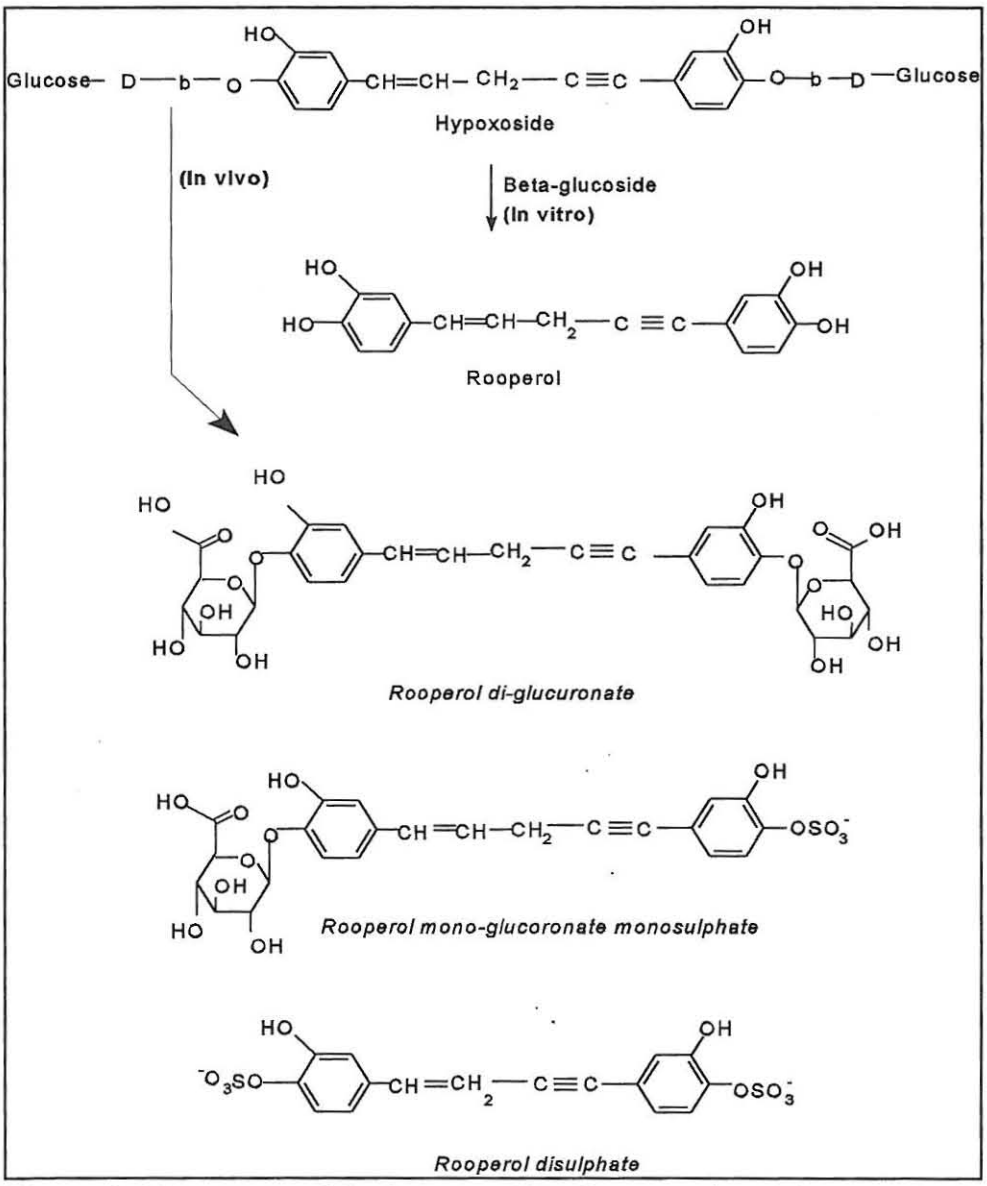


Figure 3: Structures of the hypoxoside of rooperol and the metabolic products. To obtain rooperol tetra-acetate, the four hydroxyl groups of rooperol are each replaced by an acetate moiety.

The active hypoxoside component is first extracted from the corms of *Hypoxis rooperi* and *Hypoxis latifolia* [7] before being deconjugated to rooperol using the enzyme, beta glucosidase. The mechanism by which rooperol tetra-acetate effects its cytotoxic activity is believed to be through the disruption of chromosomal structures during the mitotic stage of cell development [5]. Problems associated with the delivery of clinical amounts of rooperol tetra-acetate into the skin from a topical vehicle necessitated the development of an effective delivery system for the drug. The criteria used in selecting this delivery system were stability, compatibility of the system with the drug and the potential for effective drug delivery.

1.3. THEORY OF TRANSDERMAL DRUG DIFFUSION

1.3.1. SKIN PHYSIOLOGY AND HISTOLOGY

The skin is one of the most readily accessible organs of the human body. Its varied functions are vital to the survival of human life. Skin tissue consists of aggregates of closely packed cells that have both lipid and aqueous regions [9] [FIG 4].

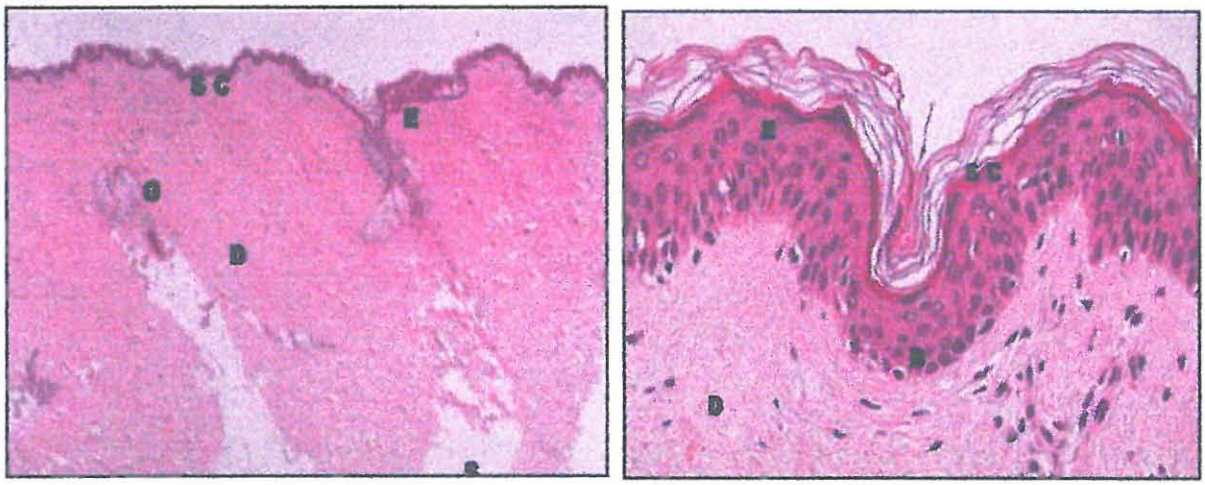


Figure 4: The structure of the skin. (SC), stratum corneum; (E), epidermis; (B), basal layer with melanocytes; (D), dermis; (S), subcutaneous fat and (G), glands.

The regulatory functions of the skin include the maintenance of temperature and blood pressure [10,11] while its protectional function offers defence against physical and chemical damage and invasion by micro-organisms. The skin is a heterogeneous structure consisting of stratified levels which can be divided into three distinct tissue layers, [3,4] the epidermis, dermis and subcutaneous fat. Adding to the complex nature of skin are blood vessels, hair follicles, glands and nerves which traverse the layers of this organ. To understand the utilization of the skin as a route for drug delivery, knowledge of its structure and enzymatic activity is essential, especially when investigating the transdermal diffusion properties of a novel drug like rooperol tetra-acetate.

Epidermis

The epidermis, which is in contact with the external environment, is a non-vascular layer pierced by hair follicles and sebaceous glands that allow the passage of nutrients and waste products from the dermis to the skin surface [11]. Anatomically, the epidermis varies in thickness, with the palms and soles being the thickest regions while the eyelids are the thinnest area of the skin [9,12]. The outermost layer of the epidermis comprises stratified epithelial squamous cells [13] called the stratum corneum. It is this external layer which forms the physical barrier of the skin to exogenous material. The diffusitivity of topical chemicals is dependent on the physiological condition of the stratum corneum. Immediately below this keratinised epithelial tissue lies a monolayer of constantly dividing cells called the stratum basale. The stratum basale produces keratinocytes which migrate towards the skin surface where they ultimately form the cells of the stratum corneum. Additionally, the stratum basale contains melanocytes the function of which is to transport melanin granules to the keratinocytes. The melanin produced is transported by the keratinocytes to the stratum corneum where it provides pigmentation to the skin and offers protection against radiation [14]. Oxygen and nutrient deprivation of the mitosing cells from the stratum basale causes shrinking and a loss in metabolic activity in these cells. As the cells reach the skin surface, they become flattened keratinised laminates which are eventually removed from the surface by sloughing. It takes approximately 4 weeks for

a keratinocyte to migrate from the basal region to the stratum corneum and eventually to the outer layer where it is removed by abrasion. Although the stratum corneum is highly impervious to the ingress and egress of water, when it is immersed, it is capable of absorbing large quantities of water. Various intercellular lipid processes such as cholesterol, fatty acid and sphingolipid synthesis are known to occur within the epidermis. The barrier function of the stratum corneum is influenced by the presence of these intercellular lipids and inhibition of the synthesis of these components can result in delayed barrier formation, abnormal lamellar bodies and progressive barrier perturbation. Enzymes are also involved in skin barrier homeostasis. Diminished enzyme production is said to arrest hydrolysis and the resultant water loss from the skin surface may lead to diminished barrier efficacy.

Linking the dermis to the epidermis is an anatomic structure called the dermo-epidermal junction [10,14]. This junction provides chemical support for the dermis and in addition, it controls the passage of cells and some molecules across the two layers.

Dermis

The dermis, which constitutes the bulk of the skin structure consists of a matrix of connective tissue made from fibrous proteins [9]. The dermis interacts with the epidermis via a network of blood vessels, nerves, lymphatics and skin appendages existing within its structure [13,14]. The rich blood supply existing in the dermis not only regulates body temperature, but also transports nutrients to the skin, removes waste products, initiates the defence mechanism of the body and contributes to skin colour [12]. The excellent blood supply in the dermis functions as a "sink" for diffusing molecules. The sink condition keeps the concentration of penetrating molecules very low thereby maintaining high concentration gradients across the skin layers and in doing so, provides the driving force for the delivery of topical drugs.

Enervation of the skin varies from region to region with the face and extremities containing a high profusion of neural supply. The sensations of pain, touch and temperature are jointly activated by the cutaneous nerves and the blood supply within the region.

Skin Appendages

The functions of the skin appendages are also sustained by the blood, nervous and lymphatic systems present in the dermis [9,12]. The pilosebaceous unit is a duct comprising hair follicles and the sebum secreting sebaceous glands. The follicles provide an opening for the extrusion of keratinous hair filaments and they occupy a low proportion (about 0.1%) of the available skin surface. The sebaceous glands receive a rich supply of blood and high enervation of the follicles provides the hair with good sensory function. Sebum, derived from the sebaceous gland, is a mixture of lipids, mainly triglycerides, wax esters, squalene, cholesterol esters and cholesterol. The principal function of sebum is to act as a natural skin lubricant, bacteriostat and fungistat [11]. It is believed that the sebum has little, if any, effect on overall drug diffusion [15].

Sweat Glands

The sweat glands are divided into eccrine and apocrine glands [9,11-13]. Both sets of glands originate from the dermis and surface onto the stratum corneum. The eccrine sweat glands act as regulators of body temperature through the process of water elimination. Sweat production by the eccrine glands varies in composition according to the stimulus, rate of sweating and the site involved [9]. The liquid secreted is a dilute hypotonic solution containing electrolytes, trace elements and organic substances [14]. The apocrine sweat glands have no apparent physiological function. These glands, which develop as a part of the pilosebaceous unit, mainly exist in the armpits, nipples and groin where they secrete small quantities of a milky, oily fluid.

Subcutaneous Fat

Below the dermis lies a layer of subcutaneous tissue comprising adipose cells. The principal function of this subcutaneous tissue is to serve as a mechanical cushion to the outer layers from impact and its insulation properties contribute to the temperature regulatory function of the skin [16]. Collagenous fibres from the dermis traverse the fat cells and in doing so, provide a connection between the skin surface and the subcutaneous fat cells [14].

1.3.2. SKIN METABOLISM

The skin is a complex structure whose metabolic activity can significantly influence topical drug delivery [17]. The biotransformation of a drug penetrating through this organ may affect the rate of delivery and bioavailability of that drug [17-18]. Due to the complexity of skin, the rate of drug biotransformation may differ significantly at different application sites [20]. The skin contains numerous enzymes capable of metabolizing a host of drug products [21]. Phase I and Phase II enzymatic reactions are known to occur within the organ. The Phase I (functionalization) reactions include oxidation, reduction and hydrolysis, while the Phase II (conjugation) reactions involve the formation of glucuronide, sulphate, methyl and glutathione metabolites [22]. This abundance of metabolic activity can be exploited in the transdermal delivery of prodrugs such as rooperol tetra-acetate. Studies performed on rooperol tetra-acetate have shown that the drug undergoes Phase II reactions, namely glucuronidation and sulphate conjugation during its conversion from the inactive prodrug to its active form. The advantage of utilizing prodrugs is the attainment of site specific activity. Site specificity is critical in cancer treatment as toxicity by the cancericidal agent on neighbouring cellular regions is prevented while high drug concentrations at the desired site of action are achieved. Conversion of the parent drug to the active agent may occur chemically or enzymatically [23]. In this study, rooperol tetra-acetate takes advantage of the enzymatic activity of the skin to increase its pharmacological action. Studies performed on the pharmacodynamic activity of this drug have shown that it concentrates in regions containing high levels of Phase II enzymes. This high concentration of enzymes occurs because of elevated glucuronidase production observed in certain tumours. The increase in enzyme production encourages the formation of the cytotoxic rooperol metabolites, rooperol diglucuronate, rooperol monosulphate monoglucuronate and rooperol disulphate [4-6] [FIG 3]. The metabolic capacity of the skin can be further exploited to improve the transdermal penetration of hydrophilic drugs, which include rooperol and ketobemidone [24]. To ensure favourable partitioning of these hydrophilic drugs into the stratum corneum it is necessary to increase their lipophilicity through chemical processes such as acetylation and

esterification. After permeating the skin, the chemically altered drug relies on the enzymatic activity present in skin for conversion to its therapeutically active form. The full metabolic capacity of the skin has not yet been fully elucidated and the utilization of prodrugs to optimize the delivery of topical drugs for local or systemic activity requires further investigation [21].

Skin used for *in vitro* studies primarily functions as a diffusional membrane due to substantial loss in metabolic activity following excision and subsequent long term storage [25,26]. The potential influence of metabolism on the diffusant is therefore only measurable when the metabolic viability of the membrane has been maintained [12].

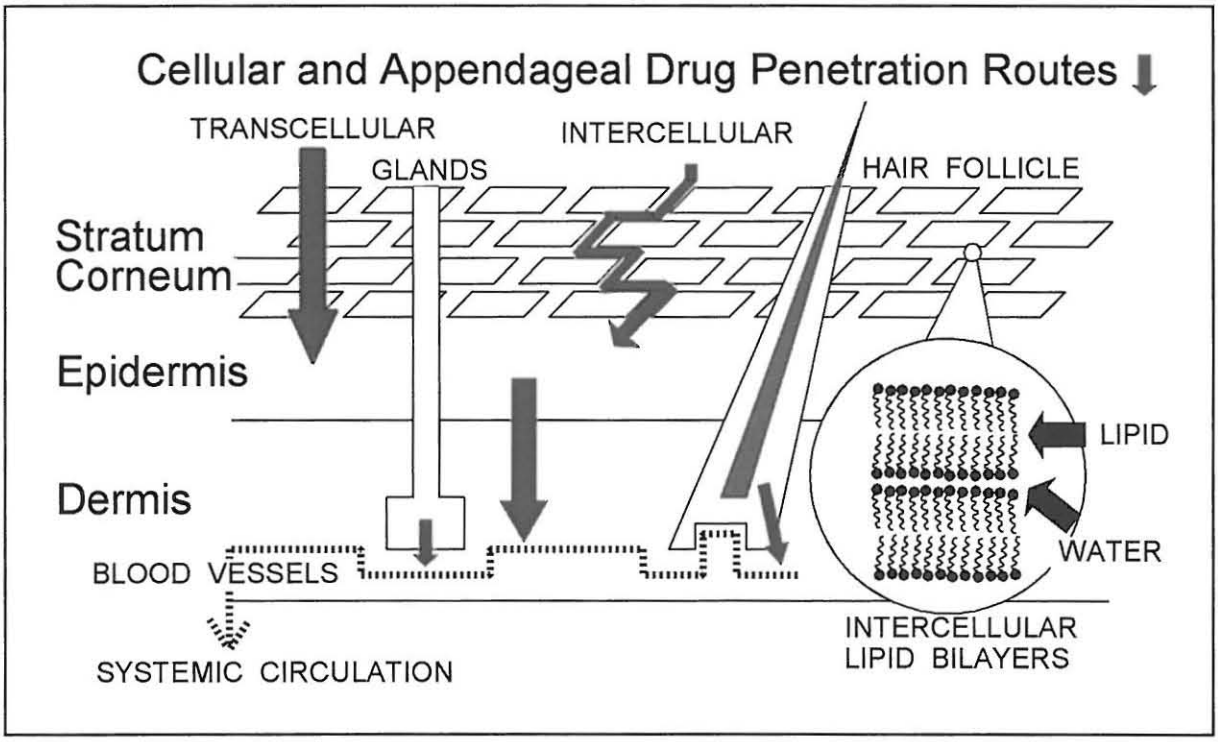
1.3.3. TRANSDERMAL ABSORPTION

Transdermal drug absorption involves the transfer of drugs from the skin surface into the stratum corneum and its subsequent diffusion through the stratum corneum and underlying epidermis. The drug is thereafter transported into the dermis and cleared via the micro-circulation. Drug absorption from the dermis into the systemic circulation is enhanced by good circulatory effects in the dermis [16] [FIG 5]. Increased blood flow at the dermal absorption site increases the concentration gradient between the dermis and the stratum corneum through constant removal of the drug from this site. The rate at which a medicament penetrates the skin is influenced by its release rate from the vehicle which in turn, depends on the physico-chemical properties of the medicament and vehicle.

The transfer of drugs across the stratum corneum is by passive diffusion and for most drugs this process occurs very slowly. Transepidermal penetration occurs via two pathways: transcellularly and intercellularly [FIG 5]. There is lower resistance to intercellular diffusion, however molecular size and chemical structure will determine substances which can be transported using this route. Due to the long pathway resulting from the molecules avoiding diffusion through cell components, this form of drug transportation through the lipid and aqueous regions of skin is more arduous [11]. To ensure effective drug permeation through the lipophilic stratum corneum and into the more hydrophilic epidermal layer, the diffusing agent needs to possess sufficient

lipophilic and hydrophilic character [27].

Figure 5: Routes of drug penetration through the skin.



The shortest diffusion pathway is the transcellular route whereby substances readily pass through and across the protein and aqueous regions of the corneal cells [1,9,28]. Further permeation through the skin can occur by means of the skin appendages which are regarded as a low resistance pathway for the rapid diffusion of drugs. The eccrine sweat glands and hair follicles are in direct contact with the dermis and the subcutaneous fat and therefore, they permit rapid drug ingress via the appendages. There is some evidence that compounds with both lipophilic and hydrophilic properties, that is, with an oil-water partition coefficient close to unity are best able to pass through the stratum corneum. The area available for absorption by means of the appendages is small compared with the total epidermal surface, hence, transappendageal drug transport is limited. Varying opinions exist over the importance

of the appendages in transdermal drug penetration experiments [29]. Fang et al. [30] attributed high plasma drug levels, obtained early after topically applying sodium nonivamide acetate, to the shunt pathways of the appendages. Investigations by Jadoul et al. [31] and Lieb et al. [32] further affirm the importance of the shunt route as a passage for drug penetration. Contrastingly, studies performed independently by Barr [33] and Hisoire et al. [34] showed that drug permeability was not influenced by skin regions containing a high proportion of eccrine glands. Barr concluded that the role of the glands as a pathway for the penetration of transdermal drugs seems insignificant. Hisoire went further to suggest that it is the structure and composition of the stratum corneum, rather than the follicular density, which influenced drug diffusion. The transappendageal route may, however, play a significant role in the diffusion of drugs with low partition coefficients [11].

The diffusional pathways of rooperol tetra acetate have not yet been elucidated. Intercellular transportation is likely to be the dominant route, however, all diffusion pathways were considered during this study. The principal route of drug diffusion will largely depend upon the physico-chemical properties of this drug, such as molecular size, pH, polarity and lipophilicity.

1.3.4. DIFFUSION KINETICS

The ability to quantitatively predict the absorption of substances through the skin is useful for determining variables such as the loss of compound from the skin and metabolism as the substance diffuses through the membrane. It is critical that the laws governing topical diffusion are understood as they assist to define the permeation properties of rooperol tetra-acetate through polydimethylsiloxane membrane and rat skin. Diffusion is defined as a process of mass transfer of individual molecules of a substance brought about by random molecular motion associated with a concentration gradient. The basic theory of diffusion is expressed in Fick's first law which states that the rate of diffusion through a unit area of a section is proportional to the concentration gradient measured perpendicular to the section. That is:

$$J = \frac{-K_m D}{h} .\delta C \quad \textit{Equation 1}$$

Where J is the steady state flux of the diffusant;

δC is the concentration difference of solute across the membrane;

h is the thickness of the membrane;

K_m is the solvent/membrane distribution coefficient;

D is the average membrane diffusion coefficient for the solute in the membrane.

The negative sign indicates that mass transfer occurs in the direction of decreasing concentration, that is, drug permeation is unidirectional due to vascular drug removal leading to the establishment of a concentration gradient.

The quantity of drug absorbed over a given unit area in a unit time depends on:

1. the solubility of the drug;
2. the concentration difference of the drug across the membrane;
3. the nature of the vehicle in which the drug is presented;
4. the thickness of the stratum corneum;
5. the partition coefficient of the drug from the vehicle into the stratum corneum;
6. the diffusitivity of the drug across the skin structure to its site of absorption.

The concentration of the dissolved drug is important for permeation across the skin as it serves as the driving force for diffusion. Fick's first law is a simplified equation representing drug diffusion in an isotropic medium and therefore will only hold for steady-state conditions where first order kinetics apply. Fickian conditions are applicable to synthetic membranes where the diffusional and structural properties of the system are the same at all points throughout the region of diffusion. The heterogeneous environment existing in skin causes a deviation from Fick's first law. Ideally, this law needs to take into account the rate of change of diffusant concentration at a particular point in the system. The equation for mass transport that emphasizes the change in concentration with time at a definite location rather than the mass diffusing across a unit area of barrier in unit time is represented by Fick's second law [35]. Fick's second law is used to represent a non-steady state of flow. This law therefore provides a fundamental mathematical statement of diffusion in a form most

useful in resolving many diffusional problems. To determine the total amount of drug that has diffused over a specified time, Fick's first law is expanded to give the following expression:

$$M = \frac{DC_o\, t}{h} - \frac{hC_o}{6} - \frac{2hC_o}{\pi^2} \sum \frac{(-1)^n}{n^2} \exp\left(\frac{-Dn^2\pi^2 t}{h^2}\right) \qquad \textit{Equation 2}$$

Equation 2 considers the diffusion of a cumulative mass of diffusant M of concentration C_o through a unit area of membrane with thickness h in time t. This equation takes into account the lag time where non steady state conditions exist. Equation 3 illustrates diffusion taking place in three dimensions thus representing the diffusion process in all spatial coordinates of x, y and z.

$$\frac{\delta C_o}{\delta t} = D\left(\frac{\delta^2 C_o}{\delta x^2} + \frac{\delta^2 C_o}{\delta y^2} + \frac{\delta^2 C_o}{\delta z^2}\right) \qquad \textit{Equation 3}$$

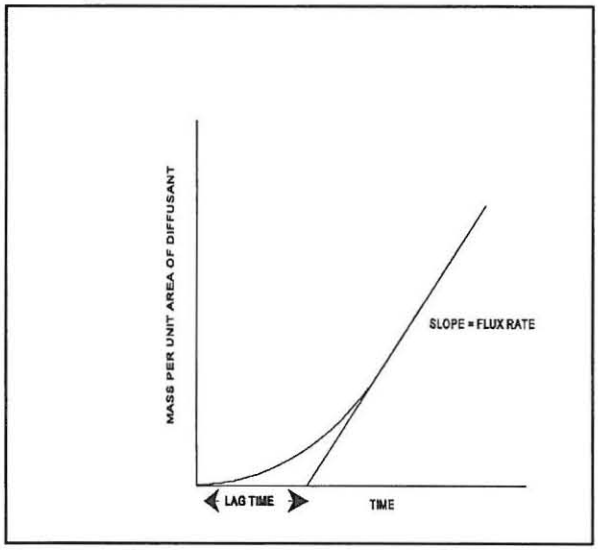
Equation 3 can be further simplified to represent diffusion of a drug of concentration C_o in the single direction, x over time t. As mentioned earlier, D is the diffusion coefficient. For the usual experimental situation in which diffusion is unidirectional, Fick's second law can therefore be written as:

$$\frac{\delta C_o}{\delta t} = D\,\frac{\delta^2 C_o}{\delta x^2} \qquad \textit{Equation 4}$$

Diffusion cells comprising a donor and receptor compartment are frequently used in *in vitro* diffusion determinations. Common to diffusion analyses is a situation of steady state. Fick's second law takes into account non-steady state flow whereby the diffusant permeating across a membrane is continuously cleared. This results in the formation of sink conditions and a concentration gradient between the donor and receiving compartments is established. The concentration gradient between these two compartments continues to exist while a state of equilibrium within the membrane is

established. At this equilibrium, the concentration of drug in either compartment remains constant however, the quantity of drug in both the donor and receptor compartments will not be the same. Therefore, at this point, the rate of change of concentration is zero. During the lag time, the system is in the process of establishing a uniform concentration gradient within the membrane separating the donor and receptor compartments. After the lag time, steady state is achieved and as one approaches infinity, linear diffusion is observed, provided sink conditions are maintained or the concentration of drug in the donor cell is substantially greater than the concentration of drug in the receptor compartment [FIG 6].

FIG 6: Plot of lag time and flux rate profiles.



A straight line equation can be derived by modifying Equation 2 to give Equation 5.

$$M = \frac{DC_o}{h}\left(t - \frac{h^2}{6}D\right) \quad \textit{Equation 5}$$

To obtain the steady state flux dM/dt expression of the straight line, Equation 5 is differentiated to give the following expression:

$$\frac{dM}{dt} = \frac{DC_o}{h} \qquad \textit{Equation 6}$$

Extrapolation of the steady state portion of the straight line to the time axis results in an intersection where M = 0. This intersection is the lag time (L), which can be derived from the lag time plot. The diffusion coefficient can therefore be calculated using Equation 7, provided that the membrane thickness is known.

$$L = \frac{h^2}{6D} \qquad \textit{Equation 7}$$

Normally, the concentration measured is the concentration of the drug which covers the surface of the membrane rather than the concentration present in the first and subsequent lamina of the membrane. Proximal concentration can be calculated by taking into consideration the partition coefficient of the drug between the vehicle and the membrane. The equation used is:

$$\frac{dM}{dt} = \frac{DC_d\,K}{h} \qquad \textit{Equation 8}$$

where K is the partition coefficient. In circumstances where the partition coefficient is not easily measurable a composite parameter of permeability which combines K and D is used. The partition coefficient can be derived by applying Equation 9:

$$P = \frac{DK}{h} \qquad \textit{Equation 9}$$

Absolute values for *D* and *K* cannot be calculated by this approach as an accurate determination of the vehicle-skin partition coefficient and diffusion coefficient are very difficult to measure. As it may not always be feasible to determine all the dimensions present in equation 9 the equation can be further simplified such that the rate of skin permeation is constant. The resulting equation is:

$$\frac{dM}{dt} = PC_d \qquad \textit{Equation 10}$$

Application of the above equations enables one to investigate the diffusion kinetics for the *in vitro* delivery of rooperol tetra-acetate from vehicles possessing different physico-chemical properties.

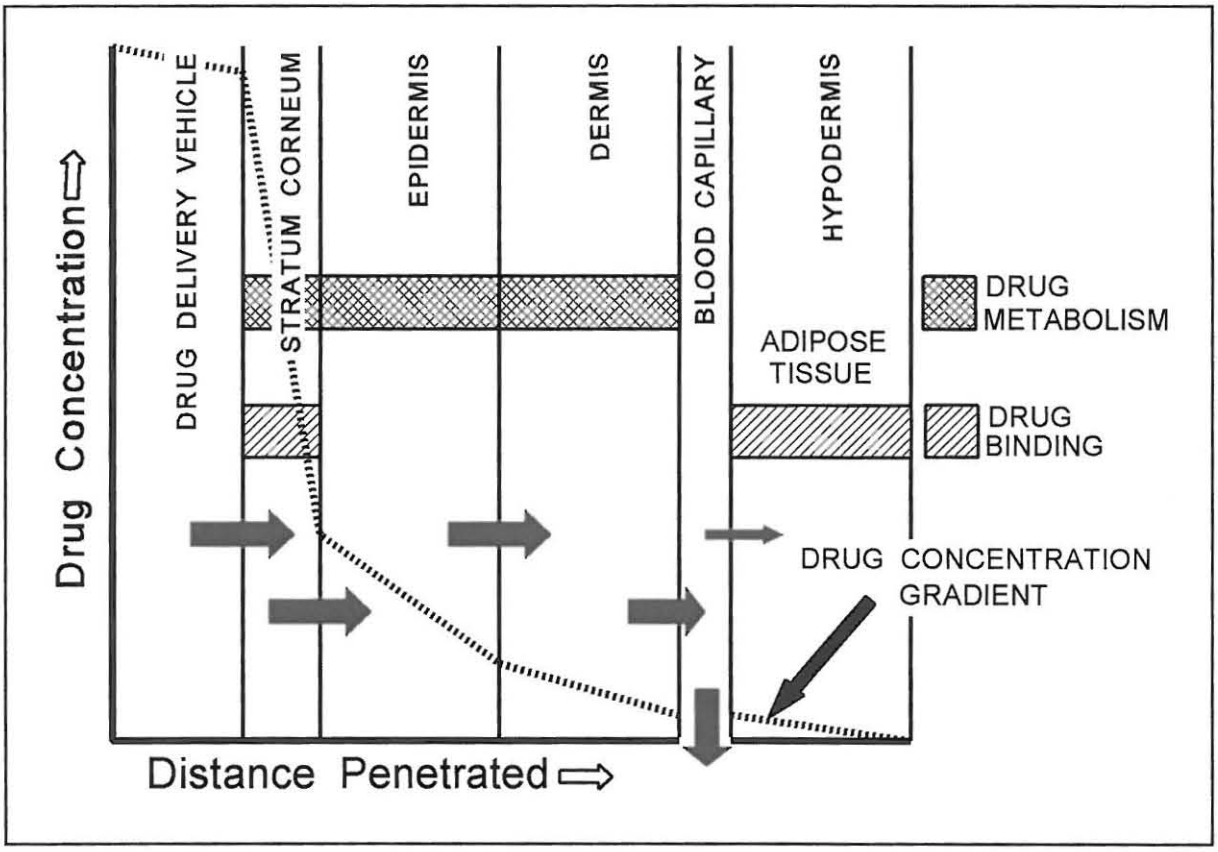
1.3.5. FACTORS INFLUENCING THE RATE OF DIFFUSION

The passive diffusion of drug molecules is largely influenced by the solubility characteristics of the drug, the concentration gradient, the composition of the vehicle and the nature of the skin membrane [9]. A change in any one of these factors can either increase or decrease the rate of diffusion of the drug through the stratum corneum. Swarbrick et al. [36] proposed two models used to describe the diffusion process. The first, a membrane-controlled system, suggests that the drug concentration in the vehicle is constant throughout diffusion and as the drug permeates through the membrane a dynamic equilibrium is established between the membrane and the vehicle. The second model, a vehicle-controlled system, postulates that the concentration of drug at the membrane surface is lower than at the surface of the vehicle and as diffusion progresses there is gradual depletion of drug from the surface of the vehicle. Other models have been suggested, however, in all cases transdermal drug diffusion is optimized by evaluating, the physical and chemical characteristics of the active ingredient with respect to the vehicle, other components in the formulation and the skin [FIG 7]. The function of the model systems is to describe the permeation characteristics of drugs and by doing so, optimize the delivery of topical agents like rooperol tetra-acetate.

Diffusant Solubility

The thermodynamic activity of a drug in a particular vehicle indicates the potential of the active substance to be released from the delivery system. A saturated solution is, therefore, preferable as a topical drug delivery system since maximum thermodynamic activity (leaving potential) is generated. The level of saturation will be dependent on the solubility of the active drug in the topical drug delivery system base [16].

FIG 7: Stages in percutaneous absorption and the concentration gradient across the skin layers.



The diffusant solubility of a drug can also be affected by the presence of a cosolvent in the formulation. Cosolvency principles were used to investigate the diffusion properties of rooperol tetra-acetate when formulated in a supersaturated system. Binary systems that increase the solubility of the active drug, can produce greater drug concentrations across the vehicle-skin interface to the extent of raising both the thermodynamic activity and the permeation rate [37]. It is the choice of vehicle used which will influence partitioning of the drug from a supersaturated vehicle into the skin. As the solubility of the drug in a topical base increases, the partitioning of that drug between the membrane and the vehicle may decrease [16]. As a result, there is a need to keep the solubility of the drug in the vehicle as near to saturation solubility as possible while maintaining the optimal partitioning potential between the vehicle and

stratum corneum. A drug that is highly soluble in the base is therefore undesirable as the release of that drug may be retarded.

Surfactants added to a base often enhance the solubility of the drug in the vehicle. Surfactants acting as solubilizers, emulsifiers and wetting agents are capable of lowering the interfacial tension thus improving binding between the vehicle and the drug [38,39]. This enhanced association is due to an increase in the solubility of the diffusant in the base, and therefore, vehicles containing high concentrations of surfactant are likely to exhibit low diffusion profiles. In the event of the surfactant penetrating into animal membrane an increase in drug diffusion is expected. This increase is due to lowered membrane integrity as a result of unfavourable chemical interactions between the surfactant and cellular lipids found in skin. The manner by which the diffusion coefficient will be influenced is largely dependent upon the degree of surfactant activity in the topical base when compared with activity in the membrane.

The thermodynamic activity of rooperol tetra-acetate in the delivery systems formulated will evidently affect its release and availability for therapeutic use [39]. Therefore, to obtain optimum drug release from the topical bases, it is essential to understand the solubility characteristics of the active ingredient in all solvents used for the preparation of the vehicle.

Partition Coefficient

The higher the partition coefficient of the drug for the membrane it is to diffuse through, the greater the concentration of drug established in the proximal lamina of that membrane [16]. The vehicle to stratum corneum partitioning often contributes to the rate limiting step in transdermal drug delivery and as a result, it has restricted the use of the topical route of administration for many drugs. Partitioning of the drug into the stratum corneum is dependent upon the binding strength of that drug for the base in which it has been formulated and the affinity of the drug for the skin [40]. Drug-to-vehicle interactions of rooperol tetra-acetate, in each of the bases formulated, will play a significant role in determining the partition coefficient of the drug into polydimethylsiloxane and rat membranes [41]. Determinations designed to assess drug solubility in the solvent systems used in the formulation can help establish the degree

of drug binding to the vehicle. The physico-chemical composition of the vehicle may control drug release by influencing the rate of drug transportation within the vehicle and the partition coefficient between the drug and the skin [35]. Many topical emulsions consist of two or more phases and drug partitioning takes place at different ratios between the phases. Rooperol tetra-acetate release from the water-in-oil and oil-in-water vehicles is influenced by the partition coefficient of the drug between the respective oil and water phases. It is vital that the concentration of drug in the external phase, in contact with the skin, is high enough to ensure penetration into the stratum corneum [13]. A high affinity of the bases for the drug is therefore undesirable [16]. Drugs that "bind" to components of the vehicle tend to be released into the skin very slowly [39]. Satisfactory release of a drug is favoured by using a vehicle that shows decreased affinity for the active agent. When performing diffusion studies under unoccluded conditions it is essential to take into consideration that the evaporation of volatile and aqueous solvents following exposure to an ambient environment may alter the ability of a drug to penetrate the skin due to gradual changes in the composition of that vehicle. Studies examining changes in the topical release of hydrocortisone butyrate propionate showed that solvent evaporation under unoccluded conditions increased the lipophilicity of the base and the resultant association between the base and the drug adversely affected the thermodynamic activity of the system [42].

Normally, the higher the partition coefficient, the greater the flux of drug diffusing through the stratum corneum. Drugs which possess a high partition coefficient for the skin tend to be highly lipophilic and therefore permeation into the stratum corneum is accelerated but, permeation into the aqueous viable tissue may be delayed [13]. Due to regional variations in skin physiology, it may be necessary to establish a balance between effective drug partitioning and satisfactory pharmacological drug uptake from dermal tissue at different skin sites. Certain vehicles are known to alter the barrier membrane and thus alter the skin's resistance to diffusion [11,43]. It is well documented that solvents such as propylene glycol and ethanol enhance the partitioning and delivery of several drugs [44-51]. The degree to which membrane resistance is lowered is proportional to the concentration and amount of solvent present [52].

The bioavailability of rooperol tetra-acetate will be controlled by the partition coefficient of drug between the vehicle and the skin. Partition coefficient determinations can predict the permeability characteristics of this drug through skin and in doing so, help establish thermodynamic parameters necessary to overcome the barrier properties of the stratum corneum.

Diffusion Coefficient

The diffusion coefficient of a drug, either in a topical vehicle or in the skin, depends on the properties of the drug, the diffusion medium and the extent of interaction between the two [14]. Diffusion decreases as the viscosity of a vehicle increases. Factors influencing the diffusion rate will govern the percutaneous delivery of drugs from a topical formulation through the skin layer. During the design of a topical delivery system for rooperol tetra-acetate, possible physical interactions which the active ingredient may undergo when in solution were taken into consideration. If such interactions between the drug and the solvent are high then the diffusion coefficient is expected to be low and, hence, the release of the active substance to the stratum corneum will decrease. On the other hand, if a solvent system which has low attraction for the drug is selected then increased topical drug diffusion can be achieved. Concentration independent factors which may alter the diffusion coefficient include changes in drug mobility through the skin brought about by physical changes or chemical interactions of the solvent with the tissue. These interactions modify the diffusion environment and in doing so, change the diffusion characteristics of the drug. Ostrenga et al. [53] and Sathyan et al. [40] reported elevated drug permeation rates in propylene glycol solvent systems as a result of changes to the barrier properties of the stratum corneum due to delipidization of the skin by the vehicle used. Rooperol tetra-acetate is a drug of high molecular weight and like other bulky compounds, may exhibit low diffusitivity due to spatial size causing a physical impediment to drug diffusion. Additional factors which are likely to alter the diffusion coefficient include, the polarity of the permeant in relation to the polarity of the skin [28,45], drug solubility in the different skin layers [15] and the lipophilicity of the active agent [1]. Effective treatment of solar keratosis is dependent upon optimizing the diffusivity of the drug to

ensure that it reaches its site of action in quantities which will guarantee therapeutic efficacy.

1.3.6. BIOLOGICAL FACTORS INFLUENCING TRANSDERMAL DELIVERY

The best method of approach to designing a vehicle which will provide optimum bioavailability of a topical drug is to take into account that disease, the age of the skin and the site of topical drug application usually violates the constraints of simple diffusion theory. One of the major difficulties encountered when designing a good delivery system is the inherent biological variation of the skin resulting in regional variations in the permeability of the stratum corneum [13]. Developing the ideal vehicle for the transdermal treatment of solar keratosis is a particularly complicated task as the condition affects individuals of a wide age group and presentation of the disease may vary from small keratotic spots to penetrating cancerous lesions located on different body sites. Investigation into the physicochemical nature of the different formulation bases and consideration of those components in the topical product which will influence the bioavailability of the drug under different physiological conditions is essential for achieving suitable dosage form design.

Skin Age

The design of a transdermal drug delivery system must be adapted to suit the physiological conditions under which it may be used. It is known that with increasing age, the elasticity and hence the permeability of the skin alters [54]. Furthermore, changes in the chemical composition and barrier properties in ageing skin may alter the diffusion properties of a topical drug. Lipid content in the stratum corneum is age dependent and with increasing age, lipid content normally decreases. In addition, there is an age related decrease in enzyme production with increasing age. The decreasing skin lipid and enzyme content results in unfavourable changes to the barrier properties of the stratum corneum. When investigating drug permeation in the very young and the old, factors such as dissimilarities in blood concentration, differences in the development of epidermal metabolic enzymes and variations in the integrity of the

stratum corneum will alter the permeation kinetics of the diffusing material. More detailed studies into the effect of skin age on drug permeation are required as knowledge on this topic is limited.

Skin Condition and Sites

Intact skin presents a barrier to absorption that can be reduced considerably when the skin is damaged or is in a diseased state. Therefore broken, damaged and inflamed skin has increased permeability [55], while calluses and corns have decreased permeability. As the condition of the skin approaches normality, its barrier function becomes more competent. Hence, decreased drug permeation may be observed in previously broken skin or increased permeation may occur in formerly hardened skin. A comparative study performed by Bronaugh et al. [55] using *in vitro* diffusion cell techniques, effectively demonstrated the theorized increase in drug permeation following mechanical and chemical damage of animal skin models. The competence of the barrier properties of the stratum corneum can be further lowered by water loss, delipidization by surfactants and denaturisation [56].

Transdermal absorption is not only influenced by the physical state of the skin but also the area to which the topical preparation is applied [55]. The composition of the lipid bilayer and the number of cellular layers will affect the extent of transdermal drug delivery. The diffusion of a substance across the skin is inversely proportional to the thickness of the stratum corneum. Regions with a thin epidermal layer, as found on the abdomen, have less bilayer lipid content and therefore, the permeability of drugs through these surface layers of the skin is greater. Investigations into skin site variability were performed by Bronaugh et al. [57], who reported structural differences in the dorsal and abdominal regions of rat skin. To produce an effective dermatological preparation one needs to consider the extent to which the site and condition of the skin will affect drug absorption through the stratum corneum. An attempt can be made to assess the effect of regional and physiological variations in permeability by measuring a pharmacologic response and/or determining the rate and quantity of drug diffusion through the skin.

1.3.7. PHYSICOCHEMICAL FACTORS AFFECTING TRANSDERMAL DRUG DELIVERY

Skin Hydration and Temperature

Compounds that hydrate the outer layers of the stratum corneum cause it to swell making it increasingly permeable. Many topical formulations cause increased skin hydration by reducing evaporation with an occlusive layer and by supplying the skin with hydrophilic fluids. For example, oils in ointments and in water-in-oil emulsions prevent water loss and in so doing, conserve skin hydration [58]. Changes to the penetration rates resulting from changes in hydration are dependent upon the physico-chemical properties of the vehicle and the diffusant [56]. A characteristic feature of keratotic skin is increased keratin production, resulting in dry patches in regions where the condition exists. The state of skin hydration is therefore an important consideration and will influence drug penetration through the hyperkeratotic regions. The influence of occlusion on the state of skin hydration and permeation rates has been reported by several researchers. Wurster et al. [59] observed an increase in the diffusion of several topically applied salicylates subjected to occlusive conditions. To obtain the desired degree of hydration and diffusion conditions, careful selection of the topical base is essential [60]. For instance, topical bases containing paraffin retard moisture loss from the skin while those which contain propylene glycol are distinctly hygroscopic and may withdraw water from the skin, especially when used in high concentrations.

The penetration rate of compounds through human skin can also be accelerated by raising the surface temperature of the skin [58]. As its surface temperature increases, the kinetic activity of the molecules within skin increases, resulting in increased drug permeability across the stratum corneum. This is because with a rise in temperature, the lipid layer of the stratum corneum becomes less viscous and therefore, the activation energy for diffusion is decreased. Clinically, skin temperature increases following the application of an occlusive base or when the skin is in a diseased state. For example, under occlusion, sweat cannot evaporate nor can heat radiate as readily from the skin surface and therefore, the skin temperature may rise marginally. The penetration rate of a material through the skin therefore varies

according to the type of topical base used (occlusive or non-occlusive) and the clinical condition of the skin.

In practice, limited control can be enforced on the temperature changes which influence drug diffusion, especially where ambient temperatures vary widely according to different geographical, diurnal and seasonal conditions.

Drug-to-Skin Binding

Prolonged binding of the drug to the molecules in the stratum corneum reduces the penetration of the drug into the viable epidermis and dermis. Recent studies have demonstrated that drug-skin binding gives rise to the so-called reservoir effect where topically applied agents form a depot or reservoir in the stratum corneum [12,13]. The mechanism of reservoir formation most probably arises from the physicochemical nature of drug solubility and diffusion within the stratum corneum. The therapeutic potential of reservoirs is restricted to highly potent compounds because of the relatively small amounts that can be retained in the stratum corneum. Reservoir formation predominately takes place in the upper layers of the stratum corneum and loss of this depot effect can occur via evaporation, chemical and mechanical removal or absorption into blood. Abrasion of the stratum corneum leads to mechanical removal while solvation of the compound and subsequent rinsing leads to chemical removal of the reservoir effect. In order to understand the processes of liquid evaporation from the skin surface and subsequent topical absorption of certain drugs it is important to acknowledge the presence of the surface reservoir. The therapeutic effects of the depot are still to be clarified through further investigations. However, studies have indicated that the depot effect is likely to occur with drugs, such as rooperol tetra-acetate, that exhibit low aqueous solubility or have a poor partition coefficient.

1.3.8. IN-VITRO DIFFUSION METHODS

Transdermal absorption is frequently studied via *in-vitro* techniques by the use of a diffusion cell system. In this system, the intact skin serves as a permeable membrane placed between the drug-containing vehicle and the receiving fluid. The rate of diffusion of the drug across the membrane is evaluated through periodic sampling and subsequent analysis of the receiving fluid.

Research workers may argue that *in vitro* experimentation does not take into account some pharmacological and physiological changes which occur within the skin when it is exposed to certain xenobiotics [9]. Such changes include vasoconstriction, vasodilation, inflammation and erythema. Nonetheless, the diffusion cell system has proved to be effective in simulating *in vivo* conditions [61]. To achieve good correlation between *in vitro* and *in vivo* diffusion systems, it essential that the experimental methods used are validated and are, therefore, reliable. A major advantage of *in vitro* tests is that they allow increased control over the environmental conditions thus enabling workers to screen drugs and determine their absorption properties before application on living skin [14,62].

Diffusion Cell Design

The diffusion cell design is important to *in vitro* percutaneous absorption studies performed to evaluate the permeation of topically applied rooperol tetra-acetate. To date, the most regularly utilized diffusion apparatus for *in vitro* drug permeation studies is the Franz cell [FIG 8] [44,63-67]. There are three basic diffusion cell designs, namely, the flow through, vertical and horizontal types: each of which serve a specific purpose and may possess different sampling and measuring methods [67,68] [FIG 9]. Gummer et al. [69] evaluated design flaws such as receptor compartment shape, volume and diameter, in the original Franz diffusion and made recommendations for a modified Franz diffusion cell which was selected for use in this study.

Figure 8: The Franz diffusion cell.

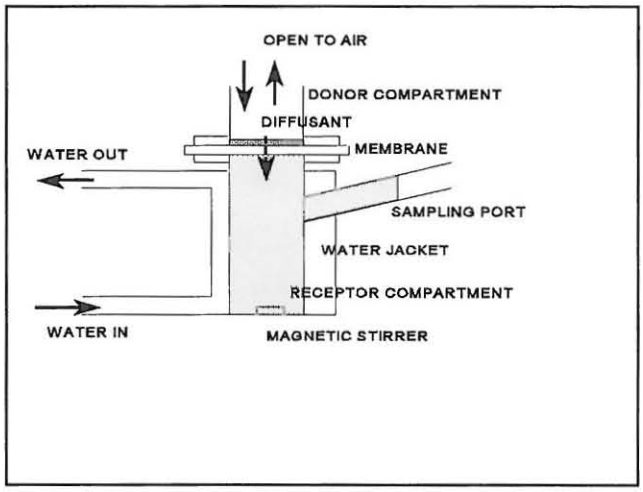


Figure 9: Flow through and horizontal diffusion cells in use.

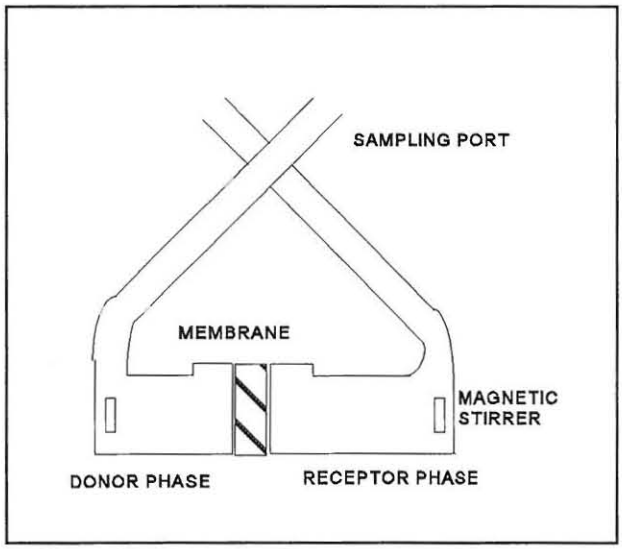


FIG 9a: Horizontal diffusion cell for solution-solvent diffusion studies.

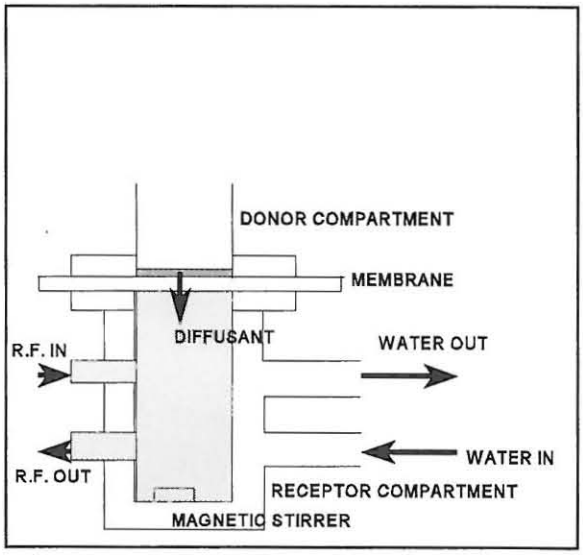


Figure 9b: Vertical flow through diffusion cell. R.F., represents the receptor fluid.

The modified Franz diffusion cell was selected on the basis of its simplicity, the close contact of the membrane with the donor vehicle and the receptor fluid and its potential to closely simulate *in vivo* situations through the generation of sink conditions. Furthermore, versatility of the cell allowed for interchangeable use of rat and synthetic membrane. Rapid, controlled and uniform stirring gave rise to uniform hydrodynamic conditions within the receptor phase [69-71] while uniform temperature distribution throughout the cell was effected by the water jacket which extended to the level of the membrane. There was minimum interference with the diffusion system during the sampling procedure and good contact of the receptor fluid with the diffusional membrane was attained after the elimination of air bubbles in the receptor chamber.

Recent publications demonstrate that good qualitative and quantitative correlation exists between *in vitro* and *in vivo* data obtained from diffusion cell analyses [9,56,62,64,72-74]. In these studies, the Franz cell was able to adequately determine drug release profiles and evaluate permeation kinetics. Furthermore, the system exhibited good reliability and reproducibility.

Receptor Fluid

The receptor fluid selected for *in vitro* diffusion studies needs to fulfil three criteria. Firstly, the integrity of the diffusion membrane must be maintained throughout experimentation. Secondly, acceptable dissolution of the diffusant must occur [18,56]. Thirdly, the receptor phase should maintain drug stability and demonstrate good compatibility with the diffusant. The most commonly selected receptor fluid for water soluble compounds is normal saline buffered at pH 7.4. Organic fluids, in the presence or absence of a non-ionic surfactant, are often preferred for the diffusion of lipophilic diffusants. Dal Pozzo [75] explored the suitability of different receptor media for the diffusion of lipophilic compounds. The results obtained from this investigation showed higher diffusant concentrations of lipophilic compounds in bovine serum albumin than in aqueous saline solution. Similar results were published by Bronaugh [76] who analysed the solubility of hydrophobic compounds in aqueous and non-aqueous receptor phases. A low driving force for topical diffusion is undesirable as it may result in inadequate collection of the permeant [77]. To maintain the driving force for

permeation, the thermodynamic activity of the drug in the receptor phase should not exceed the thermodynamic activity in the donor compartment. In addition, the receptor phase used should interfere as little as possible with the analytical procedure selected to quantitate the amount of drug present in the receiving fluid.

Membrane Systems

Animal skin required for diffusion experiments may not always be readily obtainable at the time it is needed. To alleviate availability problems associated with animal skin it is frequently stored at reduced temperatures following excision. One of the most important considerations, when using animal membrane, are the conditions under which the skin is stored. Studies performed independently by Harrison et al. [26] and Higo et al. [78] have demonstrated that percutaneous penetration is largely unaffected by freezing and thawing skin. Furthermore, investigations performed on rat skin frozen at -20°C then thawed have provided sufficient evidence to show that drug permeability is minimally affected by freezing the skin [74]. It is generally accepted that freezing alters the metabolic activity of the dermis and epidermis. Higo illustrated that the metabolic activity of frozen skin is markedly reduced by the freezing and thawing process.

Alternatively, synthetic membranes can be employed in place of animal skin. The principal objective for using synthetic membranes would be to determine drug-vehicle interaction, the release characteristics of the drug from the vehicle and the suitability of the receptor phase in relation to other components of the system.

Rat Skin

The use of animal models to predict the percutaneous diffusion of drugs in human skin is a widespread practice [79-82]. Human skin is not always readily available and problems associated with the storage of cadaver skin makes the use of animal skin a more viable option for *in vitro* diffusion experiments. Due to species differences and experimental variations, percutaneous absorption studies which utilize animal skins can only be approximations of activity in man [13,80].

The structure and biochemical composition of animal skin differs from human skin [79]. Pig skin is said to have similar physiological and histological properties to human skin and, as a result, it is often recommended as a suitable substitute for human skin permeability analyses [10,82]. However, due to the low maintenance needs and affordability of laboratory rodents, rat skin was selected in this study for the preliminary diffusion studies on rooperol tetra-acetate. Rat skin has a greater concentration of hairs per unit area than human skin. The greater number of hair follicles can provide a rapid and convenient shunt pathway for certain diffusing molecules [11]. Table 1 lists the thickness values of different layers of rat and human skin as reported by Bronaugh et al. [57]. Skin thickness variations suggest a difference in the lipid content between animal and human skin which makes direct extrapolation of diffusion results between these two species difficult. Furthermore, Bronaugh showed that the permeability of topical drugs through the dorsal and abdominal regions of a male rat is substantially lower than permeation through the same regions of a female rat.

Table 1: Human and rat skin thickness values.

Species	*Stratum Corneum (μm)*	*Epidermis (μm)*	*Whole Skin (mm)*
Human	16.8	46.9	2.97
Rat (female)			
back	18.4	32.1	2.09
abdomen	13.7	34.8	0.93
Rat (male)			
back	34.7	61.1	2.80
abdomen	13.8	30.4	1.66

He attributed these differences in permeability to variable thicknesses at different skin sites. The faster diffusion rates observed with rat skin when compared with human skin may be a result of biochemical differences and, to a lesser extent, a product of combined appendageal and stratum corneal diffusion [31].

Although full thickness skin is normally selected for diffusion experiments, the use of split-thickness skin prepared by dermatome or stripping is equally common [26,76]. The decrease in the hydrophilic dermal tissue of dermatomed or stripped skin reduces the distance for diffusion resulting in modest increases in the absorption of hydrophobic compounds.

It is important to note that the correlation of diffusion results between human and animal membrane may differ according to the physico-chemical properties of the drug being analysed. It does not always follow that if a drug tested on a single animal species shows similar diffusion characteristics to human skin diffusion, then all other drugs will produce similar penetration results when tested on that particular diffusion system [79-81].

Synthetic Membranes

The use of artificial membranes is common in laboratory percutaneous absorption studies. Synthetic membranes are not intended to mimic either the barrier properties or the heterogeneous nature of the skin but, instead, to serve as predictive models for topical drug release [83,84]. Artificial skin membranes may be used in place of animal skin for several reasons, such as the unavailability of animal skin or they may be used in preformulation studies designed to determine the leaving potential of the drug from its vehicle [85]. The stability and batch-to-batch uniformity of synthetic membranes makes them desirable for use in diffusion experiments [86]. Polydimethylsiloxane (silicone) membranes are hydrophobic and exhibit a relatively high permeability to lipophilic diffusing molecules. Drug diffusion through silicone membrane is via the dissolution of the permeant in the barrier matrix and subsequent diffusion across the barrier. In addition, mobility of the polymer chains, which make up the barrier matrix, results in the formation of passages through which drugs can easily diffuse [14,87]. The aporosity of silicone membranes provides some rate limiting function to the system. Drug permeation through the polymer membrane is, therefore, largely determined by the polarity of the drug, the molecular weight of the diffusant and its molecular volume [88,89]. Results obtained from the use of synthetic membranes can be used to elucidate diffusion mechanisms and establish conditions which will optimize drug release.

1.4. FORMULATION CONSIDERATIONS

Effective treatment of diseased skin largely depends on the selection of an acceptable vehicle for the active agent. During the formulation of a vehicle designed for the topical use of rooperol tetra-acetate, many factors were considered. Specific product use, the site of application and product type needed to be combined in a dosage form which will readily release the drug when applied to the skin. A list of the general factors which were used to evaluate the state of the formulated semisolids, both during developmental studies and as a function of time on storage, follows:

1. Drug stability
2. Stability of the excipients
3. Rheological properties of the preparation
4. pH effects
5. Effect of volatility and loss of water
6. Stability of the vehicle eg. phase separation, homogeneity
7. Particulate and microbial contamination

The objective in selecting a suitable base is to optimize drug delivery through the skin. In reality, optimum delivery is rarely achieved as one needs to consider the aesthetic appeal of the formulation, its conditions of use and the convenience of application. This will require that the physical and chemical properties of each base be assessed. However, one would ultimately like to ensure that the drug in the final preparation is therapeutically effective. It was, therefore, essential to develop a vehicle in which the drug was stable and caused no irritation to sensitive skin areas. Of primary concern when selecting the bases for rooperol tetra-acetate was that safety, stability and effective preservative activity were maintained while optimum drug delivery in the total formulation was achieved [13,16].

1.4.1. CLASSIFICATION OF TOPICAL SEMI-SOLIDS

Creams

Creams are viscous semi-solids and are usually oil-in-water emulsions (aqueous creams) or water-in-oil emulsions (oily creams). The type of cream used will depend on the solubility of the drug between the aqueous and oily phases. Water soluble drugs are more easily dispersed in oil-in-water emulsions while lipid soluble drugs are better dispersed in water-in-oil emulsions [9]. Due to the influence of excipients, the dispersion properties of a drug may change following formulation into a comprehensive dosage form. Hence, rooperol tetra-acetate was prepared in both an oil-in-water and a water-in-oil emulsion to ensure that the diffusion properties of the drug from both preparations were examined. Creams are used to apply solutions or dispersions of medicaments to the skin for therapeutic or prophylactic purposes where a highly occlusive effect is not always necessary. Bland creams may also be applied for their emollient, cooling or moistening effects on the skin. Creams are frequently chosen for wet and weepy skin conditions. They are desirable to the user because they have an elegant appearance, are more readily spread, are less greasy and they rub into the skin leaving little or no trace of their former presence.

The method used for the preparation of the extemporaneous cream was one initially utilized to formulate rooperol tetra-acetate into a topical base. Cetomacrogol Cream B.P., on the other hand, was specifically selected for use in this study because of its potential for effective drug release. In addition, the base exhibits good compatibility with numerous medicaments, stability over a wide pH range and resistance to hydrolytic processes, thus decreasing the potential for vehicle-induced toxicity through chemical degradation. The manufacture of Cetomacrogol Cream B.P. is uncomplicated and an agreeable final product, acceptable to the user, is obtainable.

Gels

Gels are transparent or translucent semisolid or solid preparations consisting of solutions or dispersions of one or more active ingredients in suitable hydrophilic or hydrophobic bases. As vehicles for the presentation of medicaments which are minimally soluble in water, gels are ideal because their high water content ensures increased partitioning of the drug into the lipophilic environment of the stratum corneum. Gel products tend to be smooth, elegant and produce cooling effects because of the evaporation of water. The water loss causes drying out of the gel formulation resulting in the formation of a film over the site of application. These films adhere well to the skin and are usually easily removed by washing. For the presentation of insoluble materials, hydrophilic gels have the limitation that the resultant products may lack clarity and smoothness. Due to the non greasy nature, transparent appearance and easy removal of gels they are ideal for use on hairy parts of the body and, more importantly, the face.

The main objective of this study is to optimize rooperol tetra-acetate release from the formulated vehicles. Diffusion theory predicts that appreciable release of a lipophilic drug is more feasible from a hydrophilic base. As a result of their potential to deliver appreciable quantities of the active agent, gels are commonly used to maximize drug release. In addition, these vehicles have shown superior drug availability over creams and ointments. The gel formulation selected for this study was specifically chosen because of its simple preparation procedure. Furthermore, the compatibility of sodium carboxyl methyl cellulose with organic solvents such as propylene glycol contributes towards maintaining the stability of the formulation. The function of propylene glycol in the gel formulation was to promote the dissolution of rooperol tetra-acetate resulting in uniform dispersion of the drug to give a homogenous base.

Ointments

Ointments are semisolid preparations intended to adhere to the skin or certain mucous membranes; they are usually solutions or dispersions of one or more medicaments in non-aqueous bases. There is a greater emphasis on the emollient and

protective functions of ointments because of their highly occlusive nature. Ointments prevent water loss from the surface of the stratum corneum resulting in increased skin hydration and drug permeability. Due to the presence of a lipid continuous phase, ointments are particularly suitable for chronic dry skin lesions as found in keratotic conditions and are disadvantageous in moist skin conditions.

Simple Ointment B.P. is a non emulsified base which is capable of absorbing large quantities of water and aqueous solutions resulting in the formation of water-in-oil emulsions. The physical nature of ointments often make them undesirable to use. However, non emulsified bases are more acceptable to the patient and were selected for use in this investigation because they show adequate occlusivity, good emolliency, improved penetration of oil-soluble medicaments and good spreadability [90]. In addition, the fact that non emulsified bases are stable, chemically inert and can be mixed with various chemical substance makes them ideal vehicles for drugs such as rooperol tetra-acetate.

Supersaturated Systems

A major problem encountered in the delivery of topical drugs is the effectiveness of the barrier system imposed by the stratum corneum. To overcome the skin's resistance to the ingress of exogenous substances, various innovative techniques requiring complex delivery systems have been studied [86,87,91,92]. Other methods used to promote percutaneous absorption involve altering the barrier properties of the skin [44]. Supersaturation, in comparison to other known transdermal systems, is a simple delivery technique which does not intend to modify either the physical structure or the chemical composition of the stratum corneum, yet may effectively overcome the barrier properties of the skin [93-95]. Supersaturated systems make use of the enhanced thermodynamic activity of the drug resulting in increased flux rates across diffusion membranes. These systems have a high capacity to promote the delivery of transdermal agents and due to their contribution towards optimizing drug permeation their role in the topical diffusion of rooperol tetra-acetate was investigated. Supersaturated systems are developed by increasing the relative solubility of the drug such that its transient concentration in a given vehicle is greater than the saturated

concentration in that vehicle [96,97]. Therefore, an increase in the free energy of the diffusant is achieved and the rate of transdermal delivery is increased as partitioning into the membrane is favoured. The thermodynamic activity of the drug will correlate with the degree of supersaturation achieved. Due to the existence of an unusually high free energy, supersaturated systems are susceptible to drug crystallization with time which reduces the diffusion potential of the active drug to that of a saturated solution. It is possible to retard drug precipitation and, therefore, control crystal growth within these systems by polymer addition [98].

CHAPTER 2: EXPERIMENTAL AND ANALYTICAL TECHNIQUES

2.1. HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS

In recent years, rapid expansions in analytical technology have enhanced the reliability, utilization and applicability of HPLC analysis resulting in the formation of an indispensable separation, detection and quantitation procedure [99,100]. Factors that have made HPLC a popular analytical method among researchers working in diverse environments include high selectivity and specificity, even when low concentrations of the test substance are used. Rooperol tetra-acetate is a novel compound and therefore, a new HPLC method had to be developed and validated for the quantitative analysis of the drug in the aqueous receptor phase of *in vitro* diffusion cells. Limited analytical work has been performed previously on rooperol tetra-acetate and these studies concentrated on the chromatographic analysis of the biotransformation of the hypoxoside and rooperol analogues in man by the use of an in-line sorption enrichment HPLC technique [4-8]. The adaptability of high performance liquid chromatography has made it possible to quantitatively determine the amount of rooperol tetra-acetate that diffuses through membrane systems from different topical bases. System validation of the chromatographic method, developed for the analysis of rooperol tetra-acetate, was conducted. Precision and limit of quantitation studies were performed. From the results obtained in the limit of quantitation studies, a calibration curve was constructed from which the sensitivity and linearity of the system were determined. Further studies included determining the range of the system which in turn made it possible to assess the accuracy of the constructed calibration curve. The objective of performing validation tests is to confirm the reliability and reproducibility of the analytical procedures selected for this study [101,102]. Through system validation, an acceptable analytical method based on sound scientific principles can be applied to the quantitative analysis of rooperol tetra-acetate.

2.1.1. MATERIALS AND METHOD

Reagents and Chemicals

A mobile phase of spectral grade acetonitrile obtained from Burdick and Jackson, USA, and HPLC grade water purified through a Elgacan system (Elga, England) was filtered using a 0.45 µm membrane filter (type BD, Millipore, USA) and simultaneously degassed under vacuum at ambient temperature. Rooperol tetra-acetate was obtained from the Department of Pharmacology at the University of Stellenbosch, South Africa, and the prazepam was obtained from Parke Davis (South Africa).

Apparatus

A solvent pump, (Model SP8810, Spectra-Physics, USA) was connected to a manual injection valve (Model 7126, Rheodyne, USA) equipped with a 20 µl injection loop. The 10 µm C_{18} analytical column, custom packed with octadecylsilane, had a column length of 25 cm and an internal diameter of 4.9 mm. A variable U.V. detector (Linear 200 Model, Spectra-Physics, USA) was coupled to a strip chart recorder (Perkin Elmer, Model 561-1002, Japan) and a datajet integrator (Model SP4600, Spectra-Physics, USA) set at a wavelength of 260 nm and sensitivity of 0.002 A.U.F.S. All liquid chromatographic determinations were performed utilizing the above mentioned apparatus.

HPLC Conditions

Selective chromatographic separation is highly dependent upon establishing the correct HPLC conditions for the drug in question. Lengthy investigations were carried out into developing a solvent system that generated chromatograms with good resolution and efficient selectivity. An HPLC solvent system which was compatible with the detector and system components and above all, in which the drug exhibited good solubility was required. Normally, it is possible to obtain complete peak resolution within a short analytical period through the manipulation of pH in buffer solutions [103]. However, complex buffer solvent systems often cause analytical problems. To avoid

such problems, a simple mobile phase of acetonitrile/water was selected for the study. No ionic or buffer component was added to this mobile phase. The mobile phase was prepared by mixing 70 parts acetonitrile with 30 parts water in a stoppered flask. The mixture was first allowed to equilibrate to room temperature before being filtered and degassed under vacuum. The flow rate was maintained at 1.5 ml/min and all operations were carried out at ambient laboratory temperature.

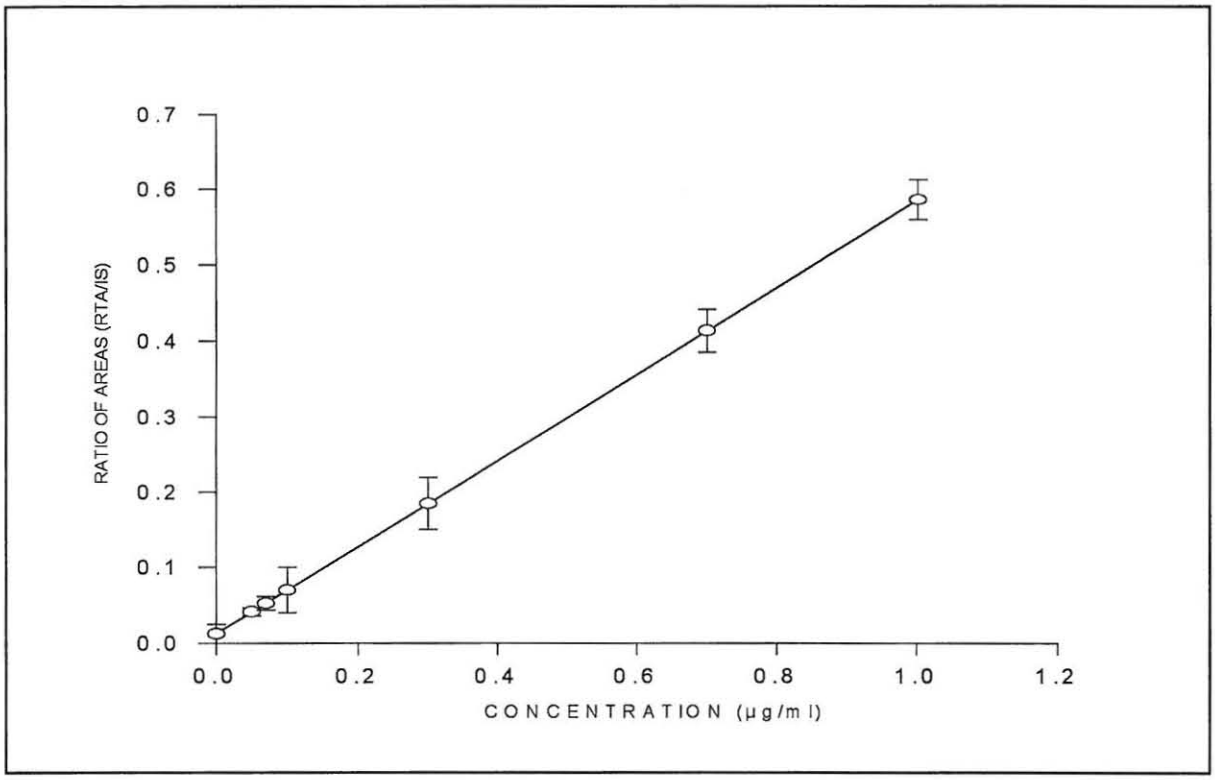
Changes in the resolution of the chromatographic traces generated when the composition of the acetonitrile/water mobile phase was altered have been studied in other laboratories and was explored in this investigation [104,105]. In addition, the changes brought about by substituting acetonitrile for methanol and adding isopropanol to a mixture of acetonitrile and water were also examined. Following the extensive study into solvent system optimization it was found that a mobile phase of acetonitrile/water in the ratio 70:30 resulted in the formation of chromatograms that exhibited good selectivity, separation and resolution. The utilization of acetonitrile in place of methanol is further supported by suggestions that an extended column life is achieved when acetonitrile is used as the organic component in the mobile phase [106].

2.1.2. SAMPLE PREPARATION

A calibration curve was constructed on each day of analysis. From a 0.1% stock solution of rooperol tetra-acetate in acetonitrile, serial dilutions containing 0.0501 µg/ml, 0.07014 µg/ml, 0.1002 µg/ml, 0.3006 µg/ml, 0.7014 µg/ml and 1.002 µg/ml of active drug were prepared. A 2 ml aliquot of 1 µg/ml prazepam solution in acetonitrile was mixed with 2 ml of the prepared rooperol tetra-acetate dilutions. A final internal standard concentration of 0.5 µg/ml was injected into the HPLC system. A minimum of six assays was performed for each calibration sample. Typical calibration curve data is presented in Chapter 3 (Table 5) and Figure 10 illustrates a characteristic calibration plot.

Figure 10: Calibration curve for rooperol tetra-acetate.

EQUATION: y = 0.5727X + 0.0125 and CORRELATION COEFF. = 0.998



Precision Studies

At the beginning of each of the three precision runs performed, three individual samples of 0.01 g rooperol tetra-acetate were weighed out and each sample was dissolved in acetonitrile in a 10 ml volumetric flask. Each rooperol tetra-acetate sample was then diluted to a concentration of 0.1 µg/ml in acetonitrile. The internal standard solution, which was diluted to a concentration of 1 µg/ml in acetonitrile was prepared by dissolving 0.025 g prazepam in acetonitrile in a 25 ml volumetric flask. A 2 ml sample of the internal standard was added to a 2 ml sample of rooperol tetra-acetate and 10 µl of this final standard solution was injected into the HPLC system. A total of ten replicate injections of each of the three standard solution samples prepared were made on each of three occasions. Over the three days of analysis 90 injections were made and the data obtained was used to calculate the percent relative standard deviation.

Limit of Quantitation Studies

A 1002 µg/ml solution of rooperol tetra-acetate dissolved in acetonitrile was prepared. Using this 1002 µg/ml stock solution, six dilutions ranging in concentration from 0.0501 µg/ml to 1.002 µg/ml were made. A 2 ml aliquot of each dilution solution was mixed with 2 ml of internal standard solution. A minimum of six, 10 µl, replicate injections of the standard solution were introduced into the HPLC system. From the data obtained the ratio of areas was determined and was thereafter used to calculate the mean and percent relative standard deviation. Data obtained from the limit of quantitation studies was also used to determine the sensitivity and linearity.

Range Studies

Range studies were performed by preparing six samples of rooperol tetra-acetate in acetonitrile whose concentrations covered the extremes of the range expected. The masses of rooperol tetra-acetate ranged from 14 mg to 30.6 mg. Suitable dilutions of each solution were made and a 2 ml aliquot of the diluted solution was mixed with 2 ml internal standard. A minimum of six replicate 10 µl-injections of standard solution were introduced into the HPLC system. The ratio of the areas was calculated from the data obtained. The corresponding drug concentrations were determined by solving the regression equation for concentration and substituting the peak areas ratio for the sample.

Internal Standard

When developing a new HPLC method for a drug, it is often necessary to find an appropriate internal standard, the function of which is to improve the accuracy of the results [107]. Experimental variations during the analysis of rooperol tetra-acetate were controlled by using the internal standard prazepam.

Stability tests performed on rooperol tetra-acetate demonstrated that the drug and its degradation products all eluted within a retention time of 3.2 minutes. To avoid undesirable interactions between the internal standard and the active agent, it was essential to find an internal standard which eluted after the retention time of 3.2 minutes. A combination of two approaches was adopted when researching for a

suitable internal standard. The first approach was to select a drug of greater lipophilic character and the second, was to alter the organic composition and thus the polarity of the mobile phase. The rationale behind these strategies is that the greater the molecular weight of the compound of interest, the greater the number of intermolecular interactions between the eluent and the hydrophobic surface of the column. As solutes are eluted in order of polarity, the non polar solutes, which are soluble in the stationary phase, will travel more slowly through the system resulting in longer retention times. Furthermore, in reverse phase chromatography where the most polar component is eluted first, increasing the mobile phase polarity will also increase the elution time for more lipophilic compounds [108-110]. Through literature studies [108,111] and a process of trial and error, several potential drugs were analysed and it was found that prazepam eluted at a retention time of 4.0 minutes when injected into a system comprising a mobile phase of acetonitrile/water in the ratio 70:30. Under these conditions, complete resolution of prazepam was achieved and interference of the drug with other peaks of interest was avoided.

2.1.3. CALCULATIONS

The percent relative standard deviation gives the percentage variation of the standard deviation of the peak area ratios calculated about the sample mean while the percent relative error is the degree of correlation of the test results to a theoretical value. Here, the experimental value is a value calculated from the equation of the calibration curve.

$$\text{Percent Relative Standard Deviation} = \frac{\text{Standard Deviation}}{\text{Mean of Sample Population}} \times 100$$

$$\text{Percent Relative Error} = \frac{\text{Experimental - Theoretical}}{\text{Theoretical}} \times 100$$

2.2. STABILITY- INDICATING STUDIES

Knowledge on the stability of a drug is vital in analytical chemistry. Not only can an unstable drug alter the credibility of an analytical procedure, but more importantly, it can cause compatibility problems when combined with the formulation excipients [99,112]. Moreover, safety, purity and bioavailability cannot be assured if the quality of the product is questionable [113,114]. It is therefore critical that stability tests which result in chromatographic characterization of the degradation process of the drug of interest are performed as part of the validation procedure of an assay method [101,102,112]. Liquid chromatography, which is routinely employed to detect the presence of degradation products during stability tests, was used in the stability-indicating analysis of rooperol tetra-acetate [112,113,115]. Prior to designing and performing stability analyses, it is essential to define the criteria for the stability of the active drug. Hence, a study had to be undertaken to, firstly, determine the factors which promote the degradation of rooperol tetra-acetate, secondly, to establish the probable degradation routes of the drug and thirdly, to develop an assay method which will result in selective detection of the active drug and its degradation products [115-117].

The stability of a drug can be influenced by several parameters including pH, heat, light, gases and storage time [101,102,117-120]. Due to the requirement of gentle heat application during the preparation of the topical vehicles used to deliver rooperol tetra-acetate to the skin, heat degradation was therefore the most relevant variable that required investigation. To determine the stability-indicating capacity of the developed HPLC method, rooperol tetra-acetate had to be degraded under extreme heat conditions [99,121]. In assessing the drug stability, two samples of rooperol tetra-acetate, one stored in a glass vial and the other stored in a plastic vial were exposed to extreme temperatures while the third sample was allowed to stand at room temperature for the twelve week duration of the stability studies. During this period significant degradation occurred in both samples stored at elevated temperatures and within one week of heat exposure, degradation of the rooperol tetra-acetate was apparent. Acceptable separation of the degradants was attained by chromatographic

means. The heat-accelerated stability test on rooperol tetra-acetate has provided useful indication of the possible deterioration of the drug. However, it must be noted that this does not directly imply that performance of the drug, when used under normal conditions, will be identical.

2.2.1. EXPERIMENTAL PROCEDURE

Investigations on the stability of rooperol tetra-acetate involved subjecting the drug to elevated temperatures. The United States Pharmacopoeia guidelines for stability testing were used to determine the conditions under which accelerated tests were to be conducted [122].

A 1 g sample of rooperol tetra-acetate was measured into a teflon plastic vial and the same amount was measured into a glass vial. Both containers were placed in the oven set at 40°C. At weekly intervals, a 0.1 µg/ml solution of rooperol tetra-acetate in acetonitrile was prepared from each of the two samples stored in the oven. A 2 ml aliquot of the rooperol tetra-acetate solutions was mixed with 2 ml internal standard solution and six replicate injections of the mixture, each measuring 10 µl, were introduced into the HPLC system. For comparative purposes, a 0.1 µg/ml solution of rooperol tetra-acetate in acetonitrile was freshly prepared from a sample maintained at room temperature. Experimental manipulation of the fresh sample was identical to that of the samples stored at higher temperatures. The retention times and areas of each peak produced were recorded. Statistical evaluation of the results collected over twelve weeks made it possible to assess the storage stability of rooperol tetra-acetate.

2.2.2. CALCULATIONS

Data evaluation involved the application of the *t*-distribution test used to determine if a significant difference at a 95% confidence level exists between the drugs stored under heat and the sample stored at room temperature. Statistical assessment of the stability data makes it possible to evaluate the significance of the changes taking place in the degrading product. That is, it provides a more meaningful interpretation of stability which is frequently expressed in vague terms [118].

2.3. DRUG PERMEATION STUDIES

Percutaneous drug diffusion can be enhanced or retarded by altering the vehicle in which the active ingredient has been formulated. To achieve optimal drug penetration into the skin it is necessary to determine the release characteristics of the drug in relation to the physico-chemical properties of the vehicle [12]. For adequate release of rooperol tetra-acetate into the skin, an effective delivery system needed to be developed. *In vitro* permeation studies were performed to assess the delivery of rooperol tetra-acetate from a gel, oil-in-water, water-in-oil and ointment vehicle across polydimethylsiloxane and rat membrane mounted in Franz diffusion cells.

The key issue in percutaneous drug absorption studies is the ability to quantitate the amount of drug penetrating through the skin layers and into the receiving fluid. Successful topical penetration of rooperol tetra-acetate is dependent upon selecting the correct *in vitro* test system for the simulation of *in vivo* drug permeation. A modified Franz diffusion cell system was utilized for the quantitative analysis of the topical penetration of the drug across rat skin and synthetic membrane. The objective of the permeation studies was to compare the permeation characteristics of diffusant from the four topical bases formulated. The assessment also involved a comparison of the diffusion properties of the drug under occluded and unoccluded conditions.

2.3.1. MATERIALS AND METHODS

Membrane

Of the wide variety of commercially available synthetic membranes, a silicone-based (polydimethylsiloxane) membrane was utilized to study the *in vitro* diffusion of the active compound from different topical vehicles. The polydimethylsiloxane membrane, which had a thickness of 0.12 mm, was obtained from Atos Medical Laboratory, Sweden (ref. 7458). Animal membrane was obtained by excising rat skin from the abdominal region of male Wistar rats. Prior to excision, the rat hairs were clipped using Whal clippers (Model SC, USA). A micrometer screw gauge was used to measure the thickness of the excised skin and while taking the measurements,

caution was taken as not to damage the skin surface. The average abdominal skin thickness was 1.24 ± 0.03 mm (n = 8). Storage of the skin was accomplished by freezing it at -18°C for a period not exceeding three weeks. When required for use, the skin was thawed. Pieces of full-thickness abdominal skin measuring approximately 4 cm^2 were placed between the donor and receptor compartments of the Franz diffusion cells with the dermis in contact with the receptor fluid. The polydimethylsiloxane membrane was soaked in the receptor fluid for one hour and preparation of the rat membrane involved first soaking the skin in the receptor fluid for 15 minutes then rinsing with water to flush out impurities on the membrane surface. The wash procedure for the rat skin was repeated four times to ensure sufficient removal of endogenous substances diffusing from skin which may interfere with chromatographic identification of the diffusant.

Diffusion Cells

A ground glass Franz diffusion cell (Crown Glass, USA) with an open cap was utilized for the topical penetration studies. Each cell, of the four diffusion cell assembly, had a diffusional area of 1.767 cm^2 . The receptor phase was agitated by means of a star-head magnetic stirrer while the temperature of the cells was maintained at 30°C. In the unoccluded studies the epidermal side of the skin was exposed to ambient laboratory conditions. To attain occluded conditions, the donor compartment of each diffusion cell was covered with parafilm for the duration of the test. The receptor phase comprised of 12 ml ethanol/water in the ratio 60:40. Preparatory studies showed that rooperol tetra-acetate was suitably soluble and stable in the ethanol/water solvent system. Furthermore, there was minimal interference by the receptor phase with the identification and quantitation processes employed. The effects of ethanol on membrane integrity are discussed in detail in subsequent chapters. Ethanol was obtained from BDH Laboratories, England, while the water was purified by passing it through the Elgacan system (Elga, England).

Topical Formulations

Four topical formulations were selected to deliver rooperol tetra-acetate across the diffusional membrane. The selection of each base was based on its safety, stability and the potential to transport an efficacious quantity of a lipophilic drug through a membrane effectively. Each preparation was formulated according to the procedures published in literature and the British Pharmacopoeia [123,124]. The formulation of each vehicle is shown in Table 2. Ten grammes of each topical base were prepared in triplicate. The vehicles used in the synthetic membrane permeation contained 0.01 g rooperol tetra-acetate while the vehicles used in the animal membrane permeation experiments contained 0.1 g of the active compound. At the lower drug concentrations no penetration of the permeant was observed through animal membrane. As a result, the drug concentration was increased in an attempt to obtain measurable quantities of diffusant in the receptor phase. In addition to the preparations containing the active drug, topical bases in which the active ingredient was absent were prepared in triplicate for each formulation. Permeation studies of these blank preparations were performed to ensure that chromatographic peaks, resulting from the diffusion of excipients in the base, did not interfere with the peak representing the drug product. All topical vehicles were stored in air tight jars at ambient laboratory temperature. Prior to each permeation study, 0.25 g of the test base was measured and evenly distributed over the entire surface of the membrane in the donor compartment, using a spatula. The resultant formulation loading quantity of the drug was approximately 0.14 mg/cm^2 for the polydimethylsiloxane analyses and 1.4 mg/cm^2 for the skin permeation studies.

2.3.2. EXPERIMENTAL PROCEDURE

Each diffusion cell was filled with 12 ml of the receptor fluid using a pipette. The membrane was placed between the donor and receptor chambers where it was secured by a clamp. At the start of each diffusion test air bubbles between the receptor fluid and the membrane were eliminated by gently tipping the cell such that the bubble was expelled via the sampling port. At regular intervals 500 µl of the receptor fluid was withdrawn through the sample port of the diffusion cell. The sampled fluid was placed in a vial containing 500 µl of 0.1 µg/ml prazepam dissolved in acetonitrile. After

withdrawal from the receptor chamber, the diffusion cell was refilled with the 500 µl of fresh receptor phase thus maintaining sink conditions.

Table 2: Formulae of topical preparations.

GEL FORMULATION:	
Rooperol tetra-acetate*	1%
Sodium carboxyl methyl cellulose	2%
Propylene glycol	25%
Water ad	100%
CETOMACROGOL CREAM B.P. (Oil-in-water)	
Rooperol tetra-acetate*	1%
Cetomacrogol emulsifying ointment	15%
Propylene glycol	25%
Water ad	100%
EXTEMPORANEOUS CREAM (Water-in-oil)	
Rooperol tetra-acetate*	1%
Cetomacrogol 1000	12%
Cetostearyl alcohol	10%
White soft paraffin	16%
Liquid paraffin	25%
Propylene glycol	25%
Water ad	100%
SIMPLE OINTMENT B.P.	
Rooperol tetra-acetate*	1%
Wool fat	5%
Hard paraffin	5%
Cetostearyl alcohol	5%
Propylene glycol	25%
White soft paraffin	59%

*0.1% rooperol tetra-acetate was used for polydimethylsiloxane diffusion analyses

All permeation studies were performed in triplicate for each batch of formulation. To evaluate the permeation of the excipients in the vehicle, initial diffusion studies were performed using the control topical vehicles in which there was no active drug present. Thereafter, the bases containing rooperol tetra-acetate were analysed. All studies were performed under occluded and unoccluded conditions. All permeation studies were performed in triplicate for each batch of formulation. To evaluate the permeation of the excipients in the vehicle, initial diffusion studies were performed using the control topical vehicles in which there was no active drug present. Thereafter, the bases containing rooperol tetra-acetate were analysed. All studies were performed under occluded and unoccluded conditions.

HPLC Analysis

The chromatographic techniques used to identify and quantify the active drug have been described previously. The percent relative standard deviation values were calculated by determining the closeness of the ratio under the peaks of interest and the internal standard, prazepam to a mean value. The mobile phase was modified to acetonitrile/water, 60:40, for the elution of the samples collected during drug permeation across the animal membrane. The increased organic phase allowed for more selective separation of the test sample which often contained interfering substances released from the animal membrane. All other HPLC conditions and evaluation procedures were performed as described in the validation methodology. The concentration of rooperol tetra-acetate present in the sample was calculated using a standard of known concentration. The cumulative amount of rooperol tetra-acetate was thereafter calculated and the quantity of drug released per unit area was determined.

2.4. SUPERSATURATION STUDIES

The principles governing supersaturation techniques were applied to enhance the penetration and subsequent permeation of rooperol tetra-acetate through polydimethylsiloxane and rat membranes. Higuchi was the first to recognise the potential use of supersaturated solutions to enhance topical delivery [125]. In most early studies, supersaturation was achieved by evaporating the volatile phase of volatile/non volatile systems [126-128]. Since Higuchi's work, there has been growing interest in the utilization of these systems as a method for increasing the thermodynamic activity of topical medicaments [129-131]. To overcome the inherent instability of supersaturated solutions, recent studies have explored the effect of polymer addition to these systems [128,132-137]. It has been found that the inclusion of an antinucleant in a supersaturated solution greatly increased the stability of the system thus enabling increased drug transport across a membrane structure over a given period. The aim of the study was to increase the driving force for drug diffusion resulting in enhanced percutaneous penetration of rooperol tetra-acetate beyond the limiting transport values of saturated solutions [95].

Due to the inherent thermodynamic instability of supersaturated preparations, these systems are formed *in situ* using any one of the following methods:

1) heat application followed by cooling
2) solvent removal through evaporation
3) crystallization of the drug from a given solution
4) co-solvent systems resulting in reduced solubility of the drug.

The supersaturated solutions used in the study were prepared by using a procedure previously described by Davis and Hadgraft which makes use of the formation of binary co-solvent systems [95]. The thermodynamic activity of a supersaturated rooperol tetra-acetate solution was explored by adding excess drug to a binary co-solvent system of propylene glycol and water. Chromatographic analysis of the formulated solutions made it possible to construct a saturated solubility curve for rooperol tetra-acetate. From the curve it was possible to determine the compositions of binary solvent-to-drug required to make subsaturated, saturated and supersaturated

systems. Of particular interest to the study were the diffusion properties of active agent from supersaturated solutions through polydimethylsiloxane and rat membrane.

2.4.1. MATERIALS AND METHODS

The materials used have been mentioned previously in this chapter. Sodium carboxyl methyl cellulose and reagent grade propylene glycol were obtained from Saarchem, South Africa. Chromatographic analyses were performed as described in the opening section of this chapter.

2.4.2. EXPERIMENTAL PROCEDURE

Solubility Studies

The solubility of rooperol tetra-acetate in a propylene glycol/water system was determined over a range of 0 to 100% v/v by adding excess drug to the binary co-solvent system. The solubility studies were performed in triplicate. To ensure complete dissolution of rooperol tetra-acetate, the drug was first dissolved in propylene glycol through gentle heating then cooled before adding the aqueous phase. The binary solutions were agitated for 72 hours at a rate of 100 rpm using an electronic shaker (Model 3521, Lab-line Instruments, USA.). The resulting suspensions were thereafter centrifuged at 3000 rpm for 30 minutes using a centrifuge (Model NH-SII, USA.). A 500 µl aliquot of the supernatant was withdrawn, diluted in a suitable quantity of acetonitrile and chromatographically analysed. A saturation solubility profile for rooperol tetra-acetate was plotted from the results obtained.

Stability Studies

To minimise nucleation of the supersaturated solution all glassware used was thoroughly cleaned and dried before use. In order to determine the stability of supersaturated solutions of rooperol tetra-acetate over a 3-hour period, excess drug was prepared in co-solvent systems of propylene glycol/water and propylene glycol with water containing 0.1% polymer. Each supersaturated solution of rooperol tetra-acetate was prepared in triplicate and allowed to stand for a predetermined period.

After the designated time intervals, the test-tubes were shaken for 5 minutes and centrifuged at 3000 rpm for 30 minutes. A 500 µl sample of the supernatant was diluted in acetonitrile and assayed by HPLC. The saturation solubility of the drug in the presence and absence of polymer was recorded as a function of time. The degree of supersaturation of the solutions was calculated by dividing the concentration of drug in the solutions analysed by the saturation solubility in the same propylene glycol/water ratio [132]. The results obtained from stability studies showed that supersaturated solutions in 60% v/v propylene glycol yielded the most stable system which maintained a reasonable degree of supersaturation throughout the investigation. Accordingly, supersaturated solutions in 60% propylene glycol were used for further investigations.

Permeation studies

Membrane permeation studies of supersaturated and saturated solutions of rooperol tetra-acetate were performed in order to characterise drug delivery from thermodynamically dissimilar systems. The diffusion of drug from saturated and supersaturated solutions in 60% propylene glycol across rat skin and polydimethylsiloxane was investigated. For comparative purposes, the supersaturated solutions were prepared in the presence and absence of polymer. Evaporation of the donor phase was prevented by covering the donor compartment with parafilm. At designated time intervals, 500 µl aliquots of the 60:40 ethanol/water receptor phase were withdrawn and the receptor compartment was replenished with fresh receptor phase. The samples were assayed by HPLC techniques previously outlined.

CHAPTER 3: RESULTS AND DISCUSSION

3.1. HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS

3.1.1. MOBILE PHASE DETERMINATIONS

These experiments were designed to develop a suitable mobile phase for the identification and analysis of rooperol tetra-acetate in the receptor phase of diffusion systems. The results showed that a decrease in the organic component ratio resulted in longer elution times, peak tailing and broad peaks. The retention times obtained from analysing different mobile phase compositions are listed in Table 3.

Table 3: HPLC Retention times for rooperol tetra-acetate using different mobile phase compositions

Mobile Phase	Ratio	Retention Time (min) (IS)*
ACETONITRILE: METHANOL: WATER	60:20:20	2.6 (2.9)
ACETONITRILE: METHANOL: WATER	60:10:30	3.4 (3.6)
ACETONITRILE: METHANOL: WATER	75:20:5	2.4 (2.5)
ACETONITRILE: METHANOL: WATER	75:24:1	1.8 (2.1)
ACETONITRILE: ISOPRAPANOL	80:20	8.9 (10.2)
ACETONITRILE: ISOPRAPANOL: WATER	70:20:10	7.3 (9.5)
ACETONITRILE: WATER	75:25	2.7 (2.8)
ACETONITRILE: WATER	70:30	3.2 (4.1)
ACETONITRILE: WATER	65:35	4.1 (5.5)
ACETONITRILE: WATER	60:40	5.6 (6.8)
ACETONITRILE: WATER	55:45	7.9 (10.1)

*IS: Internal Standard

Figures 11 to 16 represent typical chromatograms of rooperol tetra-acetate obtained during the study. These figures display that an increase in the organic phase resulted in narrower peaks of equal band width which eluted at shorter times. Above concentrations of 70% acetonitrile, the peaks of interest were not adequately spaced and therefore, complete peak resolution was not always possible.

FIGURE 11

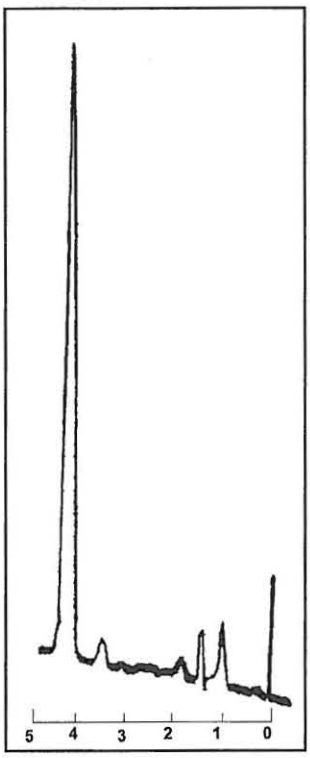


FIGURE 12

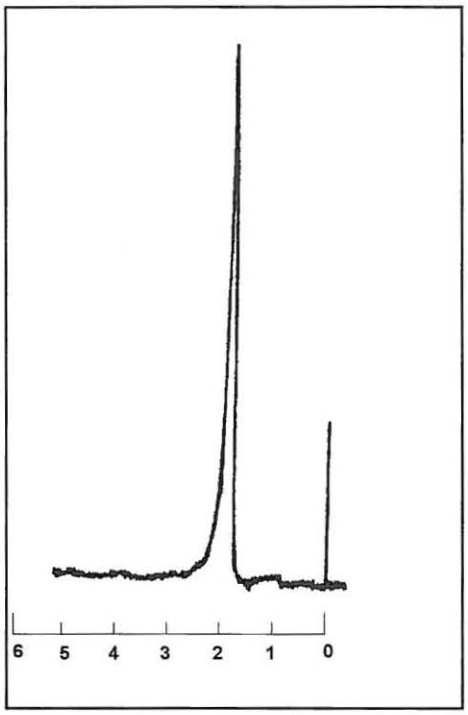
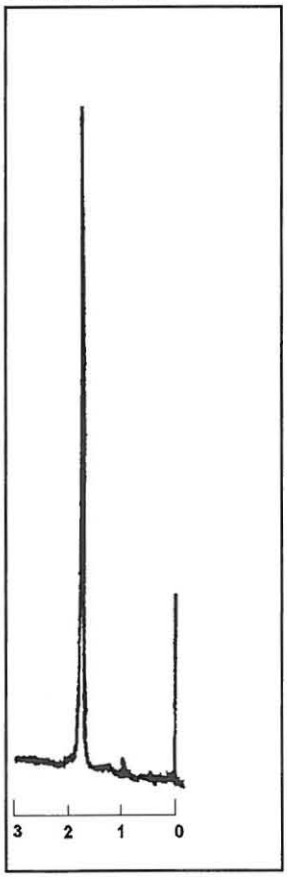


FIGURE 13



Figures 11 to 13 illustrate changes in the chromatographic traces when the ratio of acetonitrile to water is altered.

Figure 11: Mobile Phase; acetonitrile:water; 60:40

Figure 12: Mobile Phase; acetonitrile:water; 70:30

Figure 13: Mobile Phase; acetonitrile:water; 75:25.

FIGURE 14

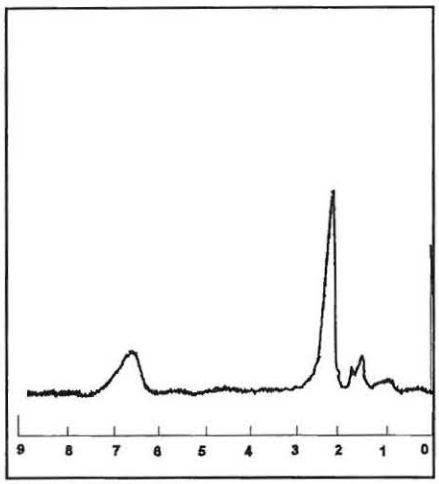
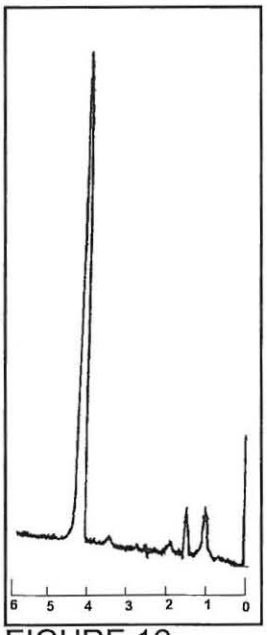


FIGURE 15



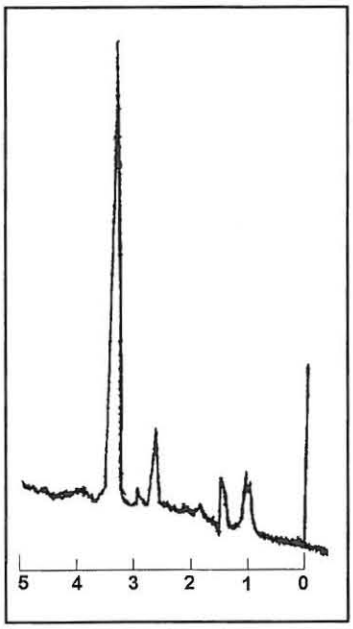
Figures 14 to 16 illustrate changes in the chromatograms when the composition of the organic phase is altered.

Figure 14: Mobile Phase; acetonitrile:isopropanol:water; 70:20:10

Figure 15: Mobile Phase; acetonitrile:methanol:water; 60:20:20.

Figure 16: Mobile Phase; acetonitrile:methanol:water; 70:10:20

FIGURE 16



The inclusion of an additional organic solvent such as methanol or isopropanol to an acetonitrile/water composition decreased the polarity of the mobile phase resulting in a decrease in the retention times as illustrated in Figures 14-16. These figures display the change in the chromatographic traces resulting from mixing several organic solvents during the preparation of the mobile phase. In addition, wider chromatographic peaks were noted due to the less organic compounds weakly binding to the column [Fig 14]. Increasing the flow rate decreased the analysis time. However, at the higher flow rates, the resolution of late eluting chromatographic peaks was inferior.

3.1.2. VALIDATION PROCEDURE

Precision Studies

Precision studies are concerned with the repeatability of a given analytical method [107,138]. Precision is often expressed as the percent relative standard deviation and the data obtained from these studies reflects the distribution of test results around their mean value. Most reported chromatographic methods have percent relative standard deviations of between 5% and 10%. However, the commonly accepted limit for inter-day relative standard deviation values for a given HPLC assay is less than 5%. The percent relative standard deviation for the nine sets of ten injections had an overall average of 2.4%. The percent relative deviation values for day one to day three are presented in Table 4. It is evident from the results that the assay method is sufficiently precise for the quantitative analysis of rooperol tetra-acetate.

Table 4: Percent relative standard deviation values for rooperol tetra-acetate injections (n = 10 for each trial on each day).

	DAY 1	DAY 2	DAY 3
TRIAL 1	2.71%	2.25%	1.89%
TRIAL 2	4.23%	3.36%	2.10%
TRIAL 3	1.42%	1.45%	2.33%

Limit of Quantitation

Limit of quantitation studies are used to quantify the lowest concentration of drug that may be accurately determined in a given sample [107,138]. Results for this test were obtained by calculating the percent relative standard deviation of the instrument response for progressively lower concentrations of rooperol tetra-acetate. The lowest concentration quantified from the ratio of areas was 0.0501 µg/ml. However, at these low concentrations the percent relative standard deviation value was as high as 15.2%, thus indicating that the reliability of the system at this level is significantly reduced.

Sensitivity Studies

Sensitivity studies are dependent upon the degree of response, by the system, to concentrations of analyte covering the entire range of analysis [109]. The gradient of the analytical calibration curve provides a measure of the sensitivity of the system. In the analysis of rooperol tetra-acetate, the gradient and therefore the sensitivity of the absorbance against concentration response was calculated as 0.5727 (FIG 10). In addition, the standard deviation about the mean of each point of the calibration curve is an indication of the accuracy with which the system is able to respond to decreasing concentrations of analyte. The presence of narrow standard deviation bars indicates that the sensitivity of the system is satisfactory.

Table 5: Calibration curve data for rooperol tetra-acetate in an acetonitrile: ethanol: water (30:35:35) system (n = 10).

	RATIO OF AREAS (ROOPEROL TETRA-ACETATE/INTERNAL STANDARD)		
CONC. (µg/ml)	DAY 1	DAY 2	DAY 3
0.0501	0.024 ± 0.002	0.027 ± 0.004	0.035 ± 0.002
0.07014	0.052 ± 0.006	0.037 ± 0.0008	0.058 ± 0.005
0.1002	0.125 ± 0.008	0.053 ± 0.003	0.074 ± 0.003
0.3006	0.206 ± 0.006	0.181 ± 0.002	0.263 ± 0.004
0.7014	0.437 ± 0.007	0.396 ± 0.003	0.464 ± 0.007
1.002	0.603 ± 0.014	0.542 ± 0.006	0.593 ± 0.007
Gradient	0.5738	0.5478	0.6012
Corr. Coefficient	0.9958	0.9992	0.9909
y-intercept	0.0328	0.0024	0.0215

Table 5 presents data obtained from calibration standard assays performed over three days. It is evident from the data presented in this table that for all calibration curves generated, acceptable intra- and inter-day precision was achieved.

Linearity Studies

When examining the calibration curve (FIG 10), the ratio of areas is evidently proportional to the concentration of rooperol tetra-acetate present in the samples analysed. The correlation coefficient of the regression line, which gives a measure of linearity, was calculated to be 0.9977 (FIG 10). A correlation of one indicates perfect linearity and therefore, the value obtained experimentally certifies that the system is acceptably linear for quantitative analytical purposes.

Range Studies

The range of an analytical method is the interval between the upper and lower analyte levels for which a suitable degree of precision, accuracy and linearity has been displayed [107,138]. The results obtained during this test clearly indicate that both the system and method employed are reliable. Acceptable precision was obtained when the method used was applied to samples containing concentrations of analyte which encompass the entire range of analysis [138,139]. The percent deviations of the masses weighed range from -0.09% to +2.6% (Table 6).

Table 6: Range data representing the different masses of rooperol tetra-acetate analysed (n= 10).

MASS RTA WEIGHED(g)	MEAN MASS RTA ASSAYED(g)	PERCENT STANDARD DEVIATION
0.00014	0.000143	-2.1
0.0017	0.001798	-1.6
0.002276	0.002246	-0.037
0.00042	0.000409	+2.6
0.02209	0.021587	+2.3
0.03057	0.030596	-0.09

3.1.3. CONCLUSION

The precision, linearity, sensitivity and range studies data indicate that the method selected is suitable for the quantitative analysis of rooperol tetra-acetate. The validation results have demonstrated that HPLC is a highly selective and specific analytical technique which exhibits good reliability and reproducibility. Therefore, the method developed can be used to determine the diffusion characteristics of the drug through membrane systems.

3.2. STABILITY- INDICATING STUDIES

The overall stability test results indicate that there was significant rooperol tetra-acetate degradation in the glass- and plastic-stored samples following exposure to high temperatures during the three months of analysis. After a week of storage at high temperatures, degradation of the drug in the glass vial was more pronounced than in that of the plastic vial. Chromatograms generated after analysing the plastic-stored samples are shown in Figure 17. Peaks A and B represent the parent drug and the internal standard, prazepam, respectively. It is important to note that at week one [FIG 17(I)], the concentration of drug used was a third less than the concentration of drug used in subsequent weeks [FIGS 17(II) and (III)]. The reason for decreasing the rooperol tetra-acetate concentration in the first week was to avoid plotting chromatograms which had a drug:internal standard peak height ratio that was greater than the generally accepted 3:1 limit. Peaks C, D, E and F [FIG 17] were not present in the chromatograms of the freshly prepared samples, but began to appear in both the plastic- and glass-stored samples following one week of exposure to elevated temperatures. Initially, a single peak (A), representing rooperol tetra-acetate was detected. However, with time, additional peaks (C, D, E, F) were detected, thus signifying advanced deterioration of the drug. The stability profiles of rooperol tetra-acetate display a significant percentage mass decrease in peak A and a percentage mass increase in peaks C, D, E and F in both the glass vial [FIG 18] and the plastic vial [FIG 19].

FIG 17: Chromatographic traces of RTA (peak A), the internal standard (peak B) and degradants (peaks C to F): I, II and III represent thermal degradation of drug in the plastic vial after 1, 2 and 8 weeks, respectively.

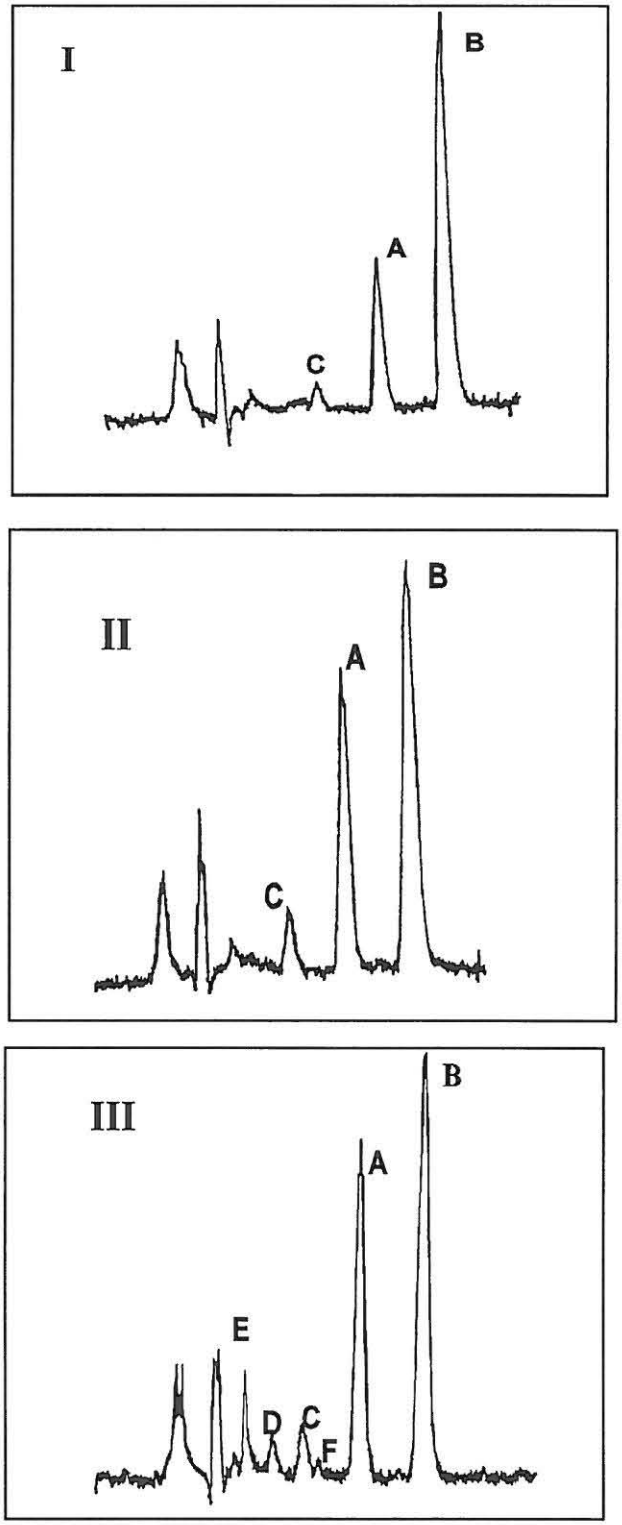


Figure 18: Stability profile of glass-stored sample.

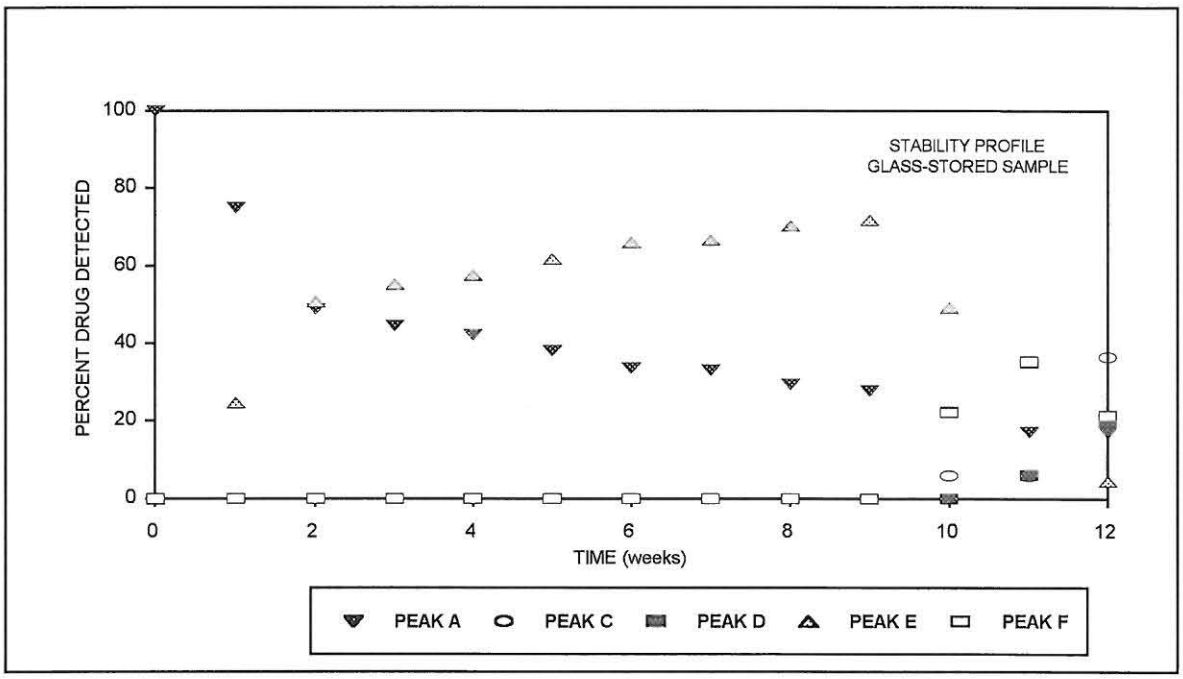
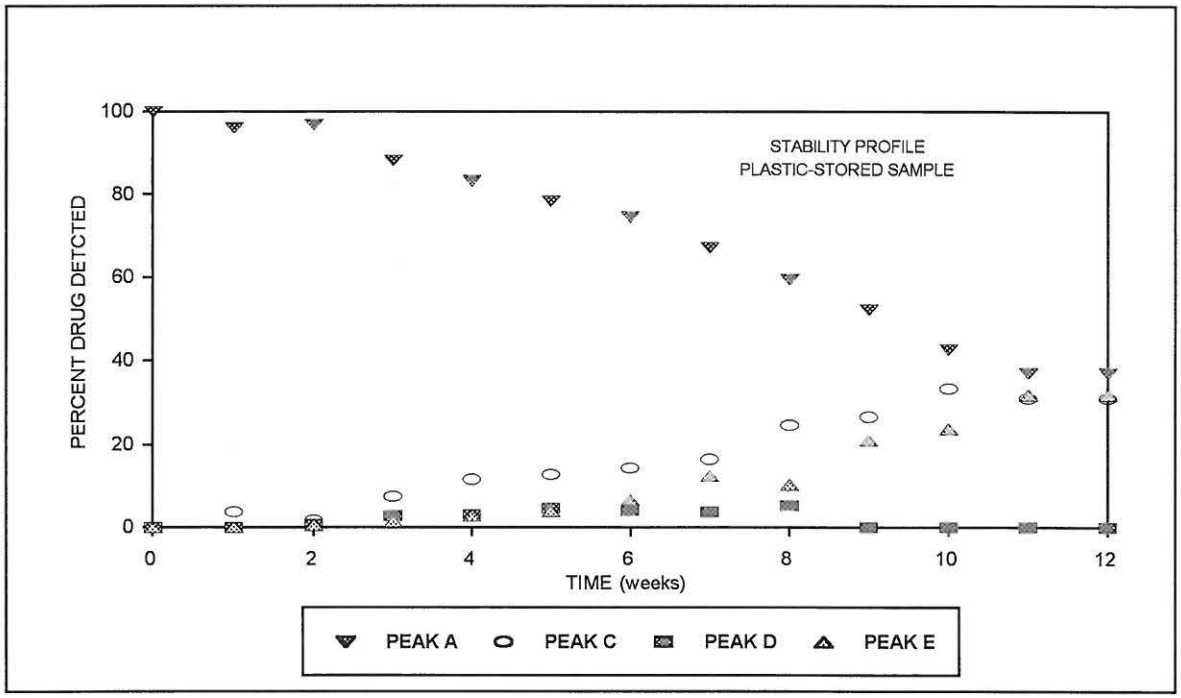


Figure 19: Stability profile of plastic-stored sample.



At weeks eleven and twelve the baseline of the chromatograms was very jagged thus suggesting the emergence of several additional degradation products whose concentrations were too low for the detector to quantitate. Slight variations from week to week in the mobile phase composition, injection standard and temperature during chromatographic analysis may well have contributed to changes in the sensitivity of the method which resulted in the collection of variable data for the degradants with low concentrations. The early detection and emergence of numerous break down products observed with the plastic-stored sample may be due to unfavourable interactions between the drug and plasticizers released from the vial upon heat exposure. In contrast, fewer degradation peaks emerged from the glass-stored drug during the first ten weeks of analysis, but, as seen in Figure 18, the initial rate of drug deterioration was faster and after twelve weeks, approximately 20% less parent drug remained in the glass vial than in the plastic vial. It is possible that chemical agents in the vials may affect the stability of rooperol tetra-acetate. The mechanism by which these chemicals influence degradation is unknown however, it is probable that in their presence, deterioration of the parent drug is faster and more pronounced.

The student's *t*-distribution test was applied to the peak area ratios obtained for all peaks measured during the stability study analyses. The results showed that at a 95% level, there is a significant difference in composition between the freshly prepared samples and the heated samples. These statistical results further confirm that thermodegradation of rooperol tetra-acetate is a significant physical property of the drug. From a study of the structure of rooperol tetra-acetate [FIG 3], it is safe to presume that the metabolites formed when the drug is stored under elevated temperatures, arise from successive cleavage of the acetate chemical groups. A pungent scent of acetic acid was detected from the vials containing the degrading drug. This positive identification of acetic acid further supports the postulate that initial thermodegradation is due to loss of the acetate groups. These acetate groups, situated on the periphery of the structure, experience decreased electronic attraction from the strong pi and triple bonds present at the core of the molecule resulting in relatively easier detachment of the groups from the core structure. Subsequent degradation may arise as a result of cleavage of the bonds which bind the two aromatic rings of the

rooperol tetra-acetate structure. Future studies could incorporate the use of mass spectrophotometry and nuclear magnetic resonance to fully elucidate the degradation pathways.

3.2.1. CONCLUSION

The stability profiles of rooperol tetra-acetate show that the drug is significantly unstable and its degradation upon exposure to elevated temperatures for extended periods of time is an important physical property of the drug. In addition, the results suggest that the type of chemicals present in the storage container may compound the deterioration of the active drug. The inherent instability of the drug at elevated temperatures impacts on the manufacturing and diffusion processes where heat application may be necessary. For this reason it is essential that the possible emergence of degradation peaks is acknowledged, particularly during the analysis of the diffusion properties of the drug.

3.3. DRUG PERMEATION STUDIES

Results obtained from the chromatographic analysis of the receptor phase show that no detectable drug diffused through full thickness rat skin into the receiving fluid throughout the 24 hours that followed application of the topical vehicles to the stratum corneum. In contrast, an appreciable quantity of rooperol tetra-acetate permeated through polydimethylsiloxane, hence, the results presented in this section describe drug diffusion through this synthetic membrane. Figures 20 and 21 give the composite profile for rooperol tetra-acetate diffusion through polydimethylsiloxane under occluded and unoccluded conditions. Each figure is a typical illustration of the cumulative permeation rate for the active drug from each topical vehicle of each batch analysed under the conditions stated above. Each point represents the mean ± SD of nine determinations. The steady flux rates obtained experimentally are listed in Table 7 for the occluded state and Table 8 for the unoccluded state. The lag time listed in the tables was obtained by applying linear regression analysis to extrapolate the linear

steady-state portion of the permeation profiles of each batch of formulation to the time axis [FIG 6]. In all cases the calculated correlation coefficient (*r*) was not less than 0.97. The diffusion coefficients, which are a function of the lag time and membrane thickness, were thereafter calculated using Equation 7. The gradient of the linear region of the plots of mass of permeant in the receptor fluid against time give the flux rates presented in Tables 7 and 8. Once the permeability and diffusion coefficients had been determined, it was possible to calculate the partition coefficient using Equation 8. It must be noted that the partition coefficients were calculated from data obtained from the occluded diffusion experiments as greater control over experimental conditions was possible and therefore, the influence of external variables, capable of altering drug release, was minimised.

Figure 20: Total amount of drug permeation-occluded (n=9).

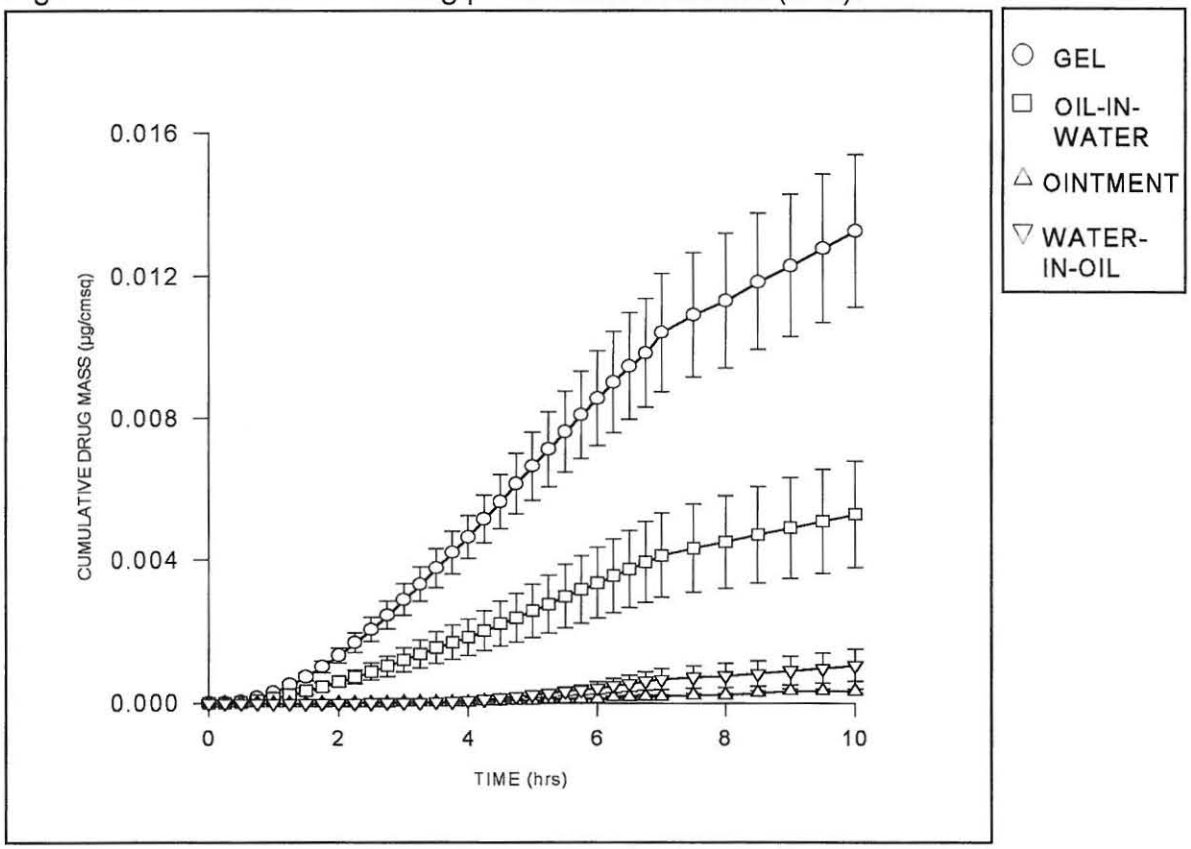
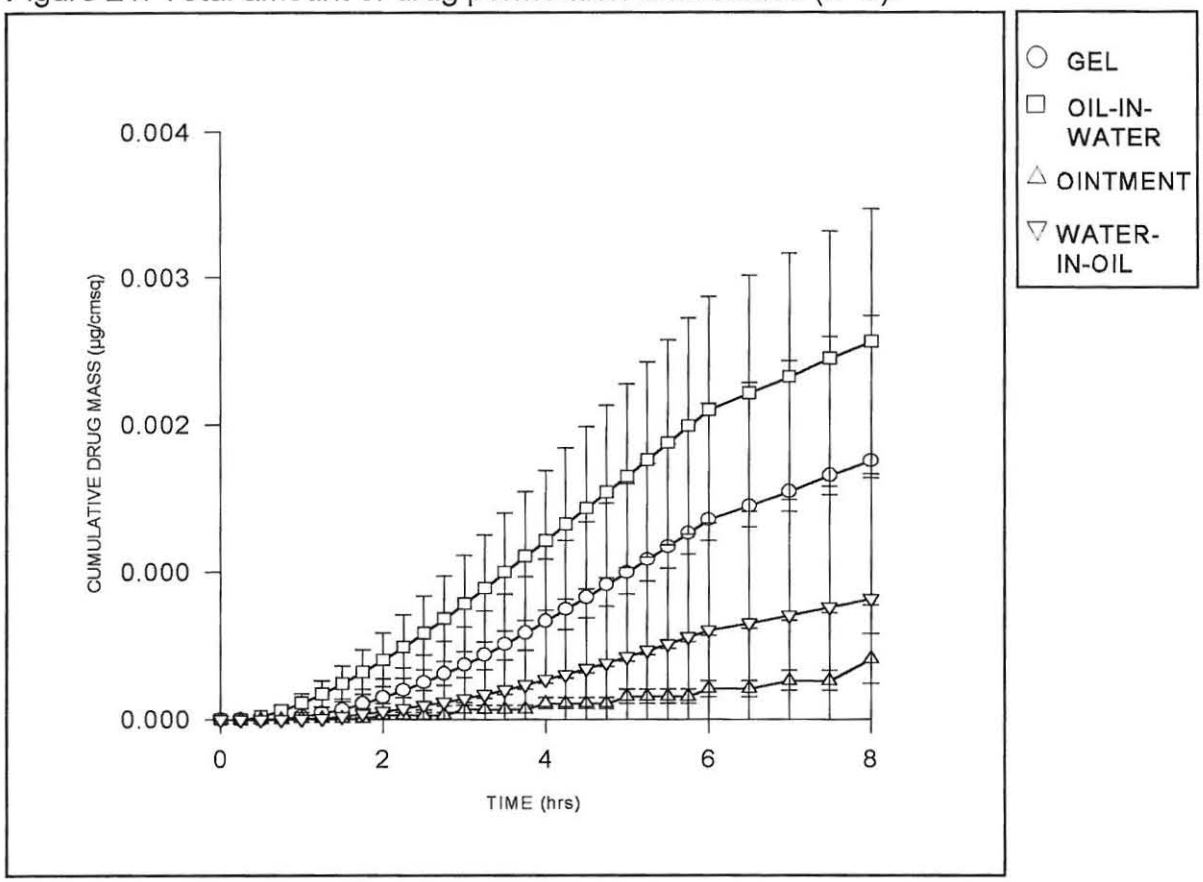


Figure 21: Total amount of drug permeation-unoccluded (n=9).



It is apparent from Figures 20 and 21 that the greatest drug permeation occurred from the more hydrophilic vehicles and drug penetration decreased as the lipophilic content of the vehicle was increased. The standard deviation bars of the occluded plots suggest that there is a significant difference between each base analysed. The *t*-distribution test, which was applied to the results obtained from each base, indicated that at a 95% confidence level the drug delivery rates from all vehicles are significantly different except from the ointment and water-in-oil bases. This is the rank order of diffusion that one would expect based on thermodynamic leaving potential principles. The results presented in Figure 21 deviate slightly from the theoretically expected diffusion profiles due to nonocclusion of the donor compartment. Greater release of rooperol tetra-acetate is noted from the oil-in-water preparation than from the gel vehicle. The ointment and water-in-oil profiles under occluded and unoccluded

conditions are similar. The wide standard deviation bars seen in Figure 21 suggest that there is no significant difference in the vehicles tested. However, application of the *t*-distribution test to the results presented in Figure 21 showed that at a 95% confidence level, drug delivery from the gel and oil-in-water bases were not significantly different while drug delivery from each of these bases were different from all other vehicles analysed.

Table 7: Mean permeation parameters for rooperol tetra-acetate diffusing from all vehicles applied onto the polydimethylsiloxane membrane under occluded conditions.

Formulation	Js (μg/cm^2/h)	L (h)	D(cm^2/hx10^5)	K
Gel				
Batch 1*	24.9 ± 1.55	1.30 ± 0.08	1.84 ± 0.13	7.40 ± 0.80
Batch 2*	24.86 ± 4.40	1.72 ± 0.39	1.46 ± 0.31	9.38 ± 1.14
Batch 3*	17.76 ± 3.06	1.45 ± 0.22	1.70 ± 0.29	5.95 ± 1.74
Mean (n=9)	22.5 ± 4.65	1.49 ± 0.32	1.67 ± 0.30	7.57 ± 1.91
Oil-in-water				
Batch 1*	10.34 ± 2.28	1.18 ± 0.18	2.09 ± 0.33	2.84 ± 0.96
Batch 2*	9.60 ± 2.37	1.37 ± 0.20	1.79 ± 0.25	2.89 ± 0.43
Batch 3*	4.68 ± 1.53	1.22 ± 0.12	1.98 ± 0.18	1.33 ± 0.54
Mean (n=9)	8.23 ± 3.23	1.44 ± 0.29	1.95 ± 0.29	2.35 ± 0.99
Ointment				
Batch 1*	0.65 ± 0.05	2.23 ± 0.47	1.12 ± 0.21	0.33 ± 0.05
Batch 2*	0.73 ± 0.14	3.26 ± 0.46	0.75 ± 0.12	0.53 ± 0.12
Batch 3*	0.44 ± 0.02	2.50 ± 0.23	0.97 ± 0.1	0.26 ± 0.03
Mean (n=9)	0.61 ± 0.15	2.66 ± 0.59	0.95 ± 0.21	0.37 ± 0.14
Water-in-oil				
Batch 1*	2.80 ± 0.24	3.79 ± 0.39	0.64 ± 0.07	2.42 ± 0.44
Batch 2*	3.18 ± 0.57	3.74 ± 0.44	0.65 ± 0.07	2.75 ± 0.82
Batch 3*	0.95 ± 0.44	6.05 ± 2.83	0.58 ± 0.40	1.08 ± 0.3
Mean (n=9)	2.31 ± 1.06	3.66 ± 0.46	0.62 ± 0.24	2.07 ± 0.92

* n = 3

Table 8: Mean permeation parameters for rooperol tetra-acetate diffusing from all vehicles applied onto the polydimethylsiloxane membrane under unoccluded conditions.

Formulation	Js (µg/cm^2/h)	L (h)	D(cm^2/hx10^5)	K
Gel				
Batch 1*	1.90 ± 0.30	1.99 ± 0.21	1.21 ± 0.12	1.03 ± 0.17
Batch 2*	6.45 ± 0.28	1.72 ± 0.01	1.40 ± 0.009	3.05 ± 0.13
Batch 3*	2.56 ± 0.07	2.17 ± 0.27	1.12 ± 0.16	1.53 ± 0.21
Mean (n=9)	3.64 ± 2.02	1.96 ± 0.27	1.25 ± 0.16	1.87 ± 0.87
Oil-in-water				
Batch 1*	5.87 ± 0.19	1.03 ± 0.03	2.32 ± 0.06	1.66 ± 0.05
Batch 2*	6.71 ± 0.23	1.21 ± 0.02	1.99 ± 0.03	2.22 ± 0.07
Batch 3*	2.54 ± 0.07	1.62 ± 0.14	1.50 ± 0.13	1.12 ± 0.07
Mean (n=9)	5.04 ± 1.81	1.29 ± 0.26	1.93 ± 0.35	1.67 ± 0.45
Ointment				
Batch 1*	0.64 ± 0.04	1.55 ± 0.15	1.56 ± 0.16	0.27 ± 0.01
Batch 2*	0.60 ± 0.04	1.45 ± 0.17	1.69 ± 0.22	0.24 ± 0.02
Batch 3*	0.42 ± 0.06	2.44 ± 0.45	1.01 ± 0.17	0.28 ± 0.07
Mean (n=9)	0.55 ± 0.11	1.82 ± 0.53	1.42 ± 0.35	0.26 ± 0.05
Water-in-oil				
Batch 1*	2.03 ± 0.26	1.88 ± 0.12	1.28 ± 0.08	0.97 ± 0.22
Batch 2*	2.44 ± 0.05	1.97 ± 0.02	1.22 ± 0.01	1.32 ± 0.03
Batch 3*	0.69 ± 0.09	3.91 ± 0.60	0.72 ± 0.04	0.73 ± 0.03
Mean (n=9)	1.72 ± 0.76	2.59 ± 1.00	1.04 ± 0.30	1.00 ± 0.27

* n = 3

Drug penetration is dependent upon the influence of the vehicle on the thermodynamic activity of the active ingredient. Rooperol tetra-acetate is a lipophilic drug, hence its release is favoured from hydrophilic bases such as the gel and the oil-in-water formulations which are poor solvents for the drug. Consequently, there are fewer drug-vehicle interactions leading to improved partitioning of the drug into the membrane. Figures 20 and 21 clearly demonstrate the superior drug release from the hydrophilic vehicles when subjected to occluded and unoccluded conditions.

Furthermore, the flux rates from the gel and oil-in-water bases shown in Tables 7 and 8 are notably higher than those from the hydrophobic bases. The lower thermodynamic activity and therefore slower release of rooperol tetra-acetate from the ointment and water-in-oil bases is a result of the enhanced affinity between the drug and the lipophilic vehicles. The longer lag time values of the ointment and water-in-oil vehicles [Tables 7 and 8] give further support to the hypothesis that lipophilic drugs are preferentially retained by hydrophobic bases. Furthermore, a lag time is noted in the plots of cumulative amount of drug released versus time [FIG 20 and 21]. The presence of this lag time suggests the existence of a rate limiting factor caused either by resistance of the membrane to penetrating molecules or delayed release of the drug from the vehicle. The membrane-vehicle partition coefficients provide an indication of the extent to which drug-vehicle interactions influence drug release. A high partition coefficient suggests that the drug favours the membrane environment over the vehicle environment resulting in enhanced penetration. In these experiments the greatest partition coefficient was calculated for the drug in the gel/membrane system under occluded conditions. This corroborates the high thermodynamic leaving potential proposed for this system and is exemplified by the very high flux rates measured from the gel vehicle. An equally important factor which influences drug partitioning from a topical vehicle into a membrane, especially biological tissue, is the degree of hydration. Hydration of the polydimethylsiloxane membrane may result in swelling of the polymer matrix and a consequent loss in the rigidity of the matrix unit of the membrane. Due to increased flexibility of the polymer structure a less arduous route for drug diffusion is created [140]. Maintaining adequate membrane hydration is therefore essential to the dissolution of the drug into and subsequent diffusion through the polymer matrix. During the diffusion experiments, the receptor fluid was in continuous contact with the membrane resulting in complete hydration of the membrane. This heightened state of hydration was maintained through the application of external occlusion which prevented the evaporation of water and volatile constituents from the formulations. Moreover, the composition of the vehicles in the occluded mode remained relatively constant throughout the delivery period. In contrast, immediately after the application of a topical base onto a membrane under unoccluded conditions, the base begins to

undergo physical changes which alter the composition of the vehicle. In the unoccluded mode, evaporation of the aqueous and other volatile components of the base takes place throughout the diffusion process. Consequently, the thermodynamic leaving potential of the drug is constantly changing as a result of the gradual increase in the lipophilicity of the vehicles that gives rise to conditions which do not favour the delivery of rooperol tetra-acetate. It is apparent when comparing Figure 20 with Figure 21 that the unoccluded hydrophilic bases, especially the gel formulation, are more affected by this steady change in vehicle composition. The inability to control the evaporation process of the volatile components from the vehicles resulted in erratic drug release from the unoccluded bases with marked variability in the drug release rates. This irregularity in drug diffusion is reflected in Figure 21 where broad standard deviation bars are evident. A close study of the permeation parameters in Tables 7 and 8 will reveal distinct differences in the partition coefficients of the drug for each base analysed, while, as expected, the diffusion coefficients values do not vary widely. This observation implies that the release of rooperol tetra-acetate from the vehicles is not rate limiting and instead, it is the presence of the membrane between the formulation and the receptor phase which is causing the finite time delay to drug diffusion [141]. During the initial non steady state, it is postulated that solvents from the vehicle, and in this case the receptor phase, penetrate the membrane resulting in changes to the structure of the polymer matrix. The extent to which the polymer matrix is altered is dependent upon the type and concentration of solvent used. During the lag time agents such as propylene glycol and ethanol are believed to cause swelling of the polymer matrix thus creating a preferential route for drug diffusion. This change in membrane structure therefore facilitates the steady state release of drug molecules at periods beyond the lag times reported [140].

3.3.1. CONCLUSION

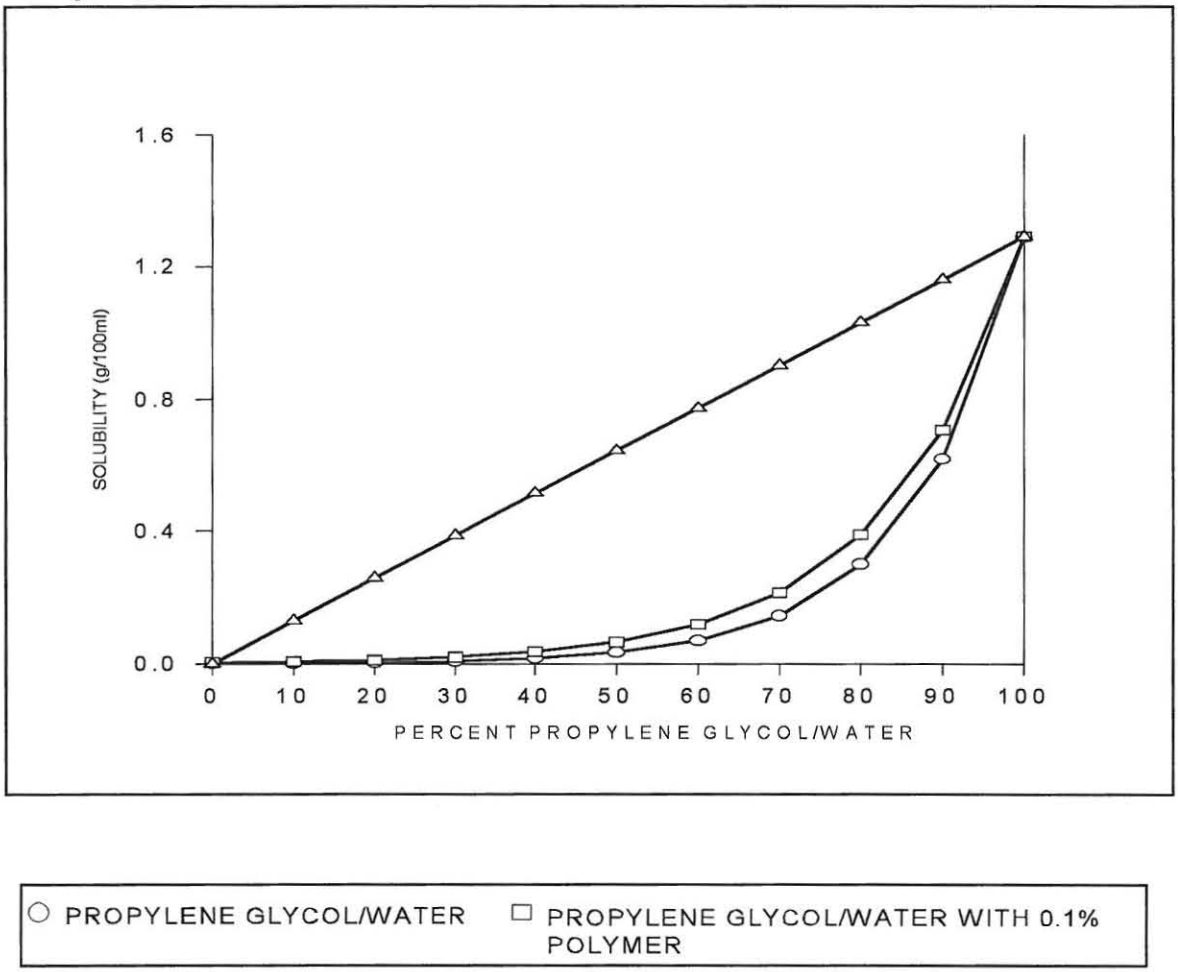
In vitro diffusion experiments of this type are useful in that they provide valuable insight into the performance of potential therapeutic formulations. The studies performed adequately demonstrated batch-to-batch uniformity of the topical vehicles manufactured and tested in the occluded mode. It is clear from these results that quality control tests for rooperol tetra-acetate are best performed in the occluded diffusion mode. The use of polydimethylsiloxane membrane allowed for controlled research into the physico-chemical factors that influence rooperol tetra-acetate release from hydrophilic and lipophilic bases. The results obtained can be used to elucidate the mechanisms by which the release of rooperol tetra-acetate occurs from various topical bases and thus predict the ideal conditions required for optimum percutaneous delivery of the drug. As the clinical administration of topical products is more likely in the unoccluded mode, the final formulation developed for this therapeutic agent will need to maximise delivery potential in spite of the evaporation of volatile components.

3.4. SUPER-SATURATION STUDIES

Solubility Studies

Saturation solubility tests serve as the basis for supersaturation investigations and the results generated from the solubility experiments are essential to the development of a successful supersaturated system. These studies define the solubility properties of the drug in the binary solvents used and in doing so, prescribe the thermodynamic potential of a given system at an early stage of analysis. The saturation solubility profiles of rooperol tetra-acetate in propylene glycol/water and propylene glycol/water with sodium carboxyl methyl cellulose are shown in Figure 22. The graph clearly demonstrates the increase in rooperol tetra-acetate solubility as the propylene glycol proportion is increased. The concave solubility profiles indicate that supersaturated systems can be created by mixing suitable drug/co-solvent mixtures. This characteristic exponential increase in drug solubility has been reported by numerous researchers working in this field [95,132,133,142].

Figure 22: Theoretical analysis: Saturated solubility profile of rooperol tetra-acetate in propylene glycol/water in the absence and presence of 0.1% sodium carboxyl methyl cellulose.

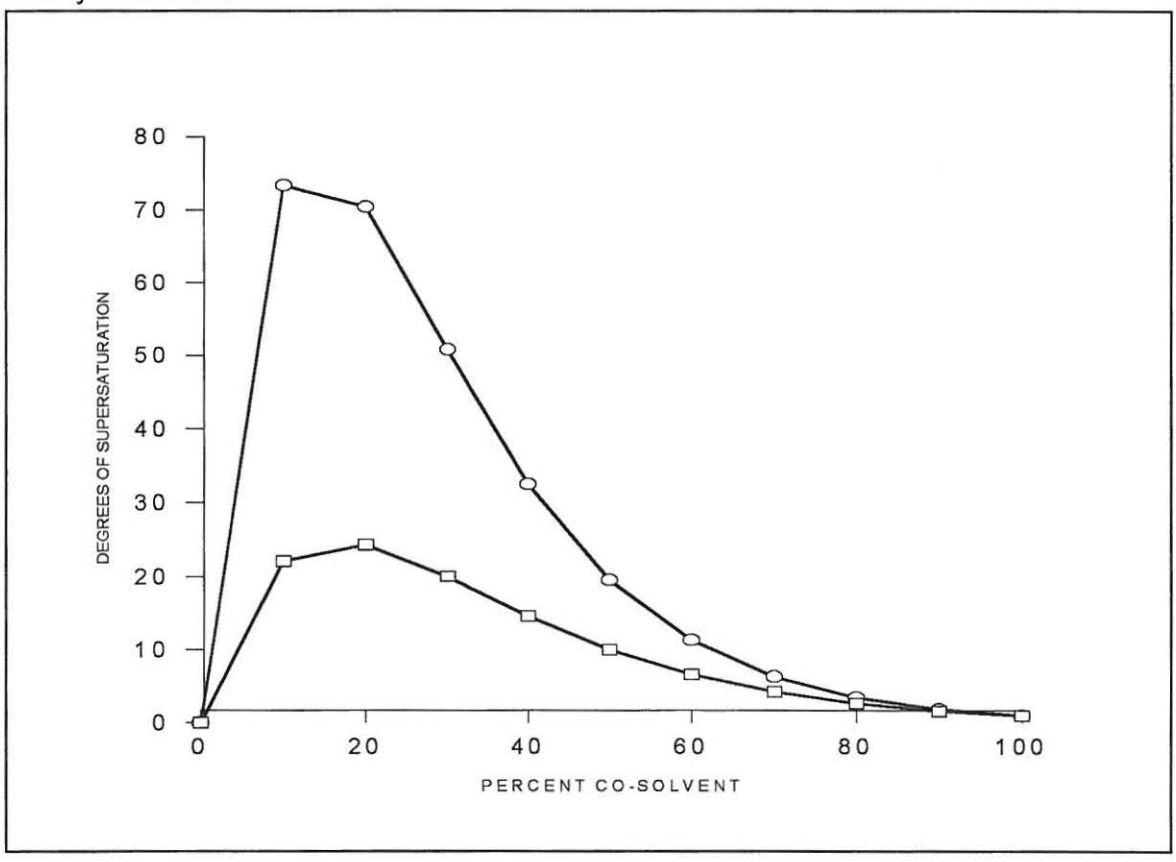


One of the principal functions of surfactants such as sodium carboxyl methyl cellulose is to improve the stability of drugs in a given solvent. The higher saturation solubility profile for rooperol tetra-acetate in the presence of sodium carboxyl methyl cellulose displayed in Figure 22, graphically illustrates the increase in drug solubility resulting from the inclusion of a polymer.

A linear relationship between solubility and volume fraction of co-solvent was found to exist in binary aqueous systems [142,143]. Using data obtained from the solubility studies, the linear line displayed in Figure 22 was plotted. Systems with points along this line are said to be metastable supersaturated solutions. The drug

solubility of metastable solutions is higher than normal and the stability of these solutions is maintained until an external influence initiates drug precipitation. Once nucleation is initiated, the drug exhibits a time dependent decrease in solubility until saturation levels are reached. Systems with points that lie above the linear line are said to be labile supersaturated solutions and within such systems, nucleation occurs immediately upon establishing a supersaturated state [142]. Subsaturated solutions have points existing below the concave lines.

Figure 23: Theoretcal analysis: Degrees of supersaturation for rooperol tetra-acetate in propylene glycol/water in the absence and presence of 0.1% sodium carboxyl methyl cellulose.



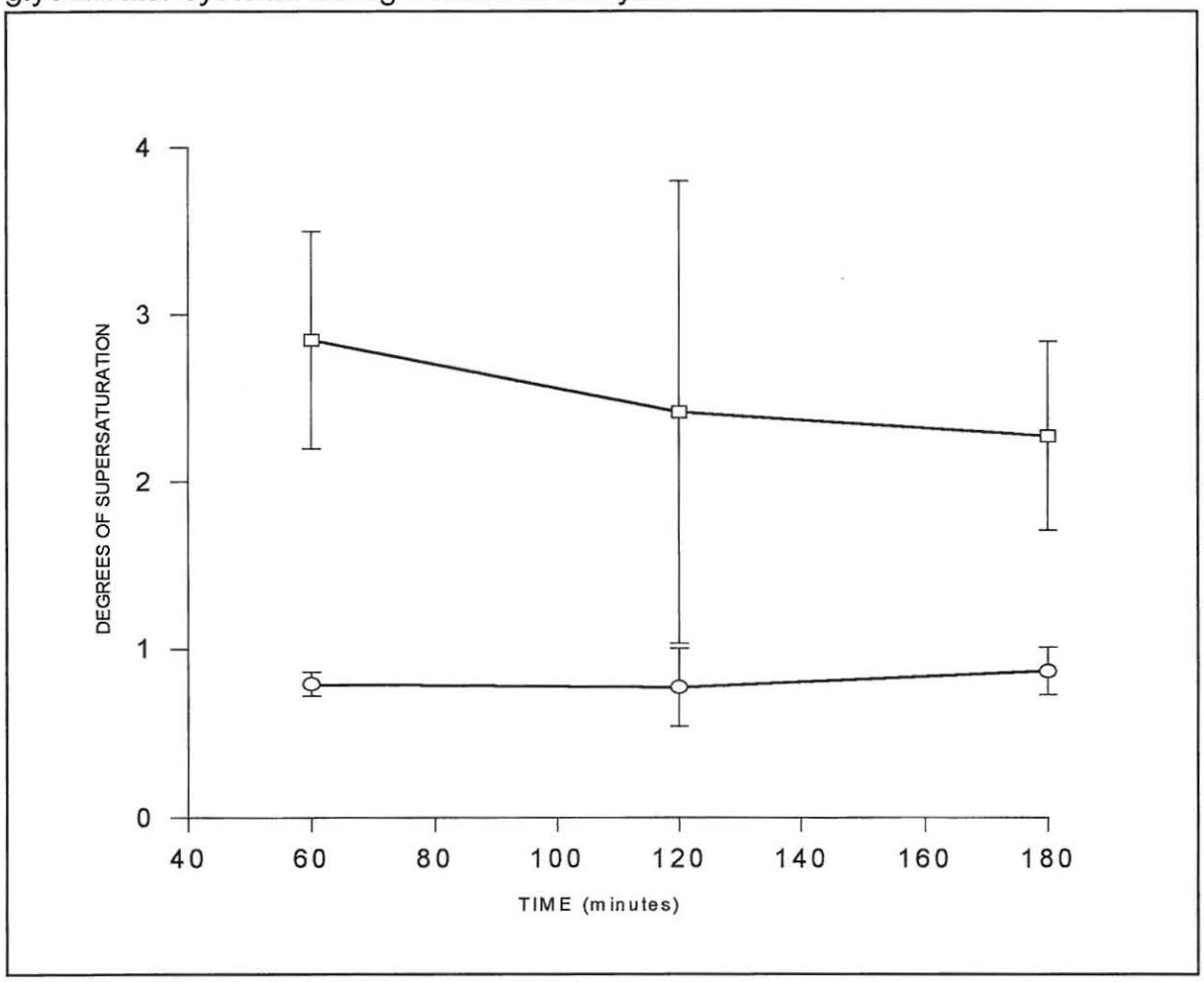
The profiles generated from the saturation solubility experiments can be used to determine the potential degree of supersaturation that a system can achieve when appropriate solute/co-solvent combinations are mixed. The degree of supersaturation is the ratio of the actual drug concentration of a given solution to the saturation solubility for the same vehicle combination [95]. The degrees of supersaturation which can be achieved from adding 0.35 g/100 ml rooperol tetra-acetate to a co-solvent combination of propylene glycol with and without polymer are expressed in Figure 23. The graph shows that the highest degree of supersaturation is normally obtained at low propylene glycol ratios of between 10% to 30%. Thereafter, as the propylene glycol ratio increases and the system becomes more stable, the degree of supersaturation decreases. A similar profile is noted when a polymer is added to the system, however, the difference between the two profiles is that at each solvent ratio, the degrees of supersaturation reached are lower than when the polymer is excluded. A high degree of saturation is desirable for enhanced drug diffusion, however, systems with high thermodynamic activity often exhibit extreme instability.

Stability Studies

Although solutions with a high degree of supersaturation are desirable for good thermodynamic action and optimum diffusion, such systems are highly unstable and will therefore decrease the quantity of drug in solution in an attempt to gain greater stability. As increasing amounts of drug are precipitated out of a supersaturated solution, the degrees of supersaturation decrease until saturation levels are reached. The time taken for a supersaturated solution to reach a saturated state varies among different systems. Ideally, a system should maintain the elevated supersaturation levels for, at minimum, the duration of its use.

Experiments designed to determine the stability of rooperol tetra-acetate dissolved in a solvent system of 60% propylene glycol/water (with and without polymer) for three hours were conducted and the results obtained are presented in Figure 24. This figure shows that the experimental degree of supersaturation for the propylene glycol/water without polymer system reached a maximum of 0.78 (plot B). This value hardly allows for an adequate magnitude of supersaturation over a three-hour period.

Figure 24: Stability determinations of rooperol tetra-acetate in 60% propylene glycol/water systems during 3 hours of analysis.



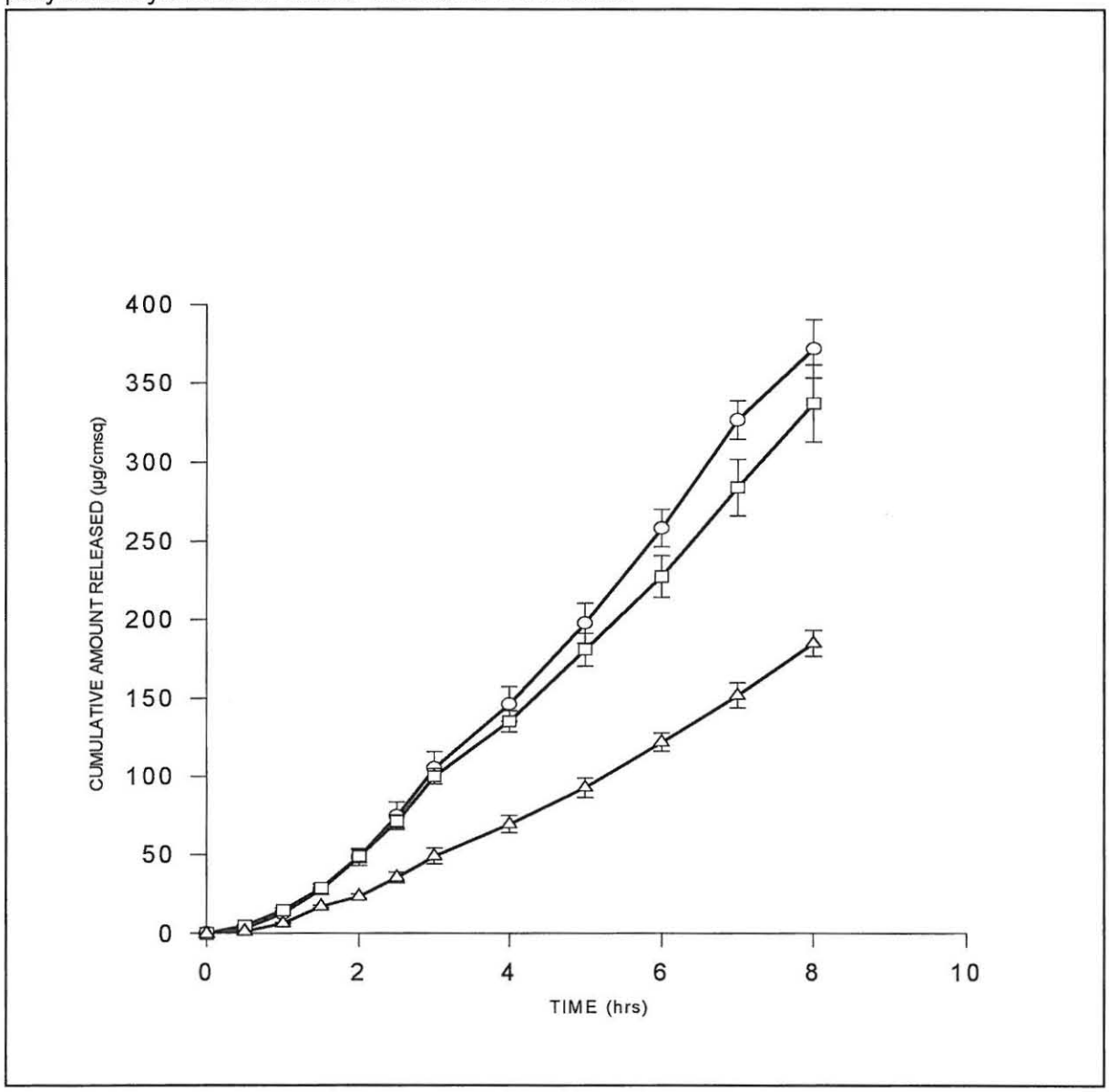
Rapid drug nucleation within the first hour of analysis is a likely cause for the low levels of supersaturation observed in the non polymer containing system. In comparison, the degrees of supersaturation reached in the polymer containing solvent are notably higher. This elevated thermodynamic state is attributed to improved stability of the

system throughout the three hours of analysis. The maximum degrees of supersaturation obtained when an antinucleant was included in the binary solution was 2.7 and after three hours of analysis, the initial concentration had only decreased by approximately 25% (Figure 24). Experiments performed for over 8 to 12 hours are likely to show a more distinguishable decrease in the initial drug concentration. Nevertheless, the graph adequately demonstrates the effect that antinucleating agents have on the stability of supersaturated systems.

Permeation Studies

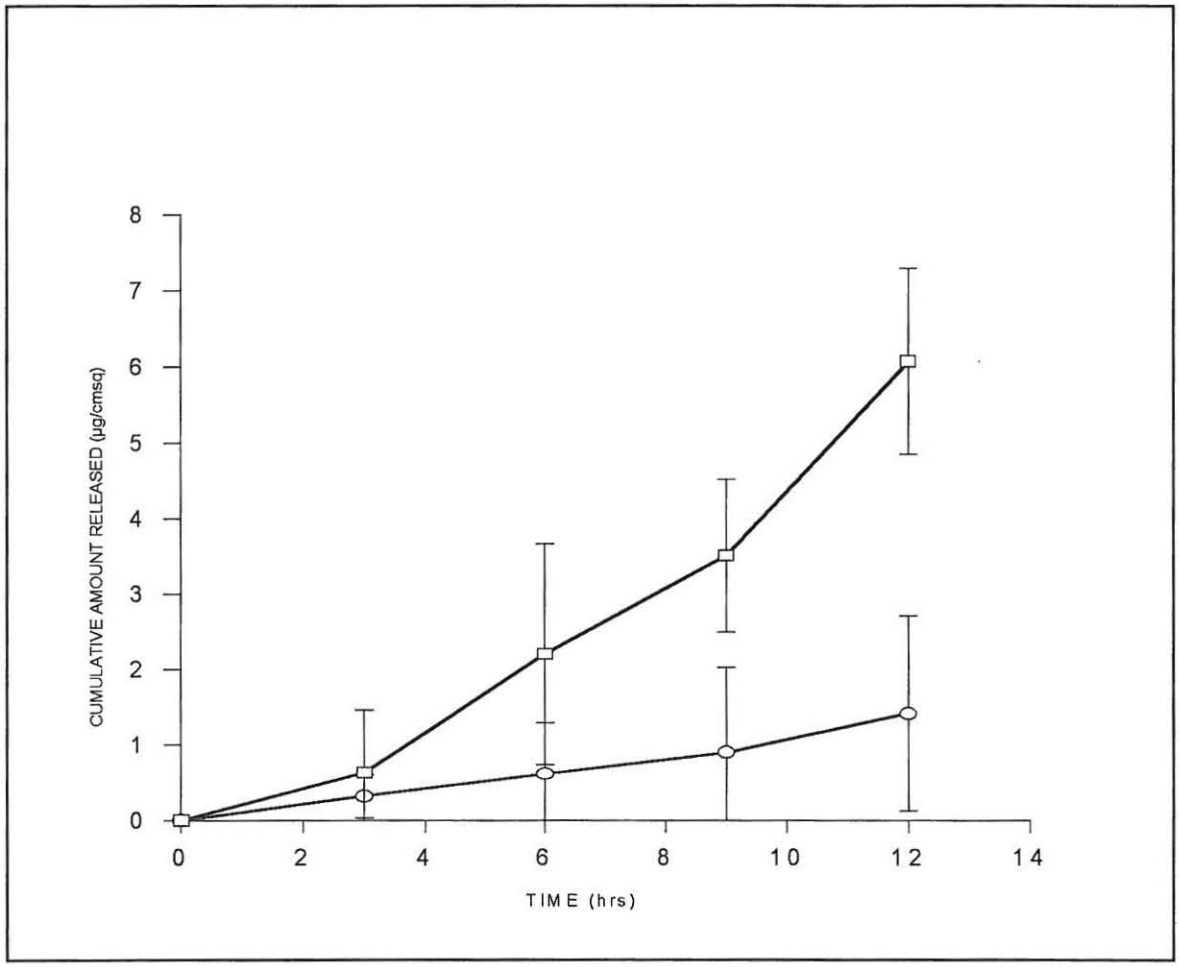
Once a supersaturated system under investigation has displayed adequate stability at an acceptable degree of supersaturation, then a study of the permeation properties of the system can be conducted. The permeation study results for this analysis are expressed in Figures 25 and 26 as the cumulative amount of rooperol tetra-acetate diffusing from the saturated solution and the supersaturated solutions through polydimethylsiloxane and rat membrane. Supersaturation diffusion results across polydimethylsiloxane are illustrated in plots A and B of Figure 25. The profiles display the differences in diffusion brought about by the inclusion and exclusion of a polymer in the propylene glycol/water binary system. The results show that rooperol tetra-acetate diffusion is not significantly influenced by the presence of the polymer. Application of the *t*- distribution test to each point of plot A and B indicates that at a 95% confidence level, the rate of drug permeation is significantly different only at the 6 hour time point and beyond. When comparing plot (A) and (B) with plot (C), drug release from the supersaturated systems is evidently appreciably greater than drug diffusion from the saturated system.

Figure 25: Plot of drug diffusion from supersaturated solutions through polydimethylsiloxane under occluded conditions.



The diffusion results illustrated in Figure 26 show that rat membrane has a significant influence on the diffusion properties of rooperol tetra-acetate from supersaturated solutions with and without an antinucleating agent. For reasons discussed in detail later, significantly lower drug diffusion rates are observed with the solution containing the polymer, sodium carboxyl methyl cellulose, than with the solution in which the polymer was excluded. No drug diffusion was detected from the saturated solution.

Figure 26: Plot of drug diffusion from supersaturated solutions through rat skin under occluded conditions.



○ (PLOT A):SUPERSATURATED SYSTEM WITH 0.1% POLYMER

□ (PLOT B):PROPYLENE GLYCOL/WATER WITHOUT POLYMER

△ NO MEASURABLE DIFFUSION OCCURED FROM SATURATED SYSTEM

Table 9 represents the diffusion parameters of the saturated and supersaturated rooperol tetra-acetate solutions when polydimethylsiloxane and rat membranes were used. As predicted, drug diffusion through both membranes from the saturated system exhibited a lower flux rate and partitioning than from the supersaturated solution. When comparing the two supersaturated systems developed, it is noted that in the presence of the polymer, a higher flux rate through, and partition coefficient into, the synthetic membrane is achieved. The *t*-distribution test was applied to the lag time and flux rate results of both supersaturated systems presented in Table 9.

Table 9: Mean permeation parameters for saturated and supersaturated systems.

Formulation	*Js* (µg/cm^2/h)	*L* (h)	*D* (cm^2/h x10^5)	K
Polydimethylsiloxane Membrane (n = 4)				
Saturated				
	3.22 ± 0.10	1.25 ± 0.16	1.95 ± 0.23	0.40 ± 0.05
Supersaturated (propylene glycol/water vehicle)				
	5.37 ± 0.43	0.75 ± 0.14	3.32 ± 0.56	0.41 ± 0.1
Supersaturated (propylene glycol/water with 0.1% polymer vehicle)				
	6.38 ± 0.10	1.04 ± 0.22	2.46 ± 0.68	0.66 ± 0.14
Skin Membrane (n = 3)				
Supersaturated (propylene glycol/water vehicle)				
	0.052 ± 0.008	2.72 ± 2.41	2.30 ± 1.97	0.016 ± 0.16
Supersaturated (propylene glycol/water with 0.1% polymer vehicle)				
	0.01 ± 0.009	1.41 ± 0.87	3.39 ± 1.39	0.0014± 0.001

At a 95% confidence level, the test revealed that the lag time of the supersaturated system in which the polymer was excluded is statistically similar to that of the polymer containing system. In contrast, the *t*-test showed that the flux rates of each system were significantly different. It is likely that the similarity in the lag time values arises from the fact that between 0 and 3 hours of diffusion (FIG 25), the supersaturated diffusion profiles overlap hence, the point at which the linear portion of the graph crosses the time axis does not vary widely. After three hours, the gradient of the linear

region of the diffusion profile containing the polymer increases moderately and this rise is reflected by the higher flux rates obtained for this system. In comparison, the supersaturation diffusion parameters of the rat membrane analyses differ from those of the polydimethylsiloxane membrane studies. The drug in the non polymer containing solution exhibited a higher partition coefficient into and diffusion through the rat membrane. This difference in diffusion activity is largely attributed to the complex diffusion environment encountered when animal membrane is utilized.

The stability study experiments effectively demonstrate the role of sodium carboxyl methyl cellulose in supersaturation investigations. Figure 24 displays the antinucleating action of the polymer which when present, enhances the stability of the system and in doing so, contributes towards maintaining a high thermodynamic activity for a longer time. The mechanism by which polymers inhibit or retard drug nucleation in order to maintain the stability of supersaturated solutions is largely unknown [142,132]. It was noted, however, that the sodium carboxyl methyl cellulose solutions were viscous and due to this high viscosity, it is possible that minute drug nuclei forming in the system were physically inhibited from binding with other precipitates to form large nuclei. As a result, the polymer containing systems were thus able to delay the nucleation process. It has also been suggested that the polymer is adsorbed onto the crystal surface, however, this mechanism of action has not yet been elucidated.

Drug penetration is dependent upon the influence of the vehicle on the thermodynamic activity of the active ingredient. The presence of abnormally high concentrations of rooperol tetra-acetate in the supersaturated systems enhanced passive diffusion by improving the release potential of the drug from the vehicle into the membrane. This resulted in the observed increase in the driving force for drug diffusion. In comparison, the drug release potential from saturated solutions is lower due to a decrease in thermodynamic potential brought about by limits in drug concentration.

Figure 25 shows that within the first six hours of experimentation, there was no significant difference between the diffusion of rooperol tetra-acetate from the supersaturated solution containing polymer and the control solution. However, beyond six hours of diffusion slower drug penetration was observed from the control solution containing no antinucleant. It has been reported that physical contact of the polymer

containing vehicle with the membrane may initiate precipitation of the drug resulting in donor concentrations which are less than the initial concentration [142,132]. Membrane effects on the stability of supersaturated vehicles are likely to have brought about some nucleation in both systems analysed. Evidently, the presence of sodium carboxyl methyl cellulose delayed complete nucleation within the polymer containing solution, resulting in the marginal increase in drug release noted at times beyond six hours.

Figure 26 shows greater drug release from the control system containing no polymer than from the vehicle to which the polymer was added. This deviation from normal predictions of drug diffusion may be attributed to the action of sodium carboxyl methyl cellulose as a surfactant and therefore, due to its physico-chemical nature, it is possible that the solubility of rooperol tetra-acetate in the vehicle was enhanced. The lag time for the polymer containing formulation is notably longer than for the non polymer containing system thus suggesting a delay in the release of the active drug from the former system. Similar observations have been reported by other research workers in this field. Lalor et al. [144] reported decreased partitioning and permeation of n-Alkyl p-amino benzoates as a result of the solubilizing capacity of the surfactant. A study of Figure 22 indicates that the solubility of rooperol tetra-acetate is marginally greater in cosolvent systems in which sodium carboxyl methyl cellulose is present. The result of this enhanced affinity of the active drug for the vehicle coupled with unpredictable modifications in the partitioning potential of the drug into a heterogeneous membrane may have brought about conditions which resulted in the relatively small drug diffusion into rat skin.

3.4.1. CONCLUSION

From the observations made on supersaturated and saturated systems, it can be concluded that the barrier properties of skin can be adequately overcome by creating systems with high thermodynamic activity and good stability. Application of the proposed system and the prescribed conditions resulted in drug solubility which, after the inclusion of sodium carboxyl methyl cellulose, was 2.7 times above the saturation level. As expected, these elevated solubility levels are directly reflected in the

permeation rate results where the flux of rooperol tetra-acetate from the supersaturated solution is approximately double the flux from the saturated system. An important factor is that the supersaturated systems promoted partitioning of the active drug in rat skin thus encouraging topical delivery of the drug to levels which could be quantified by the HPLC system. This level of drug delivery could not be achieved with rat skin using saturated solutions. Supersaturation technology, therefore, provides a possible means by which enhanced delivery of the drug *in vivo* may be achieved.

CHAPTER 4: CONCLUSION

An effective and efficient HPLC assay method was successfully developed for the quantitative analysis of rooperol tetra-acetate. Acceptable precision and sensitivity over the concentration range that the analyte is expected to be used, was achieved. The validation of low assay variation provides adequate assurance that the method employed is suitable for the analysis of the receptor medium of the transdermal delivery systems developed.

The heat stability tests revealed gradual degradation of the active drug when subjected to elevated temperatures. In addition, it was noted that the instability of rooperol tetra-acetate may have further been influenced by chemicals reacting with the container in which it had been placed. More detailed investigations are necessary to generate a complete stability profile of the drug, thus determining its degradation potential under all possible conditions of use. An extensive stability evaluation identifying, (1) the routes of decomposition, (2) the production of toxic substances and, (3) the effect of decreased drug potency is essential during drug development. The first stage of such a degradation analysis is a detailed study of the kinetics of drug deterioration that enables the worker to predict and elucidate degradation pathways. The second stage is to test the compatibility of the active ingredient with excipients used in its formulations. The success of stability experiments is dependent upon the use of a reliable analytical assay which will identify the heat degradation products. High performance liquid chromatography selectively isolated each degradation product, however, samples of each product represented by a chromatographic peak, can be collected and further analysed using identification techniques such as nuclear magnetic resonance (NMR). NMR is a versatile and practical analytical technique which, through the generation of a spectrum, specific to a particular compound, enables one to solve molecular conformational problems of pharmaceutical compounds under investigation [35,145]. From NMR investigations more conclusive results can be reached on the structure of the degradation products detected chromatographically following heat accelerated stability testing. Preformulation stability studies are designed to avoid poor stability of the formulated product. Figure 20, which represents the composite diffusion

results for all nine preparations formulated, shows that the permeation activity through polydimethylsiloxane membrane was uniform. The small variance about each point of the plot indicates batch-to-batch consistency and therefore, suggests that the stability of each preparation was constant throughout the study. Clearly, the accelerated stability tests provided useful information regarding the heat instability of rooperol tetra-acetate and this knowledge was successfully applied to maintain the stability of the active drug under non accelerated conditions.

The diffusion experiments performed on rooperol tetra-acetate have provided valuable insight into the diffusion properties of the drug from different topical vehicles. Rooperol tetra-acetate is a lipophilic drug and several investigations have suggested the use of a non aqueous receptor phase to promote optimal partitioning of the drug from the membrane [77,146,147]. Optimization of the receptor phase assists towards enhancing the diffusion activity of the permeant resulting in efficient permeation of the drug through the membrane. The diffusion test results show that an acceptable quantity of permeant was measured in the receptor phase thus indicating that the ethanol/water receptor fluid proved to be a suitable medium for the permeation of rooperol tetra-acetate from its different topical formulations. The *in vitro* diffusion characteristics of rooperol tetra-acetate penetrating through polydimethylsiloxane and rat membranes were adequately determined by means of liquid chromatography. Through this separation technique, it was possible to achieve rapid analysis, precise selectivity and good resolution of the compounds detected in the receptor phase of the diffusion systems. Experiments in which synthetic membrane was used provided valuable indications of the release properties of rooperol tetra-acetate from bases of varying lipophilicity. Theoretically, drug permeation across a uniform synthetic membrane is directly proportional to the concentration of active agent present in the vehicle [148]. Therefore, by using synthetic membranes, it was possible to detect interactions which reduce effective drug penetration. The permeation kinetic results (Tables 7 and 8) show that effective release of the permeant is best achieved from aqueous vehicles which interact minimally with the drug. The majority of topical preparations are designed for use under unoccluded conditions. Comparison of the occluded with the unoccluded diffusion plot of rooperol tetra-acetate through

polydimethylsiloxane, (Figures 20 and 21) indicates that an oil-in-water emulsion base should be suitable for the transdermal delivery of the active drug in the unoccluded state. Although the permeation rates of the drug in the unoccluded oil-in-water vehicle are lower than those when the vehicle is occluded, the flux values in Tables 7 and 8 show that the decrease in diffusion from this base is not as extensive as the decrease noted between the occluded and unoccluded gel formulations. The oil-in-water emulsion vehicle, which comprises a lipid phase dispersed within an aqueous phase, was able to retain sufficient aqueous properties needed for notable release of the drug from the unoccluded vehicle. Hence, due to its physico-chemical properties, this base is ideal for the delivery of rooperol tetra-acetate, particularly, under the expected conditions of use.

The failure to detect drug permeation through full thickness rat skin suggests that the percutaneous diffusion of rooperol tetra-acetate through animal membrane is more likely to be achieved by means of an enhanced delivery system. The successful attainment of data describing the permeation of rooperol tetra-acetate through polydimethylsiloxane indicates that the unsuccessful diffusion of the drug through full thickness rat skin is due to membrane properties which do not favour drug penetration combined with inadequacies in vehicle formulation. Overcoming the barrier of the skin through the application of supersaturation techniques is a major achievement in these preformulation transdermal delivery studies. The leaving potential of the rooperol tetra-acetate was elevated sufficiently enough to allow for quantifiable drug diffusion through rat skin. The supersaturation investigations, therefore, revealed that a higher thermodynamic activity of topically applied rooperol tetra-acetate is attainable. The full capacity and potential of supersaturated systems is dependent upon obtaining a high degree of supersaturation and adequate stability of the drug which is present in high concentrations within the vehicle [149]. As displayed in Figure 24 of the experimental results, satisfactory stability was only achieved at lower degrees of supersaturation. However, under the experimental conditions employed, quantifiable drug penetration through the rat stratum corneum was obtained. Numerous investigations determining the efficacy of different polymers have been performed [98,129,131,133,134,136, 142,150-152] and application of the recommendations from such research may lead to

the discovery of a more effective antinucleating agent for rooperol tetra-acetate. Figure 23 shows that in theory, it is possible to achieve an approximately six-fold increase in the degrees of supersaturation for a supersaturated rooperol tetra-acetate system containing sodium carboxyl methyl cellulose. Contrastingly, Figure 24 displays that the maximum degree of supersaturation obtained experimentally, was 2.7. The difference between the theoretical and experimental values suggests that the antinucleating activity of the polymer, in the system used, was less than optimal. Despite limitations in the performance of sodium carboxyl methyl cellulose, the final studies of this work indicate that the delivery system developed for the practical use of the proposed supersaturated drug system was acceptable.

Further research is required for the formulation of a suitable base for the effective topical delivery of rooperol tetra-acetate. The work performed here provides essential information into the physico-chemical properties of the vehicle which will favourably or adversely affect drug release. Furthermore, it has been established that through the utilization of enhanced topical delivery methods it is possible to attain satisfactory drug permeation across rat skin. The delivery systems analysed in this investigation serve as a basis for the formulation of a vehicle which will allow effective topical transport of rooperol tetra-acetate. When developed, such a delivery system will be beneficial in the treatment of solar keratosis as a more acceptable therapy option will be available to patients suffering from this condition.

CHAPTER 5: FUTURE AREAS OF RESEARCH

During a research investigation it is automatically assumed that sound scientific principles are being adhered to. Workers continually seek to optimize the use of the apparatus and techniques employed in the analysis and data processing of any given assay procedure. However, during the development and application of that assay method new research leads, which had not previously been anticipated, may arise. Such new developments serve to improve the assay procedure employed and consolidate data findings resulting in conclusive outcomes.

In order for an *in vitro* transdermal delivery study to satisfactorily reflect *in vivo* permeation across skin, it is essential that the original state of the diffusion membrane is maintained throughout the analysis. Studies have shown a decrease in membrane integrity following exposure to high concentrations of alcohol [46,48-51,153-156]. In addition, Liu et al. [157] reported an inhibitory effect, by ethanol, to the metabolic conversion of β-oestradiol to oestrone in the epidermis. In view of these reports, a more rigorous investigation into receptor phase suitability may lead to the discovery of a more effective receptor medium which maintains the barrier functions of the membrane while preserving the permeation properties of rooperol tetra-acetate.

Attaining acceptable accuracy and precision is vital in the validation of any diffusion study. The accuracy and precision of the diffusion data generated is dependent upon the reliability and reproducibility of the diffusion system utilized. The precision of topical diffusion studies is largely reliant upon the partitioning of the permeant from the membrane into the receptor phase. Diffusion cell system validation involves observing the changes to the rate and amount of drug diffusion brought about by altering experimental parameters such as the drug concentration, vehicle viscosity, temperature, agitation and the internal:external phase composition [158]. Such a validation procedure can determine the suitability of the analytical procedure and cell design and in addition, assist in defining the ability of the assay method to quantify the drug of interest [159-161]. Diffusion kinetics are determined by the concentration of drug present in the topical vehicle. To reach reliable conclusions on drug release properties, it is necessary to perform quantitative measurements of drug content in the

vehicles employed. Such evaluations can be achieved by liquid chromatographic assaying of the topical dosage forms through the utilization of column switching techniques [10]. This technique involves a simultaneous on-line clean up process of the sample which results in relatively easy and rapid separation of the agents in multicomponent systems. Once actual drug concentrations in the vehicles have been determined, it is possible to make absolute statements on the release characteristics of a drug such as rooperol tetra-acetate.

In situations where human skin is not readily available model membranes may need to be developed in order to measure drug permeation. Hadgraft et al. [162,163] investigated the correlation of a model membrane system with human skin. The study concluded that the isopropyl myristate membrane model was a reasonable model of the stratum corneum and its barrier properties provided good simulation of drug diffusion across human skin. However, despite its versatility, it was found that the model could not be applied to all compounds tested. Rohatagi et al. [164] performed similar investigations in which the percutaneous absorption and metabolism of selegeline in various animal species and human were studied. Obtaining animal membrane which can adequately simulate the permeation of rooperol tetra-acetate through human skin is useful, especially where human skin is not procurable for ethical reasons. Ideally, human skin should be utilised for *in vitro* percutaneous absorption analyses. Normally, skin from the intended site of application is used in permeation studies. Due to the nature and complexity of solar keratosis, it may be more applicable not only to perform comparative studies of skin samples from the same body site but also from different body sites. Such a study may reveal site related variations in drug permeability which may require that different drug concentrations are to be used for different application sites. The aim of performing *in vitro* permeation studies is to predict absorption in the living state and thus, an important aspect of these studies is correlation with *in vivo* results [165,166].

The diffusion cell experiments performed have been useful in analysing percutaneous absorption through polydimethylsiloxane and rat membrane. However, the detection techniques have proved insufficient in positively identifying permeants detected in the receptor phase. A problem encountered during the HPLC analysis of

the drug permeants following diffusion through rat skin was the concurrent elution of compounds derived from the membrane. A broad peak, eluting between 1.4 minutes and 1.9 minutes, was observed and although it interfered with neither the principle peak of rooperol tetra-acetate nor that of the internal standard, its presence may have prevented the detection of certain diffusion products. Attempts were made to attain greater selectivity in the chromatograms generated through altering the mobile phase composition. Changes to the organic phase made it possible to enhance selectivity, however, complete resolution of peaks such as Peaks E and D in Figure 17 was not obtained. The broad peak produced by the skin permeants could have been eliminated via a process of column switching. The column-switching technique involves sample clean-up through the use of multi-column chromatography [167-171]. Column-switching is a rapid process which avoids the time-consuming techniques of liquid-liquid and solid-liquid extractions [172-176] while simultaneously maintaining the sensitivity, precision and reproducibility of the separations [177]. A loss in efficiency when column-switching was used has been reported by Little et al. [168] and Benjamin et al. [169], however, this loss is said to be negligible.

Rooperol tetra-acetate is a pro-drug which is converted into its active form by Phase II enzymes. Elucidation of the metabolic pathways of this drug was accomplished through various clinical trials performed on the drug by Kruger et al. [4-6]. The clinical trials performed did not include an evaluation of the capacity of the skin to metabolise the active agent. Establishing the capacity of the skin to metabolise rooperol tetra-acetate is fundamental in determining the bioavailability and bioequivalence of topically delivered drugs. Such a study would require the use of biochemically viable skin tissue in conjunction with a distinctive analytical technique which will allow for precise detection of the metabolites of the pro-drug [178]. Based on the knowledge of the bioavailability of percutaneously absorbed rooperol tetra-acetate, determining the therapeutic concentrations needed for the drug to be effective is then possible.

It is acknowledged that improved management of experimental procedures would have made it possible to achieve increased precision of the test design and lower chances of experimental errors. The potential use of rooperol tetra-acetate in the

treatment of solar keratosis is likely to increase interest in this novel cytotoxic compound. Future preparations of the drug for clinical use will require that extensive and in depth investigations of the factors mentioned above be explored. The results obtained in this introductory study on rooperol tetra-acetate are promising enough to warrant further analysis into the topical absorption of the drug. Such analyses will be in association with the growing interest and research into the viability of percutaneously delivered drugs.

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