Topical delivery of acyclovir and ketoconazole

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Abstract

**Context:** Viral and fungal cutaneous manifestations are regularly encountered in immunocompromised HIV/AIDS individuals and can be treated by drugs such as acyclovir and ketoconazole, respectively. **Objective:** The aim of this study was to determine whether the novel Pheroid™ delivery system improved the transdermal delivery and/or dermal delivery of acyclovir and ketoconazole when incorporated into semi-solid formulations. **Materials and methods:** Semi-solid products (creams and emulgels) containing these drug compounds were formulated, either with or without (control) the Pheroid™ delivery system. The stability of the formulated semi-solid products was examined over a period of six months and included the assay of the actives, pH, viscosity, mass loss and particle size observation. Vertical Franz cell diffusion studies and tape stripping methods were used to determine the *in vitro*, SC-epidermis and epidermis-dermis delivery of these formulations. **Results and discussion:** Stability tests showed that none of the formulations were completely stable. Acyclovir showed a biphasic character during the *in vitro* skin diffusion studies for all the tested formulations. The Pheroid™ cream enhanced the transdermal, SC-epidermis and epidermis-dermis delivery of acyclovir the most. The average amount of ketoconazole diffused over 12 h showed improved delivery of ketoconazole, with the Pheroid™ emulgel exhibiting the best transdermal and epidermis-dermis delivery. **Conclusion:** The Pheroid™ formulae increased transdermal penetration as well as delivery to the dermal and epidermal skin layers. The Pheroid™ emulgel and the Pheroid™ cream increased the topical delivery of ketoconazole and acyclovir, respectively.
1 Introduction

The acquired immunodeficiency syndrome (AIDS) and the human immunodeficiency virus (HIV) pandemic have had a profound effect on the extent and diagnosis of cutaneous disorders. The skin is the most commonly affected organ in HIV infected individuals (Ramdial, 2000). There is an incredibly wide range of skin manifestations that presents in HIV/AIDS infected individuals. Cutaneous disorders in HIV-positive individuals can be more severe and disabling than in normal host individuals (Yen-More et al., 2000).

There are three main types of viral infections that occur cutaneously in HIV/AIDS patients; these viruses include the Human herpes viruses (cytomegalovirus (CMV), Epstein-Barr virus (EBV), herpes simplex virus (HSV) types 1 and 2, human herpes viruses (HHV) 6, 7 and 8 and varicella zoster virus (VZV), the Human papilloma virus and Poxvirus infections (Molluscum contagiosum and vaccinia). Many opportunistic viral infections in HIV/AIDS patients have similar manifestations as those in healthy individuals, but infections in this immunocompromised population are seen in greater prevalence. These infections can cause unique and more severe mucocutaneous lesions and are more difficult to treat (Yen-More et al., 2000; Ramdial, 2000).

The AIDS epidemic has also increased the worldwide prevalence of fungal infections. Fungal infections are a major cause of morbidity and mortality in patients infected with the HIV virus. The yeast Candida species and Cryptococcus neoformans, the dimorphic fungi Histoplasma capsulatum and Sporothrix schenckii, and dermatophyte fungi are the most common pathogenic fungi in patients infected with HIV (Durden & Elewski, 1997).

Acyclovir is the anti-viral drug of choice in the treatment of many types of herpes virus infections, including genital herpes simplex infections, herpetic conjunctivitis and herpes simplex encephalitis.

Acyclovir is active against herpes simplex virus (HSV) type 1 and type 2 and against the varicella zoster virus (Acosta & Flexner, 2011). Ketoconazole is a broad spectrum imidazole anti-fungal agent (Skiba et al., 2000) with antifungal activity against fungal pathogens including: Candida species, Blastomyces dermatitidis, Cryptococcus neoformans, Coccidioides immitis, Histoplasma capsulatum, Paracoccidioides brasiliensis and Sporothrix schenckii (Bennett, 2011). The target
sites for acyclovir and ketoconazole is the basal epidermis and the stratum corneum (SC), respectively. Adequate concentrations of each drug should be delivered to the target site of the skin to inhibit the growth of viral and fungal pathogens (Pershing et al., 1994; Jiang et al., 1998). The transdermal diffusion of acyclovir may be possible when considering the aqueous solubility (2.50 mg/ml in water at 25°C) and molecular weight (225.20 g/mol) of acyclovir, but the high melting point (256.5 - 257°C) and low octanol-buffer partition coefficient (log D) (-1.56) could be a preventive factor. When considering the melting point (146°C) of ketoconazole the penetration of the drug may be possible, but ketoconazole has a very low aqueous solubility (40 µg/ml in water at 25°C), high molecular weight (531.43 g/mol) and high octanol-water partition coefficient (log P) (4.31). This could have a negative impact on the transdermal penetration of the ketoconazole and poor transdermal delivery can be anticipated (McEvoy, 2002; British Pharmacopoeia, 2013). According to Thomas & Finnin (2004), compounds with high log P values exhibits low permeability, because they are not able to partition out of the SC. This can be a positive factor for the delivery of ketoconazole to the SC.

During this study the Pheroid™ delivery system, formerly known as Emzaloid™, was employed in the formulation of the semi-solids (cream and emulgel) as a carrier system for acyclovir and ketoconazole. This was done in order to establish whether the Pheroid™ will enhance the actives’ in vitro transdermal and/or dermal delivery to the target sites when compared to a control, i.e. the basic formula without the Pheroid™. The patented Pheroid™ delivery system is a novel, colloidal type drug delivery system, composed of essential and plant fatty acids emulsified in water saturated with nitrous oxide (Du Plessis et al., 2010; Steyn et al., 2011). Its colloidal system contains stable lipid-based submicron-and micron-sized structures, called Pheroid™. These Pheroids™ are uniformly distributed in a dispersion medium. The basic Pheroid™ has a vesicular structure with sizes ranging from 200 - 440 nm (Grobler et al., 2008). The main essential fatty acids used in the Pheroid™ are linolenic acid and linoleic acid as well as oleic acid (Saunders et al., 1999) oriented in the cis-formation, making it compatible with the fatty acids found in man (Grobler et al., 2008). Therefore, the Pheroid™ is a skin friendly carrier system
All registered topical Pheroid™ formulations currently contain vitamin E or vitamin E derivatives, also known as tocopherol. These molecules are used as anti-oxidants and as emulsion stabilizers.

The Pheroid™ delivery system has the ability to entrap (both hydrophobic as well as hydrophilic drugs), protect and deliver drug molecules across several biological membranes, such as the skin (Du Plessis et al., 2010). Previously conducted membrane diffusion studies have shown an increase in the percentage release of acyclovir and miconazole nitrate (anti-fungal) to human skin, in comparison to commercially available products (Grobler et al., 2008).

Skin penetration studies play an essential role in the optimization of formulation design in dermal and transdermal delivery. In vitro permeation studies and tape stripping techniques are therefore highly important (Leveque et al., 2004).

The formulated semi-solid products, creams and emulgels (with or without the Pheroid™ delivery system) were tested for stability. The purpose of stability testing is to provide evidence on how the quality of a drug product varies with time under the influence of a variety of environmental factors such as temperature, humidity and light. Stability tests should be done on all attributes that can influence the quality, safety and efficacy of the product which are likely to change during storage (ICH, 2003).

2 Materials and methods

2.1 Materials

Acyclovir 9-[(2-hydroxyethoxy)methyl]-9H-guanine and ketoconazole (cis-1-acetyl-4-[4-[2-(2,4-dichlorophenyl-2-(1H-imidazol-1ylmethyl)-1,3-dioxalon-4-yl]methoxy]phenyl]piperazine), were obtained from DB Fine Chemicals (Pty) Limited (Johannesburg, South Africa). Liquid paraffin, PEG 400, Tween 80, cetyl alcohol, methylparaben and butylated hydroxyanisole (BHA) were obtained from Merck chemicals (Johannesburg, South Africa). Sigma-Aldrich, St. Louis, USA supplied the butylated hydroxytoluene (BHT) and the glycerin. Span 60, xanthan gum and tocopherol were obtained from Fluka Analytical (Johannesburg, South Africa), Warren Chem Specialities (Johannesburg, South Africa) and Chempure (Pretoria, South Africa), respectively. Water used
during all preparations and formulations was purified by a Milli-Q® Academic purification system (Millipore, Milford, Mass., USA). In the high performance liquid chromatography (HPLC) analysis of the attributes in the formulated products methanol LiChrosolv (Merck, Johannesburg, South Africa) and octane-1-sulfonic acid sodium salt (Merck, Johannesburg, South Africa) was used as the mobile phase. The samples of the semi-solid formulated products were diluted with methanol supplied by Merck (Johannesburg, South Africa). Potassium dihydrogen orthophosphate (Merck, Johannesburg, South Africa), and sodium hydroxide (Merck, Johannesburg, South Africa) were used during the preparation of phosphate buffer solution (PBS) which served in conjunction with methanol AR (Merck, Johannesburg, South Africa) as components of the receptor phase during the in vitro permeation studies. Ammonium phosphate monobasic (Sigma, Johannesburg, South Africa) and acetonitrile LiChrosolv (Merck, Johannesburg, South Africa) were used in the mobile phase during the HPLC analysis of the receptor phase after transdermal diffusion.

2.2 Methods

2.2.1 Formulation of the cream and emulgel

In order for the Pheroid™ to be able to entrap the acyclovir crystals an average particle size of 10 µm or smaller was needed. Therefore, prior to manufacturing of the formulations, acyclovir’s particle size was decreased by grinding the active ingredient with a Retsch K-SM1 type mill (Retsch, Germany). The smaller particle size can possibly contribute to increase acyclovir’s solubility and penetration through the skin. After grinding, the particle size of the grinded acyclovir was measured with a Malvern Mastersizer fitted with a Hydro 2000SM dispersion unit (Worcestershire, United Kingdom). The average particle size obtained was 15.17 µm and it was possible for Pheroid™ to encapsulate about 38% of the particles.

The cream and emulgel formulations (with or without the Pheroid™ delivery system) were prepared in bulk for storing and testing purposes. The formula for the acyclovir and ketoconazole cream and emulgel can be seen in Table 1.

Table 1: Formula of acyclovir and ketoconazole cream and emulgel
The cream and emulgel was prepared by heating an adequate amount of water to approximately 40°C. The xanthan gum was slowly added while homogenizing at 200 rpm and 300 rpm for the cream and emulgel, respectively, until homogenous (part A). The ketoconazole and polyethylene glycol 400 (PEG 400) was added together and heated to 80°C until dissolved (part B). The glycerin and acyclovir was mixed together and the remaining water was added to this mixture (part C). The oil phase (part D) was prepared by mixing and heating the following ingredients: liquid paraffin, Span 60, Tween 80, methylparaben, propylparaben, BHT and BHA to 80°C. Part B was added to part D. The water phase was prepared by mixing part A and part C together and subsequently heated to 80°C. The oil phase (part B and D) was immediately added to the water phase (part A and C) while homogenizing at a rate of 13500 rpm until it reached a temperature of 40°C. It was then cooled with ice water and stirred at 200 rpm until room temperature was reached and left overnight. The same basic cream and emulgel formulas were used for the Pheroid™ cream and Pheroid™ emulgel, respectively, although Pheroid™ ingredients like tocopherol were added to the oil phase to form acyclovir and ketoconazole vesicles in the cream and emulgel. All formulations had a pH between 5 and 6.

2.2.2 Stability study

A stability program was followed according to the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Tripartite Guideline (2003) and according to the Medicines Control Council (MCC) of South Africa (2010). The formulated products were subjected to long-term, intermediate and accelerated stability testing by storing the formulated products in different Labcon® humidity chambers (Johannesburg, South Africa) at 25°C/60% RH (relative humidity); 30°C/60% RH and 40°C/75% RH, respectively. Formulations were evaluated for various parameters at time intervals of 0, 1, 2, 3 and 6 months.

2.2.2.1 Assay with HPLC

An HPLC method was developed and validated for the seven analytes (acyclovir, ketoconazole, methylparaben, propylparaben, BHA, BHT and tocopherol) present in all the formulations at the Analytical Technology Laboratory (ATL) of the North-West University (NWU), Potchefstroom.
Campus, South Africa. HPLC analysis was done to determine the concentrations of these analytes in all the formulated products at 0, 1, 2, 3 and 6 months of the stability testing. The HPLC analysis was performed by using an Agilent 1100 Series HPLC system, equipped with an Agilent 1100 pump, UV detector, auto sampler and Chemstation Rev. A.10.02 acquisition software (Agilent, Palo Alto, CA). A Luna C-18 (250 x 4.60 mm, 5 µm, Phenomenex, Torrance, CA) column was used during the HPLC analysis.

The mobile phase consisted of 3 vol (30%) of analytical grade methanol and 7 vol (70%) of a 0.005 M octanesulfonic acid solution, pH 3.5. This changed over 8 min to 10 vol of methanol (100%). Methanol was used in the mobile phase as acetonitrile did not fare well with the elution of tocopherol. The flow rate was set to 1.0 ml/min and the sample injection volume was 5 µl. Due to the large concentration acyclovir and ketoconazole present in the samples, these two analytes were analyzed at an absorbance wavelength where they exhibit lower UV absorption (i.e. 220 nm) rather than at their maximum UV absorbance wavelengths of 252 nm and 205 nm, respectively. This was done to obtain peaks for acyclovir and ketoconazole of a similar size compared to the peak size of the other analytes. BHA, BHT and tocopherol were also analyzed at 220 nm where they have good UV absorbance; whereas methylparaben and propylparaben were analyzed at their maximum UV absorbance wavelength of 254 nm. There was no overlap of any peaks in the chromatogram. The run time was set to 30 min. The approximate retention times of each analyte was as follow: acyclovir (3.6 min), ketoconazole (9.6 min), methylparaben (8.6 min), propylparaben (10.2 min), BHA (10.8 min), BHT (12.5 min) and tocopherol (19.1 min).

A new standard was prepared at each testing interval and analyzed as a control before the samples on stability were analyzed. The standard consisted of a solution of all analytes (500 µg/ml acyclovir, 200 µg/ml ketoconazole, 2 µg/ml BHA, 20 µg/ml BHT, 40 µg/ml methylparaben, 8 µg/ml propylparaben and 20 µg/ml tocopherol) in methanol.

The samples on stability were prepared by the weighing of 1 g of each formulation into a 100 ml volumetric flask by using a syringe with a tube attached to the tip. Methanol was then added as solvent for the semi-solids. The samples were sonicated for approximately 20 min or until
completely dispersed. The samples were filled to volume in the volumetric flask after it cooled to room temperature. All samples were filtered with a 0.45 μm syringe filter and transferred into HPLC vials for analysis.

2.2.2.2 pH measurement

The pH of a formulation may influence the stability of the actives in the formulation. During this study a Mettler Toledo Seven Multi pH meter (Mettler Toledo AG, Germany) with an In Lab 410 NTC electrode 9823 (Switzerland), which was calibrated with buffer solutions of pH 4.01, 7.00 and 10.10 immediately prior to measurements, was used to determine the pH of each formulation. To ensure accuracy three measurements were taken on each batch at different temperatures and time intervals.

2.2.2.3 Viscosity

The viscosity of the various formulations was determined at 0 and 6 months with a Brookfield Model DV – II+ viscometer (Massachusetts, USA) mounted on a Helipath D20733 stand and fitted with a T-bar spindle. The direct sample temperature was controlled by a Brookfield circulating water bath with a temperature controller (Massachusetts, USA). Measurements were done in glass containers, containing 100 ml of the product. The viscosity was only determined on the batches stored at a stability temperature of 25°C/60% RH. Viscosity was measured for 5 min, where a total of 32 viscosity readings were taken every 10 s for each formulation.

2.2.2.4 Particle size observation

The particle size was observed with a confocal laser scanning microscope (CLSM) Nikon PCM2000 with digital camera DMX1200, with a He/Ne laser-543 nm and a Argon ion laser (457 - 517 nm) as well as a CLSM Nikon D-eclipse C1 si with a violet diode laser 400 - 405 nm, a He/Ne laser - 543 nm and a Argon ion laser with 457 - 514 nm. Immersion oil for microscopy, type A (Nikon, Japan) was also utilized in conjunction with polarized light microscopy procedures. Micrographs of the formulations were taken at 0, 1, 2, 3 and 6 months. Any increase or decrease in particle size observed visually was documented.

2.2.2.5 Mass loss
The weight variations from month 0 to month 6 was determined on a Shimadzu AUW 120 D balance (Japan). Each individual container of the four batches was weighed initially. The same container was then weighed again at 1, 2, 3 and 6 months to determine if any mass loss occurred. All containers were weighed three times at each time interval to ensure accuracy.

2.2.2.6 Physical appearance
The stored batches were visually assessed at 0, 1, 2, 3 and 6 months. Texture, color, odor and skin-feel were observed and any change was noted. The color was documented by taking photos of the formulated products and by using color charts as indicators.

2.2.3 In vitro diffusion study
2.2.3.1 Preparation of receptor solutions
The receptor solution consisted of 20:80 (v/v) methanol and PBS (pH 7.4). The inclusion of an organic solvent was to ensure the solubility of the active ingredients. Methanol was chosen as it was also used to prepare the standard solutions during HPLC analysis. The PBS was prepared by weighing 13.62 g of potassium dihydrogen orthophosphate and dissolving it in 500 ml of Milli-Q® water. Sodium hydroxide (3.14 g) was weighed and dissolved in 786.8 ml Milli-Q® water. The two solutions were mixed and the pH was adjusted to 7.4 with 10% orthophosphoric acid (British Pharmacopoeia, 2013). Subsequently 20 vol of methanol were added to 80 vol of PBS. The solution was filtered through a 0.45 µm filter membrane prior to use on the HPLC.

2.2.3.2 Analysis of diffusion and tape stripping samples with HPLC
A different method was also developed and validated at the ATL of the NWU, Potchefstroom Campus, South Africa to analyze the samples collected from the in vitro experiments as well as the samples from the tape stripping experiments. An Agilent 1200 Series HPLC, equipped with an Agilent 1200 pump, auto sampler, UV detector and Chemstation Rev. A.06.02 data acquisition software were utilized. The column used was an Agela Venusil XBP C-18 (4.6 x 150 mm) with a 5 µm particle size (Agela Technologies, Newark, DE).

The degassed mobile phase consisted of a mixture of acetonitrile and a solution of ammonium phosphate (1.15 g/L), pH 7.2. The mobile phase changed by linear-gradient elution over a period
of time. The mobile phase initially consisted of a mixture of 0.5 vol of acetonitrile and 9.5 vol of a solution of ammonium phosphate (1.15 g/L). The mobile phase changed by linear-gradient elution after 2 min to a mixture of 8.0 vol of acetonitrile and 2.0 vol of a solution of ammonium phosphate (1.15 g/L) over 9 min. This was followed by re-equilibration at the starting conditions for 5 min. A volume of 100 µl of each sample was injected. The flow rate was set to 1 ml/min and the temperature was maintained at 25°C. The total run time was 14 min. Since the concentration of the actives in these samples tend to be low, the wavelengths of maximum absorbance were used, i.e. detection was set at 252 nm for the first 6 min to detect acyclovir, where after it detected ketoconazole at 243 nm. However, ketoconazole has an even higher absorbance at 205 nm, but at this wavelength too much baseline noise was experienced. The retention time of acyclovir was approximately 3.7 min and that of ketoconazole approximately 6.9 min.

2.2.3.3 Skin preparation

The permeation studies were performed on abdominal Caucasian female skin obtained from patients who had undergone abdominal plastic surgery (Leveque et al., 2004). The skin was frozen at -20°C within 24 h after surgery. Informed consent was given by all of the patients before surgery and the identities of all donors kept confidential. The Research Ethics Committee of the North-West University granted ethical approval for the procurement and utilization of the donated skin under reference number NWU-00114-11-A5. Full thickness skin containing the dermis, viable epidermis and the outermost SC skin layers was utilized during this study. Subcutaneous fat and connective tissue was separated from the skin by using a scalpel, while taking care not to damage or rupture the skin in any way. Skin damage or rupture can lead to incorrect results. The skin was punched into circles with a diameter of approximately 15 mm after successful removal of fat and connective tissue. The skin circles were then positioned onto Whatman® filter paper with the SC side of the skin facing upwards. The skin circles on filter paper were then covered in aluminum foil sheets and sealed in plastic bags after which it was frozen at -20°C until needed. The frozen skin circles were thawed at room temperature prior to each diffusion study (Pellet et al., 1999). Each
skin circle was visually examined for any imperfections before it was mounted onto the diffusion apparatus.

2.2.3.4 Franz cell diffusion

The in vitro permeation studies were conducted in vertical Franz diffusion cells with a receptor capacity of approximately 2 ml and an active diffusion area of 1.075 cm². A Franz diffusion cell consists of a donor and a receptor compartment, where the formulated product containing the drug and the receptor fluid are placed, respectively. The full-thickness skin circles were thawed and placed between the receptor and donor compartments of the Franz cells with the SC in contact with the donor phase. The two compartments were sealed with Dow Corning® high vacuum grease and clamped together with a metal clamp to prevent leakage. The donor compartments were each filled with approximately 1 ml of the formulated product which was preheated in a water bath to 32°C, corresponding to normal skin temperature. Subsequently, the donor compartment was covered with Parafilm® to avoid evaporation of the product. The receptor compartments were filled with 2 ml of the receptor solution; taking care to prevent air bubbles beneath the skin. The diffusion cells’ receptor compartments were surrounded with a water bath kept at a constant temperature of 37°C throughout the experiment. This was done in order to control the temperature of the receptor compartments (Dias et al., 1999), which in effect attains the skin surface temperature of 32°C, simulating the temperature of human skin (Leveque et al., 2004). The receptor medium was continuously stirred at 750 rpm with the aid of a magnetic stirring plate; moving the small magnetic stirring bar which was placed in each receptor compartment. This was done in order to maintain movement throughout the duration of the experiment. For the acyclovir diffusion studies the entire content of the receptor compartments for both PBS (10 Franz cells for both the cream and emulgel) and Pheroid™ (9 Franz cells for both the cream and emulgel) were withdrawn at predetermined intervals of 20, 40, 60, 80 and 100 min, as well as 2, 4, 6, 8, 10 and 12 h. Withdrawals were followed by the replacement with an equal amount of fresh receptor solution previously heated to 37°C, to ensure that the skin conditions persisted during the duration of the experiment. The withdrawal times was determined by keeping the Pheroid™ delivery system in mind. It was
expected that the Pheroid™ would promote the permeation of acyclovir, thus the withdrawal times were started earlier. This also guaranteed that the desired amount of at least 5 data points could be achieved. During the ketoconazole diffusion studies no flux was observed; therefore the entire content of the receptor phase for both PBS (10 Franz cells for the cream and emulgel formulations) and Pheroid™ (10 and 9 Franz cells for the cream and emulgel formulations, respectively) were withdrawn after 12 h. Each sample was directly assayed by HPLC to determine the drug concentration of the active ingredients (acyclovir and ketoconazole) within each receptor compartment.

2.2.3.5 Tape stripping

Tape stripping is a technique used to remove the SC as well as a portion of the epidermis layer of the skin. The tape stripping method was used to investigate the amounts of active ingredient present in the outer layers of the skin as well as in the dermis of the skin after application of a supersaturated solution or formulated product (Pellet et al., 1997). After the completion of the diffusion study (12 h), the donor and receptor compartments of the Franz diffusion cells were carefully dismounted. Each Franz cell's skin was pinned on a piece of Parafilm® to a solid surface. The exposed area of diffusion (≈ 1.075 cm²) was clearly imprinted by the indentation from the diffusion cells (≈ 11.70 mm diameter). The last of the semi-solid donor phase was removed by dabbing the skin dry with tissue. Subsequently, pieces of 3M Scotch® Magic™ Tape was cut to cover the diffusional area. The pieces of tape were cut to a specific size to assure that it did not overlap the areas outside of the diffusion cell imprints. The first tape strip was discarded because the tape may be contaminated with the drugs from the formulation and was seen as part of the cleaning procedure. The following 15 tape strips (referred to as the SC-epidermis) were placed in a vial. The viable epidermal layer that glistened indicated the complete removal of the SC. All the strips were placed in 5 ml of a mixture of PBS (pH 7.4) and methanol. The strips in solution were kept overnight at 4°C. The excess skin was cut away from the imprints of the diffusion cells, and the remaining skin (referred to as epidermis-dermis) was cut into smaller pieces to enlarge surface area. It was then placed in 2 ml of a mixture of PBS (pH 7.4) and methanol and were kept
overnight at 4°C. The epidermis-dermis samples were centrifuged the next morning at a rate of 4500 rpm and a temperature of 10°C for 10 min. The epidermis-dermis and SC-epidermis samples were then analyzed with HPLC.

2.2.4 Statistical transdermal data analysis

The average cumulative amount per area (µg/cm²) was plotted against time (h) for acyclovir for each formulation. The \textit{in vitro} permeation profiles of acyclovir showed a biphasic character for all the formulated products (flux 1 was observed between 0 – 2 h and flux 2 between 2 – 12 or 4 – 12 h). At least 5 data points on the slope of the linear portion of the curve were used (for both flux 1 and flux 2). Since the data showed a skewed distribution, the median flux was obtained statistically (Gerber \textit{et al.}, 2008) as it is not affected by outliers in the data (Dawson & Trapp, 2004). The average amount of active ingredients diffused through the minimum of 9 Franz diffusion cells were depicted as a percentage of the applied concentration for both acyclovir (5%) and ketoconazole (2%). The total amount of acyclovir and ketoconazole diffused after 12 h was also calculated for all the formulations. For the SC-epidermis and epidermis-dermis concentrations descriptive statistics were calculated and presented as median values.

Statistical analysis was determined by using computer programs SAS (SAS Institute, 2013) and Statistica (Statsoft, 2013). The Kruskal-Wallis test was used to determine the statistical significant differences between the median flux values and the tape stripping measurements of the formulae. A p < 0.05 was considered to be of statistically significant difference (Steyn \textit{et al.}, 1994). The pairwise statistical comparisons of the median steady-state flux values and tape stripping measurements between formulated products were carried out in an attempt to establish where differences between the formulated products exist. The correlation between the epidermis-dermis and SC-epidermis concentrations as well as the correlation with the flux values of each formulation was determined.

3 Results and discussion

3.1 Assay with HPLC
According to the ICH (2003) and MCC (2010) guidelines a significant change can be defined as a 5% potency loss from the initial value. In the cream formula a significant decrease (of more than 5%) in acyclovir's concentration occurred at a temperature of 40°C after 3 months on stability. Acyclovir also had a significant change in concentration in the Pheroid™ cream, at 30°C a 6.5% decrease occurred after 6 months, and at 40°C there was an 11.8% decrease in concentration after 3 months on stability. In the emulgel formulation a significant decrease of more than 5% in acyclovir concentration was observed, a decrease of 8.2% occurred at 30°C after 3 months and at 40°C a decrease of 6.2% was noted after 2 months of stability testing. The Pheroid™ emulgel depicted significant decreases at all three temperatures. At 25°C, a decrease of 9.8% occurred after 2 months, at 30°C a 5.7% decrease in acyclovir's concentration was noted after 1 month and at 40°C a 14.0% decrease occurred after 2 months of stability testing.

The concentration of the active ingredient ketoconazole depicted no significant decrease in the cream and emulgel formulations. In the Pheroid™ cream a significant decrease of 7.0% in ketoconazole concentration was seen at 30°C after 3 months, and at 40°C a decrease of 5.68% occurred after 1 month on stability testing. In the Pheroid™ emulgel ketoconazole’s concentration decreased significantly with a 7.1% and 7.5% decrease at 25°C and 30°C, respectively after 6 months on stability. At 40°C a 15.7% decrease in ketoconazole’s concentration was observed after 3 months on stability.

Methylparaben was stable at all three temperatures, in all four formulated products, for the duration of the stability testing period. In the Pheroid™ cream formula the concentration of propylparaben decreased significantly, with 12.1% en 10.9% at 30 and 40°C, respectively, after 1 month of stability testing. A 5.6% reduction occurred in the concentration of propylparaben in the Pheroid™ emulgel at a temperature of 40°C, after 2 months of stability testing. Although a decrease of more than 5% in the propylparaben concentrations occurred, no microbial growth was noticed during physical assessment.

In the cream formulation an 18.8% and 17.1% decrease in BHA concentration was observed after 3 months on stability at 25 and 30°C, respectively, and a 9.1% decrease in BHA concentration was
observed at 40°C after 2 months on stability. The BHA concentration in the emulgel formulation decreased after 3 months of stability with 14.3%, 11.0% and 11.6% at 20, 30 and 40°C, respectively. In the Pheroid™ emulgel the BHA concentration decreased with 7.0% at 20°C after 2 months, 7.0% at 30°C after 3 months and 7.2% decrease at 40°C after 1 month of stability testing. The BHT concentration in the Pheroid™ cream formula decreased significantly after 1 month of stability testing with 13.0%, 18.8% and 20.2% at 20, 30 and 40°C, respectively. In the emulgel formulation the BHT concentration decreased with 9.5% at 40°C after 2 months on stability. In the Pheroid™ emulgel the BHT concentration decreased significantly after 1 month on stability, a reduction of 19.7%, 17.5% and 26.8% occurred at 20, 30 and 40°C, respectively.

In the Pheroid™ cream formula a significant decrease of 28.6% in tocopherol’s concentration at 25°C after 2 months was observed. In the Pheroid™ cream a decrease in tocopherol of 8.2% and 14.5% occurred after 1 month of stability at 30 and 40°C, respectively. Tocopherol in Pheroid™ emulgel had significant decreases in concentration of 14.7%, 15.7% and 27.2% after 1 month at 20, 30 and 40°C, respectively.

The vast decrease in the anti-oxidant concentrations may be due to the fact that the anti-oxidants have protected the active ingredients against oxidation.

Acyclovir and ketoconazole were stable in the cream formula at 25 and 30°C for the duration of the stability test period. Ketoconazole was also stable at 40°C in the cream formula. In the Pheroid™ cream formulation ketoconazole and acyclovir were stable at 25°C, indicating stability at this temperature. Acyclovir was stable in the emulgel at 25°C and ketoconazole was stable at all three stability temperatures for the duration of the stability program. Acyclovir and ketoconazole were both unstable at the three temperatures in the Pheroid™ emulgel formulation, indicating an unstable product.

3.2 pH measurement

The pH of the formulated products had decreased slightly at a temperature of 25°C which indicated stable products. Although the pH decreased slightly; it was still between a pH of 5 and 6. It
appears that maximum stability of all formulated products will be ensured for a longer period; if it is stored at a temperature below 25°C.

3.3 Viscosity

Decreases or increases in the viscosity of semi-solid formulations are indicative of changes in the structural elements of the formulation. It is a sign of poor physical stability if these rheological changes are irreversible (Flynn, 2002). The viscosity of all the formulations decreased over the 6 month period. Comparing the cream’s initial viscosity with its viscosity at 6 months, the viscosity decreased with approximately 350000 cP (21.05%). When comparing the Pheroid™ cream’s initial viscosity with the viscosity at 6 months, the viscosity decreased to about half of the initial measurement (48.10%). The emulgel and Pheroid™ emulgel's viscosity decreased with approximately 10000 cP (12.7%) and 5000 cP (4.71%) after 6 months, respectively, when compared to their initial viscosities. As the initial viscosities were much higher than at 6 months for all the formulations, it is an indication of the instability of the products.

3.4 Particle size observation

The particle size of the cream, emulgel and Pheroid™ emulgel semi-solid products did not change significantly over the 6 month period. The size of the acyclovir crystals have increased slightly, but not significantly. The cream, emulgel and Pheroid™ emulgel formulae seemed to be stable. The particle size of the Pheroid™ cream did not change significantly over the 6 month period, but the size the acyclovir crystals however increased. This increase was significant at 25°C and 30°C, but not at 40°C. The Pheroid™ cream formulation appeared to be unstable.

3.5 Mass loss

The mass loss of the formulated products was insignificant and no significant changes occurred at any of the three temperatures. This was indicative of a stable product in a suitable container.

3.6 Physical appearance

No physical changes were noticed in the cream and emulgel formulations at the three different temperatures over the stability test period. Over the 6 months a change in color of the Pheroid™ cream and the Pheroid™ emulgel, stored at 25°C, 30°C and 40°C, occurred. The Pheroid™ cream
had a slight change in color at 25°C where the color changed from white to soft yellow to light yellow. At 30°C the color changed from bright white to light yellow to bright yellow. At 40°C the color changed from white to bright yellow to a darker yellow. The Pheroid™ emulgel had a change in color at 25°C and 30°C from a cream-color to light yellow and again to a bright yellow. At 40°C the color changed from a cream-color to a bright yellow to a dark yellow. These color changes may be due to the oxidation of BHT and tocopherol.

3.7 Franz cell diffusion
The in vitro permeation of acyclovir and ketoconazole with the aid of Pheroid™ technology within the formulated products was investigated and compared to a control (i.e. basic formula without the Pheroid™ delivery system). The median flux values of the in vitro permeation studies for acyclovir are given in Figure 1.

**Figure 1**: The median flux (µg/cm².h) values of acyclovir

Acyclovir showed biphasic flux profiles in all four formulations, with a steady-state flux between 0 - 2 h (flux 1) and also between 2 - 12 h (Pheroid™ cream, Pheroid™ emulgel and emulgel) or 4 - 12 h (flux 2) (cream).

For acyclovir the median flux 1 values of the formulations were much greater than the median flux 2 values, showing that there was a larger transdermal delivery of acyclovir between 0 – 2 h, in comparison to the delivery between 2 - 12 h (or 4 – 12 h for the cream) after application of the formulated products. The flux 1 values of the cream, Pheroid™ cream, emulgel and the Pheroid™ emulgel formulations were 5.8, 8.9, 5.2 and 5.6 times greater than their corresponding flux 2 values, respectively.

The Pheroid™ cream formula had the biggest flux 1 (989.78 µg/cm².h) and flux 2 (111.76 µg/cm².h) values for acyclovir compared to the other formulated products, followed by the cream formula with a flux 1 of 538.00 µg/cm².h and a flux 2 of 92.19 µg/cm².h. When investigating the emulgel formulae, it was seen that the emulgel had a higher flux 1 (120.26 µg/cm².h) and flux 2 (23.02 µg/cm².h) value than the Pheroid™ emulgel (flux 1 of 109.31 µg/cm².h and flux 2 of 19.29
The average cumulative amount (µg/cm²) acyclovir that permeated the skin over 12 h (Figure 2) as well as the average %diffused can be placed in the following ranking order: Pheroid™ cream (2970.03 µg/cm², 3.194%) > cream (2172.20 µg/cm², 2.336%) > emulgel (482.38 µg/cm², 0.519%) > Pheroid™ emulgel (413.56 µg/cm², 0.445%). Thus the cream formulations increased transdermal delivery of acyclovir of which the Pheroid™ technology in the cream formulation enhanced the delivery of acyclovir the most.

**Figure 2:** Average amount per area (µg/cm²) acyclovir en ketoconazole in the different formulations that diffused through the skin after 12 h

Ketoconazole on the other hand showed no flux in any of the formulations. Therefore the average concentration (µg/cm²) diffused through the skin (Figure 2) after 12 h was investigated and compared with each other for all the formulations tested. However, after the Pheroid™ cream formula was applied, ketoconazole did not diffuse through the skin after 12 h. The concentrations of ketoconazole that diffused after 12 h were 38.86 µg/cm², 25.68 µg/cm² and 447.55 µg/cm² for the cream, emulgel and Pheroid™ emulgel, respectively. The average amount of ketoconazole that diffused (%diffused) through the skin over 12 h was 1.189%, 0.069% and 0.104% for the Pheroid™ emulgel, emulgel and the cream formulae, respectively. Thus it is clear that the emulgel formulations increased transdermal delivery of ketoconazole of which the Pheroid™ technology in the emulgel formula enhanced the delivery of ketoconazole the most.

### 3.8 Tape stripping

The median concentration acyclovir and ketoconazole present in the SC-epidermis and epidermis-dermis layers of the skin is presented in Figure 3.

**Figure 3:** SC-epidermis and epidermis-dermis values for (a) acyclovir and (b) ketoconazole

From Figure 3(a) it can be seen that the cream, emulgel and Pheroid™ emulgel formulations delivered a higher concentration of acyclovir to the epidermis-dermis layer (target site) of the skin than to the SC-epidermis layer of the skin. The SC-epidermis (µg/ml) values of acyclovir differed in
all four formulations. The Pheroid™ cream (1477.59 µg/ml) had the largest SC-epidermal concentration. The emulgel (102.10 µg/ml) showed a larger concentration of acyclovir in the SC-epidermis than the Pheroid™ emulgel (70.44 µg/ml) formula. The cream formula showed the smallest delivery of acyclovir (18.60 µg/ml) to the SC-epidermis.
The epidermis-dermis values of acyclovir were compared within the four formulations. The largest delivery of acyclovir to the epidermis-dermis layer of the skin was achieved by the Pheroid™ cream (1444.74 µg/ml). The emulgel formula (248.71 µg/ml) delivered a larger concentration of acyclovir to the epidermis-dermis of the skin than the Pheroid™ emulgel (115.21 µg/ml) formula. The cream formula showed the poorest delivery of acyclovir (45.05 µg/ml) to the epidermis-dermis layer. The Pheroid™ cream formulation illustrated that Pheroid™ increased delivery of acyclovir to the epidermis-dermis layer of the skin compared to the other formulae.

From Figure 3(b) it can be seen that all the formulations delivered a higher concentration of ketoconazole to the epidermis-dermis layer of the skin than to the SC-epidermis layer (target site) of the skin. The concentrations of ketoconazole delivered to the SC-epidermis layer of the skin were closely related in the four different formulations. The cream delivered the highest concentration (2.67 µg/ml) ketoconazole into the SC-epidermis layer, followed by the Pheroid™ cream (2.60 µg/ml), Pheroid™ emulgel (2.49 µg/ml) and the emulgel (1.10 µg/ml). The Pheroid™ emulgel improved the delivery of ketoconazole to the SC-epidermis layer of the skin to a higher extent compared to the emulgel, although to a lesser extent than the cream and the Pheroid™ cream. This indicates that that Pheroid™ increased the delivery of ketoconazole to the SC-epidermis layer in the emulgel formulae.

However, the epidermis-dermis delivery varied significantly between the different formulations. The Pheroid™ emulgel delivered 42.4 times more ketoconazole to the epidermis-dermis layer of the skin than to the SC-epidermis skin layer. The cream delivered a 5.5 times greater concentration of ketoconazole to the epidermis-dermis layer of the skin than to the SC-epidermis layer. The Pheroid™ emulgel formula delivered the largest concentration of ketoconazole to the epidermis-dermis (105.69 µg/ml) of the skin. The cream formula (14.66 µg/ml) delivered more
ketoconazole to the epidermis-dermis layer than the Pheroid™ cream formula (8.05 µg/ml). The Pheroid™ emulgel illustrated that the Pheroid™ increased the delivery of ketoconazole to the epidermis-dermis layer of the skin compared to the emulgel (6.19 µg/ml) formula.

Enhanced delivery of acyclovir and ketoconazole with Pheroid™ cream and Pheroid™ emulgel, respectively, might be due to the aqueous solubility of the drugs. Ketoconazole is more hydrophobic and acyclovir is more hydrophilic (McEvoy, 2002). The cream formula was more lipophilic than the hydrophilic emulgel formula. Thus, it may be possible that acyclovir did not diffuse out of the emulgel formula, due to the fact that it had an affinity for the water surroundings and did not effortlessly diffuse to the lipid SC (Menon, 2002). Ketoconazole may have benefitted from its fatty surroundings and did not have affinity to diffuse out of the cream formula.

When comparing the flux values of acyclovir with the epidermis-dermis and SC-epidermis concentrations, it was noted that the formulation with the largest flux 1 and flux 2 values (Pheroid™ cream) also showed the highest epidermis-dermis and SC-epidermis concentrations. Thus, the higher the flux values of acyclovir the more acyclovir was delivered to the epidermis-dermis and the SC-epidermis layers of the skin. When comparing the 12 h concentrations of ketoconazole that diffused through the skin, with the SC-epidermis and epidermis-dermis concentrations; it was noted that the formulation with the largest 12 h concentration delivered transdermally (Pheroid™ emulgel), also had the largest delivery to the epidermis-dermis layer of the skin.

3.9 Statistical data

There was a statistical significant difference between the flux 1 and the flux 2 values of acyclovir in all four formulations with p < 0.0001. The flux 1 values of acyclovir were compared within the four formulations, where a statistical significant difference was found between the cream and Pheroid™ emulgel; the emulgel and Pheroid™ cream; and the Pheroid™ cream and Pheroid™ emulgel. The cream and the Pheroid™ cream as well as the emulgel and the Pheroid™ emulgel showed a statistical similarity between their flux 1 values with p = 0.377 in both instances.

When comparing the flux 2 values of acyclovir of the four formulations a significant difference (p < 0.05) was found between the cream and the Pheroid™ emulgel; emulgel and Pheroid™ cream as
well as between Pheroid™ cream and Pheroid™ emulgel. As with flux 1, the cream and the Pheroid™ cream as well as the emulgel and the Pheroid™ emulgel showed a statistical similarity between their flux 1 values with p = 0.377 in both instances.

When the total concentration of ketoconazole diffused over 12 h for each formula was compared a p < 0.0001 was found. This showed that there was a statistical significant difference between the cream, emulgel and Pheroid™ emulgel formulations’ that diffused over 12 h.

Acyclovir SC-epidermis values were compared within the four formulations, where a statistical significant difference between the Pheroid™ cream and Pheroid™ emulgel; the cream and Pheroid™ cream as well as between the cream and emulgel was observed (p < 0.05). When comparing the total of the acyclovir epidermis-dermis values of all four formulations a significant difference between these values (p < 0.0001) was found by using the Kruskal-Wallis test. The SC-epidermis values of acyclovir for all four formulations was compared with each other. A statistical significant difference between these values was found with p < 0.0001. The SC-epidermis values of acyclovir showed a statistical significant difference between flux 1 and flux 2 with p < 0.0001 in both instances. The epidermis-dermis values of acyclovir also had a significant difference between the flux 1 and flux 2 values, with p < 0.0001 and p = 0.0002, respectively.

The ketoconazole’s SC-epidermis values were compared within the formulated products; a statistical significant difference between the Pheroid™ cream and emulgel as well as between the emulgel and cream was obtained. The epidermis-dermis values of ketoconazole were compared within the four formulations; a statistical significant difference between the Pheroid™ cream and Pheroid™ emulgel; the emulgel and Pheroid™ emulgel as well as between the cream and emulgel was seen.

There was no significant difference between the SC-epidermis and epidermis-dermis values (p = 0.09). The sum of the SC-epidermis values of ketoconazole in all four formulations was compared with each other using the Kruskal-Wallis test. A statistical significant difference between these values was found with p = 0.0091. When comparing the sum of the epidermis-dermis values of
ketoconazole from all four formulations with each other a statistical significant difference between these values was found with p < 0.0001.

4 Conclusion

None of the formulated products met the criteria for stability of the MCC (2006) or the ICH (2003) Guidelines. The results of the stability tests also indicated that the cream and emulgel formulae were more stable than the Pheroid™ formulae.

When considering literature it was expected to be possible to deliver acyclovir transdermally when looking at the relatively high aqueous solubility and low molecular weight of the drug (McEvoy, 2002; British Pharmacopoeia, 2013). The in vitro permeation studies of acyclovir showed that acyclovir penetrated through the full-thickness skin after the application of all four of the formulations. When comparing the flux values the penetration of acyclovir was enhanced by the cream formulae in comparison with the emulgel formulae. Pheroid™ technology enhanced the delivery of acyclovir in the cream formula.

The transdermal delivery of ketoconazole was poor; as was expected when considering its low aqueous solubility and high molecular weight (McEvoy, 2002; British Pharmacopoeia, 2013). Ketoconazole penetrated through the full-thickness skin in the case of the cream, emulgel and Pheroid™ emulgel formulae. Ketoconazole's average amount of ketoconazole diffused at 12 h illustrated that penetration of ketoconazole was enhanced by the emulgel formulae. The Pheroid™ emulgel formulation exhibited the best transdermal delivery of ketoconazole. Thus the Pheroid™ showed to enhance transdermal delivery of acyclovir and ketoconazole due to the higher efficiency of delivering drugs across the SC (Grobler et al., 2008).

Acyclovir was detected in the epidermis-dermis and SC-epidermis layers of the skin after application of all four formulations. The Pheroid™ cream greatly enhanced the delivery of acyclovir to the epidermis-dermis and SC-epidermis of the skin in comparison to the cream formulation.

There was detection of ketoconazole in the epidermis-dermis and SC-epidermis layers of the skin after application of all four formulations. The Pheroid™ emulgel enhanced the delivery of ketoconazole to the epidermis-dermis but not to the SC-epidermis compared to the emulgel.
When comparing the flux values and the 12 h concentrations of acyclovir and ketoconazole with the epidermis-dermis and SC-epidermis concentrations, it was noted that the larger the diffusion of the actives through the skin, the more active ingredients were delivered to the epidermis-dermis and the SC-epidermis.

In conclusion, Pheroid™ technology enhanced dermal and transdermal delivery of acyclovir and ketoconazole to human skin in several instances, although the Pheroid™ formulations showed poor stability and further investigations should be undertaken in order to increase the stability of these formulations.
Acknowledgements

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Disclaimer

Any opinion, findings and conclusions or recommendations expressed in this material are those of the authors and therefore the NRF do not accept any liability in regard thereto.
References


**Table 1:** Formula of acyclovir and ketoconazole cream and emulgel

<table>
<thead>
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<th>Raw material</th>
<th>Cream (m/m)</th>
<th>Emulgel (m/m)</th>
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Figure captions

**Figure 1:** The median flux ($\mu g/cm^2.h$) values of acyclovir

**Figure 2:** Average amount per area ($\mu g/cm^2$) acyclovir en ketoconazole in the different formulations that diffused through the skin after 12 h

**Figure 3:** SC-epidermis and epidermis-dermis values for (a) acyclovir and (b) ketoconazole
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