Topical delivery of different ketoconazole and acyclovir formulations

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Great faith is the product of great fights.

Great testimonies are the outcome of great tests.

Great triumphs can only come out of great trials.

-Smith Wigglesworth

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AIMS AND OBJECTIVES

From very early in the progression of HIV/AIDS, cutaneous diseases have emerged as external markers of HIV or AIDS (Ramdial, 2010:39). A decrease in mucocutaneous immunity can often account for the clinical signs seen in HIV/AIDS on the skin. These manifestations tend to become more severe and varied as the disease progresses, causing physical discomfort, as well as psychological distress (Uthayakumar *et al.,* 1997:595).

Fungal infections can be seen in HIV positive patients from the onset. However, established cutaneous infections are frequently found in both HIV-infected and uninfected individuals. It can present in the form of simple mucocutaneous candidiasis or could develop into life-threatening contagious infections, including *histoplasmosis* or *cryptococcosis*. Fungal infections are stubborn and may lead to secondary bacterial infections, which include *staphylococcus* and *streptococcus* infections (Dlova & Mosam, 2004:13).

As a result of compromised immunity caused by HIV/AIDS, viral infections increase among HIV patients. Lesions can be mild to disfigurative, for example *molluscum contagiosum* (MCV). Viral infections can be fatal as seen with human papillomavirus (HPV). In the case of a viral opportunistic infection, it can present as the activation of a sub-clinical infection or reactivation of a latent virus such as *Herpes simplex* virus (HSV) or *Varicella zoster* virus (VCV) (Dlova & Mosam, 2005:12).

The main use of acyclovir is for the treatment and prophylaxis of viral infections, essentially caused by *HSV* types 1 and 2. Infections caused by *HSV* include *herpes keratitis*, *herpes labialis* and genital herpes. Most acyclovir products depict positive results against these infections, if it is administered as soon as the symptoms appear (Sweetman, 2011).

Ketoconazole, on the other hand, has shown activity against most pathogenic fungi, including dermatophytes and yeasts (Bethesda, 2010). The spectrum for ketoconazole activity is wide and includes: *Candida spp., Histoplasma capsulatum, Sporothrix schenckii* and *Cryptococococcus neoformans* (Sweetman, 2011). By producing a formulation containing both acyclovir and ketoconazole, a wide spectrum of treatments against these dermatological disorders can be expected.

There are many advantages of topical formulations, which include circumvention of the first-pass metabolism and higher patient compliance due to minimising the frequency of dosage. It also evades the procedure and pain associated with parental therapy (Roberts *et a*l., 2002:90).

The main problem experienced with dermal formulation is the penetration of the stratum corneum (SC). This layer of the skin has a significant barrier which protects the body against harmful effects of the environment. The SC, however, is also responsible for the difficulty experienced when delivering compounds via the skin. Structured lipids found in the intercellular channels are the main obstacle in compound penetration through the skin (Menon 2002:4). The physicochemical properties of acyclovir and ketoconazole predict that acyclovir will permeate the skin more readily than ketoconazole (Sweetman, 2011).

Skin structures vary over different sites of the body and the SC will be thicker in some areas due to load-bearing. The site most permeable for transdermal compound delivery is the genital area and has the highest potential to deliver clinical levels of compounds administered (Williams 2003:16). Labial skin also comprises of the normal three layers found in the skin, with the exception that the SC is thinner compared to other sites of the human body (Blistex, 2009).
In vitro skin permeation of acyclovir and ketoconazole through mucosa was investigated in this study. The main focus was to evaluate the delivery of these two compounds across the different skin layers with no required systemic effect by using different formulations.

Therefore, the objectives were:

- Establishing whether there are any incompatibilities between acyclovir and ketoconazole by means of microcalorimetry.
- Validation of a high performance liquid chromatography (HPLC) method to ascertain the concentrations of the acyclovir and ketoconazole.
- Determining the appropriate components to include in the different topical formulations.
- Developing three topical formulations containing both acyclovir and ketoconazole, i.e. cream, gel and lip balm.
- Determining the topical delivery of ketoconazole and acyclovir.
- Establishing whether any active ingredient was delivered through mucosa.
- Comparing diffusion results obtained from the three formulations with selected commercial products, Acitop® and Ketazol®.
- Determining the stability (intermediate stability) of the different formulations over a 3 month period.

ABSTRACT

Acquired immunodeficiency syndrome (AIDS) has shown a rapid increase in incidence over the past 25 years. Many clinical manifestations occur in patients infected with human immunodeficiency virus (HIV) due to compromised immunity caused by this virus. Dermatological disorders are almost inevitable for individuals suffering from HIV/AIDS and are seen in approximately 90% of all infected patients (Cedeno-Laurent *et al.,* 2011:5; Dlova & Mosam, 2004:12). Vulnerability of the skin causes impaired life quality by causing low self-esteem, depression or even suicide. The skin is regarded as the most visible organ due to its location and large surface area (Cedeno-Laurent *et al.,* 2011:5). Cutaneous manifestations, including viral, fungal and bacterial pathogens, can serve as markers in HIV/AIDS progression or as indicators for commencing HIV/AIDS treatment (Vusadevan *et al.,* 2012:20).

Acyclovir is an anti-viral agent showing activity against *herpes simplex* virus type 1 and type 2, *varicella-zoster* virus and cytomegalovirus to a certain extent (King, 1988:176; Beers, 2006:1061). The anti-fungal agent, ketoconazole, shows activity against the majority of pathogenic fungal infections seen in HIV/AIDS including *Candida spp*, *Cryptococococcus neoformans* and *Histoplasma capsulatum* (Bennet, 2006:1225). Ketoconazole has shown to have *in vitro* inhibitory activity against certain *Staphylococcus spp* (Pottage, 1986:217). According to Bickers (1994:89), ketoconazole shows a synergistic anti-viral activity when used in combination with acyclovir.

Using the mucosal route of administration may be beneficial for these compounds due to the location of occurrence as many of these diseases are found on mucosal surfaces such as the labial and vaginal areas. Compounds are mainly delivered via passive diffusion across epithelium membranes (Patel *et al*., 2011:107). In mucosal skin, the principle barrier function is removed by the absence of the keratinised stratum corneum as found in normal skin and is, thus, more permeable (Farage & Scheffler, 2011:117).

In this study three different formulations containing acyclovir (5% w/w) and ketoconazole (2% w/w) were formulated for topical delivery on mucus membranes, which included a cream, gel and lip balm. Topical delivery is used to target specific sites on the skin by penetration of the skin layers, but has a minimal requirement for systemic effect (Dayan, 2005:67). The aim in this study was to formulate a stable product containing acyclovir and ketoconazole that would

provide an efficient flux of both compounds when applied on mucosal membranes. *In vitro* studies were performed to determine skin permeation of acyclovir and ketoconazole by using a flow-through diffusion system. The formulated products were compared to Acitop® and Ketazol®, which are two products available on the South African market, containing acyclovir and ketoconazole, respectively. However, no product is yet available containing both acyclovir and ketoconazole.

Results obtained for acyclovir released from the different formulations during the permeation studies depicted no statistical significant differences between the different formulations in the average cumulative amount of acyclovir released ($p > 0.05$). The cream, gel and lip balm formulations depicted a decreased average cumulative acyclovir amount released through the mucosa when compared to Acitop®. The following rank order could be established: Acitop® > gel > cream > lip balm. Furthermore, the gel formulation and Acitop® produced a relatively similar percentage of acyclovir diffused. A linear relationship ($r^2 = 0.9977$) existed between the flux and the release rate of acyclovir from the different formulations, indicating that as the acyclovir was released, the flux increased correspondingly. Using the Higuchi model, the average cumulative amount of acyclovir released that permeated the mucosa per unit surface area was constructed against the square root of time ($h^{½}$). All formulations depicted a correlation coefficient (r^2) of 0.9644 – 0.9914 for acyclovir, indicating that the release of acyclovir from the different formulations could be described by the Higuchi model. No statistical significant differences could be obtained for acyclovir between any of the formulations for % diffused, apparent release constant (ARC), release rate (RR) and lag times.

The amount of ketoconazole that permeated the mucosa from the gel and cream formulations exhibited a smaller average cumulative amount that permeated the mucosa when compared to Ketazol®. The lip balm was the only formulation that showed a statistically significant ($p < 0.05$) increase in permeation through the mucosa in comparison to Ketazol®. A rank order for the average cumulative amount of ketoconazole that permeated through the mucosa could be established namely: lip balm >>> Ketazol® > gel > cream. A linear relationship ($r^2 = 0.9991$) was depicted between the average release constant and the average release rate from each of the different formulations for ketoconazole. This indicated that as the compound was released, the flux increased correspondingly which was in accordance with the acyclovir release tendency. The only statistically significant difference ($p < 0.05$) was seen for the release rate of ketoconazole from the lip balm formulation compared to that of the cream and gel formulations. Release rate and flux of ketoconazole was the highest from the lip balm formulation. The rate of

ketoconazole released from all of the different formulations obeyed the Higuchi model as the amount of compound released from each formulation was a linear function of the square root of time (r^2 = 0.9584 - 0.9899). Statistically significant ($p < 0.05$) differences were furthermore noted between the lip balm and both the cream and gel formulations when % diffused, ARC and RR were compared. The lip balm depicted the highest percentage diffused, the highest ARC as well as the fastest RR. However, no statistical differences were obtained between the cream and gel formulation even though the gel formulation performed slightly better. Considering the lag time, all the formulations presented with a relatively shorter initial time of release (less than an hour). Shorter lag time values indicate that the ketoconazole was preferentially released by the base of the formulations. Statistically significant differences ($p < 0.05$) were depicted between the lag times of Ketazol® and lip balm formulation, as well as between the lip balm and the cream and gel formulations.

The stability of the formulated products was examined over a period of three months according to the standards of the International Conference of Harmonisation (ICH) Tripartite Guidelines (2003:12) and the Medicines Control Council (MCC) of South Africa (2011:12). Each of the formulated products was stored at three different temperatures and humidities. Stability tests conducted included visual appearance, mass variation, assay, pH determination, viscosity, particle size observation, and zeta potential. Due to the different properties of the formulations, some tests could not be conducted on the gel and lip balm formulations.

The outcomes of the stability tests showed that all three formulations presented acceptable results for some of the tests conducted. No significant changes were noted in the visual appearance, mass variation and pH values of all tested formulations at the specified storage conditions.

Acyclovir is slightly soluble in water and has a solubility of 1.3 mg/ml at 25 ºC according to Bethesda (2010). Low solubility often causes crystal formation in products. All of the formulations developed in this study presented crystals on the surface. Due to non-homogenous sample preparation differences in concentrations could be obtained as the amount and size of crystals may differ. Ketoconazole did, however, not depict any significant changes in concentration for any of the formulations at all storage conditions.

The cream depicted variable changes in viscosity over the three months, showing no clear trend, whereas, the viscosity measurement results of the gel formulation depicted a definite trend. The sodium carboxymethylcellulose (Na-CMC) used as the thickening agent in this

formulation was responsible for this trend obtained in the results, due to the effects of pH, hydration and temperature on this excipient (Aqualon, 1996:10).

Results obtained from zeta potential determination for the cream formulation depicted no significant change and the values remained below 25 mV. Zeta potential values below 25 mV present the risk of coalescence due to the lower repelling forces between particles (Jelvehgari *et al*., 2010:1240).

The average size of the particles in dispersion was also observed and could be linked to zeta potential values. The cream depicted an increase in particle size over the three months stability testing. Due to the low zeta potential depicted in the cream formulation it was expected that coalescence would occur over time.

From results obtained in this study it was clear that manufacturing different formulations containing both acyclovir and ketoconazole proved difficult due to the significant differences between their physicochemical properties, which in turn influenced the stability of the formulation. Furthermore, it was evident that formulation at specific pH values, as well as the incorporation of certain excipients, played a significant role in the stability of formulations.

Keywords: acyclovir, ketoconazole, topical delivery, mucosa, stability, compatibility.

UITTREKSEL

Verworwe immuniteitsgebreksindroom (VIGS) het ‘n merkwaardige toename in voorkoms oor die afgelope 25 jaar getoon. Menige kliniese manifestasies ontstaan by pasiënte wat geïnfekteerd is met menslike immuniteitgebrekvirus (MIV) as gevolg van onderdrukte immuniteit wat deur die virus veroorsaak word. Dermatologiese disfunksies is feitlik onvermydelik vir pasiënte wat aan MIV/VIGS ly en word opgemerk in ongeveer 90% van alle geïnfekteerde pasiënte (Cedeno-Laurent *et al.,* 2011:5; Dlova & Mosam, 2004:12). Kwesbaarheid van die vel benadeel lewenskwaliteit deur verlaagde selfbeeld en depressie te veroorsaak, wat selfmoord tot gevolg kan hê (Cedeno-Laurent *et al.,* 2011:5). Die vel is vatbaar vir virale, fungale en bakteriële patogene, wat kan dien as merkers in die verloop van die MIV/VIGS toestand of as aanwysers vir die verloop van behandeling van MIV/VIGS (Vusadevan *et al.,* 2012:20).

Asiklovir is ‘n anti-virale geneesmiddel wat aktiwiteit toon teen *herpes simplex virus* tipe 1 en tipe 2, *varicella-zoster* en *cytomegalovirus* tot ‘n mindere mate (King, 1988:176; Beers, 2006:1061). Die anti-fungale geneesmiddel, ketokonasool, toon aktiwiteit teen die meerderheid van die patologiese fungale infeksies opgemerk in MIV/VIGS wat *Candida spp*, *Cryptococococcus neoformans* en *Histoplasma capsulatum* insluit (Bennet, 2006:1225). Volgens Bickers (1994:89), toon ketokonasool ‘n sinergistiese anti-virale effek wanneer dit in kombinasie met asiklovir gebruik word. Volgens Pottage (1986:217) toon Ketokonasool ook *in vitro,* inhiberende aktiwiteit teen sekere *Staphylococcus spp.*

Die gebruik van mukosale weefsel as toedieningsroete kan voordelig wees in die aflewering van hierdie spesifieke geneesmiddels as gevolg daarvan dat verskeie van hierdie infeksies op mukosale membrane voorkom, soos op die labiale en vaginale areas. Geneesmiddels beweeg hoofsaaklik deur epiteelmembrane deur middel van passiewe diffusie (Patel *et al*., 2011:107). Die weerstandsfunksie van die vel word verwyder in die geval van mukosale membrane as gevolg van die verwydering van gekeratiniseerde stratum corneum, wat verhoogde deurlaatbaarheid tot gevolg het (Farage & Scheffler, 2011:117).

Drie verskillende formulerings met asiklovir (5% m/m) en ketokonasool (2% m/m) is geformuleer in hierdie studie vir die topikale gebruik op mukosale membrane. Dit sluit ‘n room, jel en lipbalsem in. Die topikale aflewering van geneesmiddels word gebruik om spesifieke teiken-areas van die vel te bereik deur verskeie lae te penetreer, aangesien minimale sistemiese effek

benodig word (Dayan, 2005:67). Die doel van hierdie studie was om ‘n stabiele produk te formuleer wat beide asiklovir en ketokonasool bevat en ‘n effektiewe fluks sal lewer wanneer dit op mukosale weefsel aangewend word. *In vitro*-studies is uitgevoer om die deurlaatbaarheid van asiklovir en ketokonasool te bepaal deur middel van ‘n deurlopende-vloeiperfusie-apparaat. Die geformuleerde produkte is vergelyk met Acitop® en Ketazol®, twee bestaande produkte op die Suid-Afrikaanse mark, wat onderskeidelik asiklovir en ketokonasool bevat. Daar is tans egter geen produk beskikbaar wat beide asiklovir en ketokonasool bevat nie.

Vanuit die resultate verkry uit die deurlaatbaarheidstudies kan daar waargeneem word dat asiklovir geen statisties-beduidende verskil getoon het in die gemiddelde hoeveelheid asiklovir vrygestel ($p > 0.05$) deur al die formulerings nie. Die room-, jel- en lipbalsem-formulerings het ‘n afname getoon in die gemiddelde kumulatiewe hoeveelheid asiklovir vrygestel en wat gevolglik deur die mukosa beweeg het, in vergelyking met Acitop®. ‘n Rangorde kon saamgestel word vanuit die resulte: Acitop® > jel > room > lipbalsem. Soortgelyke hoeveelhede asiklovir het vanuit die jel en Acitop® gedurende die diffusiestudie beweeg. ‘n Lineêre verwantskap ($r^2 = 0.9977$) kon vir die fluks en die vrystellingstempo van asiklovir vanuit die verskeie formulerings waargeneem word. Dit is ‘n aanduiding dat die fluks ooreenkomstig gestyg het soos die hoeveelheid asiklovir vrygestel is. Deur gebruik te maak van die Higuchi-model, kon die gemiddelde hoeveelheid asiklovir vrygestel deur die mukosa per eenheidsarea gestip word teen die vierkantswortel van tyd ($h^{½}$). ‘n Korrelasiekoëffisiënt (r^2) van tussen 0.09644 – 0.9914 is waargeneem vir al die formulerings wat daarop aandui dat die vrystelling van asiklovir vanuit die formulerings beskryf kan word deur gebruik te maak van die Higuchi-model. Geen statisties-beduidende verskil kon waargeneem word tussen die formulerings vir die % gediffundeer, aanvanklike vrystellingskonstante (AVK), vrystellingstempo (VT) asook vir sleurtyd nie.

Ketokonasool het ‘n laer gemiddelde kumulatiewe hoeveelheid deurgelaat vanuit die jel- en roomformulerings in vergelyking met Ketazol®. Die lipbalsem was die enigste formulering wat statisties-beduidend ($p < 0.05$) ‘n hoër deurlaatbaarheid vir ketokonasool deur die mukosa getoon het in vergelyking met Ketazol®. ‘n Rangorde vir die gemiddelde kumulatiewe hoeveelheid ketokonasool wat deur die membraan beweeg het, kon vasgestel word: lipbalsem >>> Ketazol® > jel > room. ‘n Lineêre verwantskap ($r^2 = 0.9991$) kon waargeneem word vir die gemiddelde vrystellingskonstante en die gemiddelde vrystellingstempo (VT) vir ketokonasool vanuit die verskillende formulerings. Hierdie resultate sluit aan by die tendens vir asiklovirvrystelling en dui daarop dat die fluks verhoog het soos ketokonasool vrygestel is. Die

enigste statisties-beduidende verskil ($p < 0.05$) is waargeneem vir die VT van ketokonasool vanuit die lipbalsem formulering in vergelyking met die room- en jel-formulerings. Ketokonasool het die hoogste VT en fluks vanuit die lipbalsem-formulering getoon. Die vrystelling van ketokonasool vanuit die verskeie formulerings het ook die Higuchi-model gevolg ($r^2 = 0.9584 - 0.9899$). Statisties-beduidende verskille ($p < 0.05$) is waargeneem tussen die lipbalsem en beide die room- en jel-formulerings indien die % gediffundeer, AVK en VT vanuit die verskillende formulerings met mekaar vergelyk word. Die hoogste % gediffundeer, AVK asook die vinnigste VT is waargeneem vanuit die lipbalsem-formulering vir ketokonasool. Geen statisties beduidende verskil is waargeneem tussen die room- en jel-formulerings nie, alhoewel die jel ietwat beter resultate gelewer het. Die sleurtyd vir al die formulerings was minder as een uur en is ‘n aanduiding dat ketokonasool redelik maklik vrygestel is vanuit die basis van al die formulerings. Statisties beduidende verskille ($p < 0.05$) is opgemerk in die sleurtyd vir die lipbalsem en Ketazol®, asook tussen die lipbalsem en beide die room- en jel-formulerings.

Stabiliteit van die formulerings is oor die verloop van drie maande bepaal volgens die standaarde van die “International Conference of Harmonisation (ICH) Tripartite” riglyne (2003:12) asook die Medisyne-beheerraad (MBR) van Suid-Afrika (2011:12). Elkeen van die geformuleerde produkte is by drie verskillende temperatuur en humiditeitdtoestande geberg. Die toetse vir stabiltiteit wat in hierdie studie uitgevoer is, sluit in: die visuele voorkoms, massaverandering, gehaltebepaling, pH-bepaling, viskositeit, deeltjiegroottebepaling, asook meting van zeta-potensiaal. Sommige van hierdie toetse kon nie op die jel of lipbalsem uitgevoer word nie as gevolg van die verskillende individuele eienskappe van die formulerings.

Die resultate vanuit hierdie toetse verkry, het getoon dat al die formulerings aanvaarbare waardes vir van die toetse getoon het. Geen beduidende verskille is opgemerk in die visuele vookoms, massaverandering of pH-waardes van al die formulerings nie.

Asiklovir het ‘n oplosbaarheid van 1.3 mg/ml by 25 ºC in water en is dus effens oplosbaar, volgens Bethesda (2010). Kristalle vorm algemeen in produkte met lae wateroplosbaarheid, en gevolglik is kristalle waargeneem op die oppervlakke van al die geformuleerde produkte. Nie-homogene monsterneming kon moontlik die rede wees vir variasies in gehaltebepalingsresultate, aangesien die hoeveelheid en grootte van kristalle in elke monster kon verskil. Die ketokonasool het egter geen beduidende verskil in konsentrasie by enige van die formulerings by alle bergingstoestande getoon nie.

Die roomformulering het wisselvallige viskositeitswaardes gelewer oor die drie maande tydperk en het geen vaste tendens getoon nie. Viskositeitswaardes vir die jel-formulering het egter ‘n

duidelike tendens getoon. Die natriumkarboksiemetielsellulose (Na-CMC) wat gebruik is as verdikkingsmiddel in die formulering kan aanspreeklik gehou word vir die waargenome tendens. Die invloed van pH, hidrasie en temperatuur op Na-CMC is verantwoordelik vir die tendens van die jel se viskositeit (Aqualon, 1996:10).

Geen beduidende verskil in zeta-potensiaal is waargeneem vir die roomformulering nie en al die gemete waardes was onder 25 mV. Lae afstootingskragte word egter waargeneem in formulerings met ‘n lae zeta-potensiaal (onder 25 mV) en koalesensie kom dan algemeen voor (Jelvehgari *et al*., 2010:1240).

Die gemiddelde deeltjiegrootte van die room is ook bepaal en kan in verband gebring word met die waardes verkry tydens die zeta-potensiaalbepalings. ‘n Verwagte verhoging in deeltjiegrootte is waargeneem in die room oor die drie maande van stabiliteitstoetse en is as gevolg van die lae zeta-potensiaal waargeneem, wat koalesensie tot gevolg kon hê.

Vanuit die resultate verkry in hierdie studie word dit duidelik dat dit beduidend moeilik was om verskeie formulerings te vervaardig wat beide asiklovir en ketokonasool bevat het. Die verskille in fisiese-chemiese eienskappe van elke geneesmiddel was die oorsaak daarvan, en kon waargeneem word in die resultate verkry vanuit die verskeie stabiliteitstoetse. Die invloed van pH en hulpstowwe in die produksie het verder ook ‘n groot invloed op ‘n formulering se stabiliteit gehad.

Sleutelwoorde: asiklovir, ketokonasool, topikale aflewering, mukosa, stabiliteit, verenigbaarheid.

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CHAPTER 1: HIV SKIN RELATED DISEASES

1.1 INTRODUCTION

The first cases of acquired immunodeficiency syndrome (AIDS) were reported more than 25 years ago, yet we are still unable to fully grasp the complexity found of this disease. Dermatological disorders are almost inevitable for patients suffering from the Human immunodeficiency virus (HIV). Cutaneous manifestations associated with HIV/AIDS cause low self-esteem induced depression that is often the cause of suicide in these patients. Although the pathogenesis of most of these cutaneous diseases is not completely understood, they can be considered as markers in the progression of the disease in HIV–seropositive patients (Cedeno-Laurent *et al.,* 2011:5).

The earliest clinical signs seen in HIV patients are cutaneous lesions caused by decreased mucocutaneous immunity (Uthayakumar *et al*., 1997:595). Severity of the dermatological condition increases as the cluster of differentiation 4 (CD4) lymphocyte cell count decreases and becomes more severe as the disease progresses (Dlova & Mosam, 2004:12). Therefore, physical discomfort and physiological distress are caused in otherwise relatively healthy individuals. Challenges in diagnoses and treatment which present in advanced immonusuppression as opportunistic pathogens, can occur as atypical cutaneous lesions (Uthayakumar *et al*., 1997:595). Futhermore, many of the dermatological lesions or infections seen in HIV/AIDS patients have an altered profile compared to HIV-unaffected individuals, which also present a challenge for diagnoses and treatment (Ramdial, 2010:40).

1.2 SKIN INFECTIONS ASSOCIATED WITH HIV/AIDS

In a fully developed human, skin represents approximately 3.7% of the total body weight and has the largest surface area compared with other organs, of between 18 000 - 24 000 cm^2, depending on the individual height and weight of the body (Poet & Mcdougal, 2002:22).

Numerous pathological manifestations including fungal, bacterial and viral infections develop during HIV/AIDS. Dermatological manifestations can cause severe emotional distress in HIV/AIDS patients, due to the skin's size and visibility. Several opportunistic infections and neoplastic disorders affect the skin of HIV/AIDS patients, as immunosuppression increases dermatological vulnerability (Cedeno-Laurent *et al.,* 2011:5). Approxiamately 90% of all HIV/AIDS patients present with one or several dermatological manifestations during the time of their disease (Dlova & Mosam, 2004:12).

Since highly active antiretroviral therapy (HAART) distribution improved, patient compliance has increased. With enhanced patient compliance a decline was seen in certain skin disorders found in HIV/AIDS patients (Yen-Moore *et al*., 2000:429). However, secondary infections are still more prevalent in HIV/AIDS patients than primary infections (Cedeno-Laurent *et al.,* 2011:5).

1.2.1 Fungal infections

The most found infectious dermatological manifestations that may present in HIV/AIDS patients are fungal infections. It can range from oral candidiasis in the early onset of HIV/AIDS, to terminal infections such as histoplasmosis and cryptococcosis, as the disease progresses. Fungi produce disease in their host by adhering to target cells and penetrating them. The defective immunity of HIV/AIDS patients provides a suitable environment for pathogens resulting in tissue invasion and damage. Mycotic pathogen infections commonly found in HIV/AIDS patients include: candidiasis, dermatophytosis, cryptococcosis, histoplasmosis and sporotrichosis (Durden & Elewski, 1997:200; Dlova & Mosam, 2004:13).

1.2.1.1 Candidiasis

Candidiasis is the most commonly found mucocutaneous manifestation in individuals with HIV/AIDS, affecting 20 – 70% of patients. A marker for rapid HIV progression is an increase in the incidence of oral candidiasis as it indicates immunosuppression. Presentation of candidiasis is seen in tongue and buccal mucosa in the form of thick, white plaques. Other manifestations can be seen at the nails, vaginal or urethral areas (Durden & Elewski, 1997:204; Dlova & Mosam, 2004:13).

Candidal vulvovaginitis (CVV) may be prevalent in HIV-infected woman as first clinical expression of compromised immunity (figure 1.1). Symptoms and signs include erythema, often

white, caseous plaques on both the vulvar and/or vaginal area. Burning or itching is also known to occur (Johnson, 2000:411).

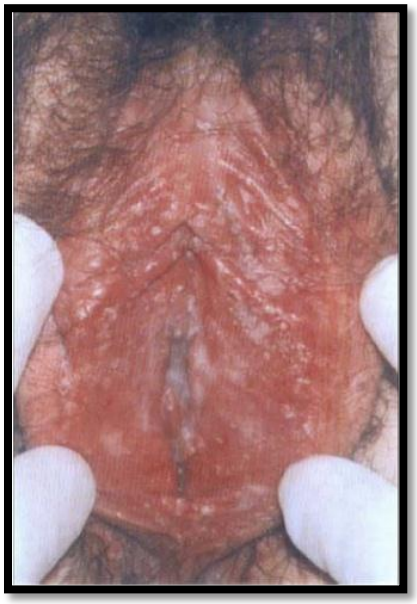


Figure 1.1: *Typical acute candidal vulvovaginitis (Higgins, 2001:184).*

1.2.1.2 Dermatophytosis

Dermatophytosis is an opportunistic infection which involves the skin, nails and hair (Trope & Lenzi, 2005:577). *Tinea corporis, T. pedis* and *T. capitis* can present in an atypical form as the "ringworm" infection with active edges, central clearing and hair loss (Dlova & Mosam, 2004:13). Although epidermal dermatophytosis usually presents as asymptomatic, some cases have reported pruritus and pain. Infection may occur on sites such as the face, neck or feet (figure 1.2). This can lead to cosmetic disfigurement (Johnson, 2000:415).

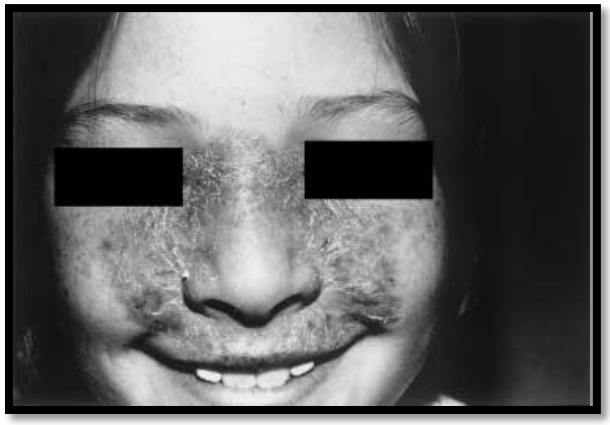


Figure 1.2: *Dermatophytosis on the facial and lip area (Zaias & Rebell, 2003:786)*.

1.2.1.3 Cryptococcosis

Cryptococcus neoformans is a yeast that is omnipresent and can be found in soil or pigeon excretement. This infection is seen as the most common life-threatening fungal manifestation in HIV/AIDS patients (Durden & Elewski, 1997:200). Skin involvement only takes place in 10% of patients infected with *Cryptococcus* as this is a systemic infection. Sites mostly affected are the head and neck. Lesions may present as papules, nodules, pustules or ulcers (figure 1.3). Cryptococcosis may be fatal if untreated and is often confused with *molluscum contagiosum* (MCV). The symptoms are often subtle which causes difficulty in early diagnosis (Durden & Elewski, 1997:201; Johnson, 2000: 419; Dlova & Mosam, 2004:14).

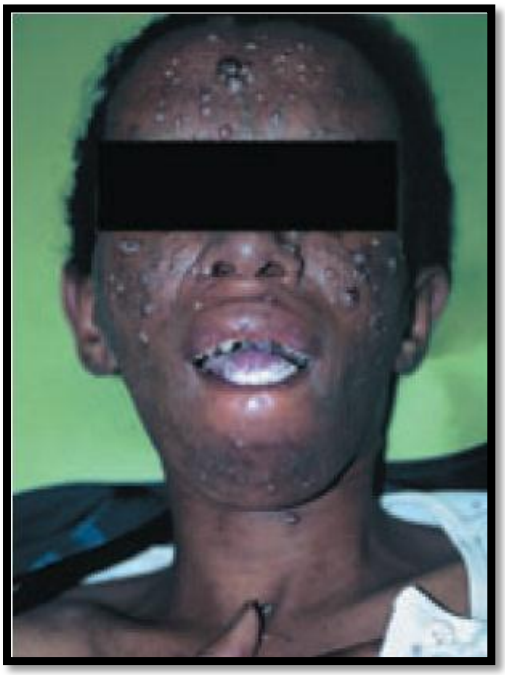


Figure 1.3: *Cryptococcosis skin lesions resembling MCV (Dlova & Mosam, 2004:14).*

1.2.1.4 Histoplasmosis

Histoplasma capsulatum is a dimorphic fungus growing intercellular as a yeast in its host (Cuncha *et al.,* 2007:250). This organism is found in caves, old buildings, school yards, parks and farms (Durden & Elewski, 1997:202). In cases of advanced immunosuppresion (CD4 cell count < 150 cells/µl) cutaneous histoplasmosis is commonly found (Johnson, 2000: 420).

A patient will present with fever, anaemia, respiratory symptoms, lymphadenopathy and skin lesions. This is often the reason why histoplasmosis is misdiagnosed as tuberculosis. Skin lesions occur in 5 – 10% of all cases and mucosal involvement appears in the form of gingival

ulcers, plaques, nodules and abscesses (Dlova & Mosam, 2004:15). Chronic mucosal ulcerations are common (figure 1.4), specifically on the tongue, palate, rectum and buccal mucosa (Durden & Elewski, 1997:203).

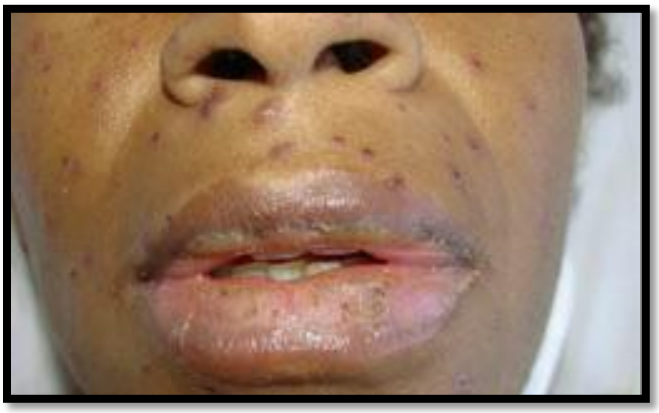


Figure 1.4: *Histoplasmosis on the facial area (Cunha et al., 2007:252).*

1.2.1.5 Sporotrichosis

Sporothrix schenckii is a ubiquitous fungus which is a mold at room temperature (± 25 ºC) and a yeast at body temperature (37 ºC). This organism, unlike other dimorphic fungi, may be present in healthy immunocompetent patients. Dissemination occurs when the fungus is inhaled (Durden & Elewski, 1997:203). The infection caused by *sporothrix schenckii* can be classified as lymphocutaneous, established cutaneous, contagious cutaneous or even systemic. Lesions on the skin (figure 1.5), sparing the palms, soles and oral mucosa, may consist of extensive ulcers, papules, nodules and plaques (Johnson, 2000: 419; Dlova & Mosam, 2004:15)

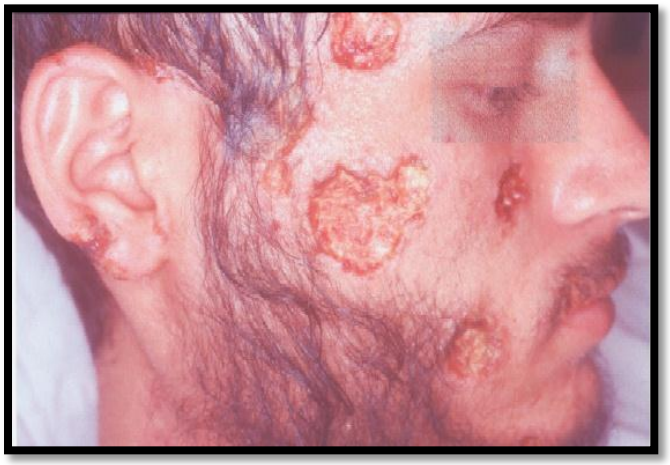


Figure 1.5: *Disseminated sporotrichosis in HIV/AIDS patients (De caso, 2002:657).*

1.2.2 Bacterial infections

Most common bacterial infections seen in HIV/AIDS patients are caused by *Staphylococcus aureus* and *Staphylococcus epidermidis* (Stulberg *et al*., 2002:119). Although it can be linked to HIV/AIDS, it is not exclusive and also occurs in the general public. If associated with HIV/AIDS infected individuals, the infections may be more prevalent, recurrent and resistant to therapy. The symptoms present can be folliculitis, impetigo, ecthyma and cellulitis (Dlova & Mosam, 2004:15).

1.2.2.1 Folliculitis

Folliculitis is a bacterial infection that may manifest as abrasive skin with papules and pustules (Dlova & Mosam, 2004:15). Hair follicles that become inflamed by injury, irritation or infection, can lead to folliculitis (Stulberg *et al*., 2002:122). The symptoms are described as multiple discrete, erythematous, crusted, follicular papules (figure 1.6), found on the head, neck and trunk (Ramdial, 2000:119). Lesions usually resolve spontaneously without any treatment. In immune compromised patients organisms such as yeast and fungi also appear as lesions (Stulberg *et al*., 2002:122).

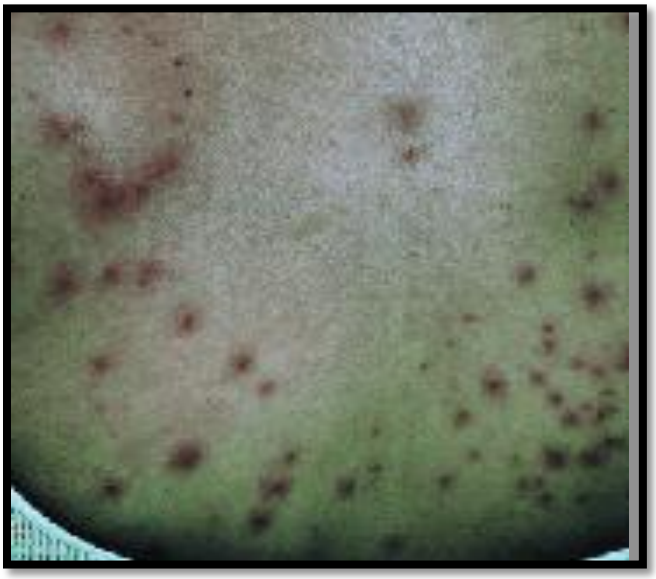


Figure 1.6: *Folliculitis caused by contaminated water (Stulberg et al., 2002:123).*

1.2.2.2 Impetigo

Impetigo is an infection seen most commonly on the perioral or perinasal area and starts off as vesicles and pustules (figure 1.7). Progression will cause a rupture, leaving an oozing honey-

coloured crust. In HIV/AIDS the lesions occur in intertriginous areas of the body (Stulberg *et al*., 2002:119; Dlova & Mosam, 2004:15). The spread of impetigo is caused by person-to-person contact and good hygiene is seen as important in management. Prevalence is higher in children aged between 2 to 5 years (Stulberg *et al*., 2002:121; Cole & Gazewood, 2007:859).

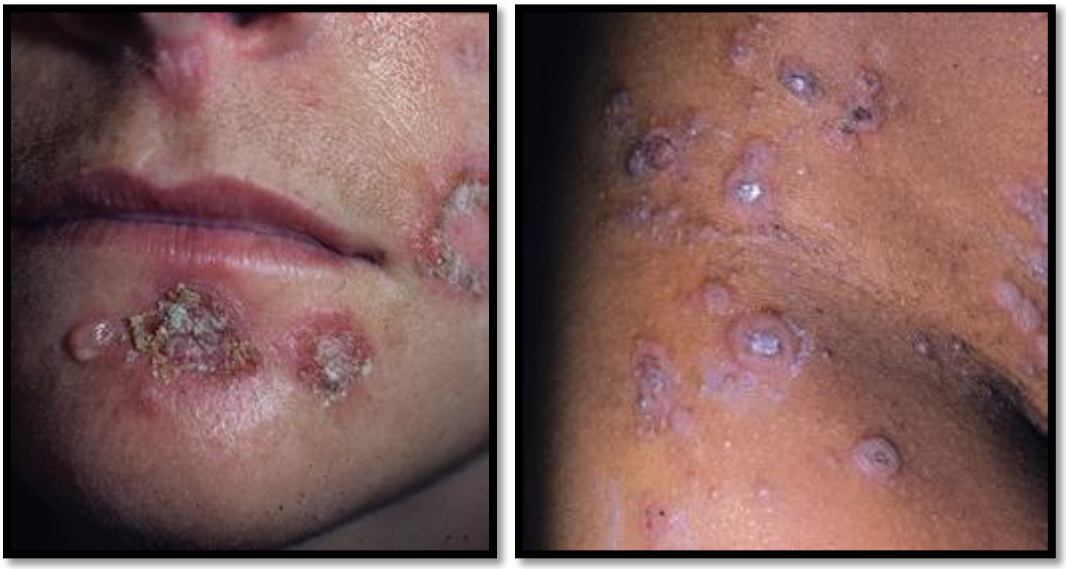


Figure 1.7: *Impetigo on the face and in the groin (Cole & Gazewood, 2007:860).*

1.2.3 Viral infections

As a result from damage caused by HIV/AIDS to the immune system, opportunistic viral infections increase among these patients. Viral infections can present as localised, extensive, restricted to the skin, or as a systemic infection. Lesions may present as mild disfiguring (*molluscum contagiosum*) or it may be life-threatening, for example the human papillomavirus (Dlova & Mosam, 2005:12).

1.2.3.1 Herpes simplex 1 and 2

The *herpes simplex* virus (HSV) presents in extensive clinical forms – necrotic, recurrent, persistent and eventually destructive (Trope & Lenzi, 2005:573). Chronic herpetic infection occurs in patients with chronic immunosuppression. Infection with HSV -1 and -2 is used as a marker in HIV/AIDS, especially in young adults. A rapid increase in *herpes* infection is noted in individuals with a CD4 cell count below 50 cells/µl. Most wet-crusted lesions present on HIV/AIDS patients can almost always be assumed to be herpetic. Mucocutaneous *herpes simplex* infection that presents for a time period of one month or more, may serve as an AIDS-

defining illness (figure 1.8). Due to an increase in immunosuprresion associated with the progress of HIV/AIDS, the changes of HSV infection are enhanced and become more persistent. Painful ulcers develop on most typical sites of infection including perioral, anogenital and digital (fingers and toes). Depending on the site of the lesion, the clinical manifestation may vary (Ramdial, 2000:113; Rigopoulos *et al*., 2004:489; Dlova & Mosam, 2005:12).

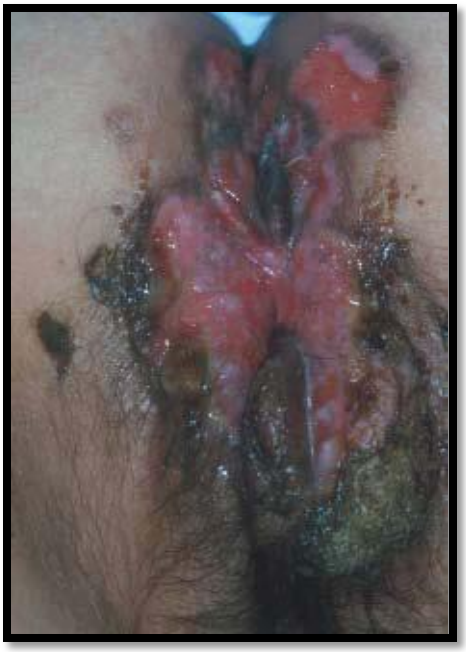


Figure 1.8: *Genital herpes in an HIV patient associated with intense pain (Martin et al., 2009:28).*

1.2.3.2 Varicella zoster

The *varicella-zoster* virus (VZV) is largely dependent on the age of a patient. In children, the infection is often severe where in adults, frequency of reactivation of latent VZV is greatly increased (Tschachler *et al*., 1996:660). The *zoster* virus (singles) is seen to be more frequent, rigorous and have a longer duration in HIV/AIDS patients than in immune uncompromised individuals (Rigopoulos *et al*., 2004:489). Approximately 8 - 13% of HIV/AIDS individuals experience at least one episode of VZV during their time of illness. Lesions may range from a vesicular eruption in a dermatomal pattern, to rigorous haemorrhagic and necrotic lacerations, followed by dissemination (figure 1.9). Chronic VZV infections may persist for several months (Tschachler *et al.,* 1996:660).

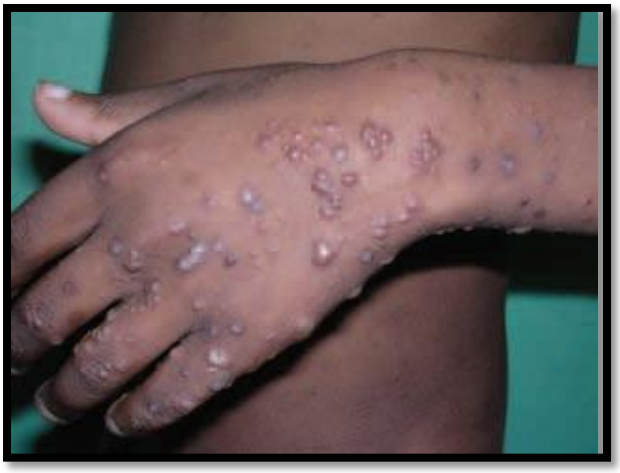


Figure 1.9: *Varicella Zoster (Dlova & Mosam, 2005:13).*

1.2.3.3 Cytomegalovirus

Cytomegalovirus (CMV) develops in 90% of HIV/AIDS patients as an acute infection. Skin involvement is rarely noted in CMV infection. Clinical signs such as maculopapular eruptions, ulcers, nodules, vesicles and hyperpigmented plaques (figure 1.10) are present in this infection (Ramdial, 2000:114). In most cases CMV is unrecognised or may cause acute mononucleosis syndrome. The virus remains in the body of the host after primary infection, causing life-long latency. Severe complications occur in immunouncompromised patients, i.e., solid organ or bone marrow transplant recipients with CMV. Clinical manifestations of CMV depend largely on the CD4 cell count, as end-organ disease is rarely observed in a count higher than 50 cells/mm^3. Opportunistic infections are also present in severe immunodeficiency (Meyer-Olson *et al*., 2010:414-415).

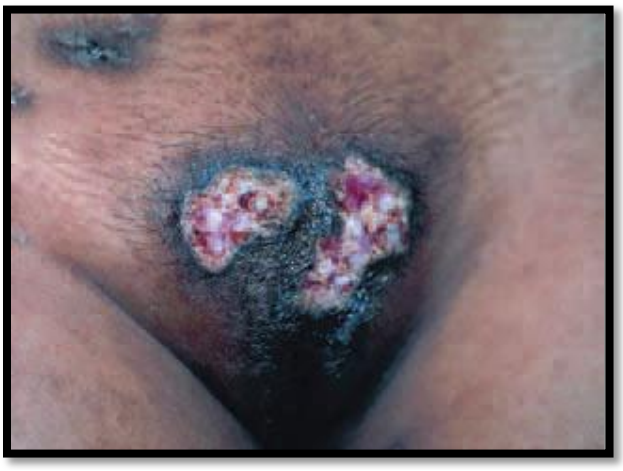


Figure 1.10: *Cytomegalovirus on the genital area (Dlova & Mosam, 2005:15).*

1.2.3.4 Molluscum contagiosum

M*olluscum contagiosum* (MC), caused by the poxvirus, is seen in 20% of HIV/AIDS patients as it is associated with immunodeficiency (Tschachler *et al*., 1996:661). Frequency of poxvirosis (double stranded deoxyribonucleic acid, DNA, virus of Poxviridae family) is more frequently noted in the pediatric population and can be linked to sexually transmitted diseases in adults (Trope & Lenzi, 2005:572; Stulberg, 2003:1233). Skin lesions occur in the face and genital regions as skin coloured papules with one or more central hyperkeratotic pore (figure 1.11). Individual lesions can grow up to a size of 1 cm in diameter and cause disfigurement if located on the face (Tschachler *et al.,* 1996:661). In advanced immune compromised patients lesions may be observed as “a plaque” or in a “beard” form (Trope & Lenzi, 2005:573). On the other hand, in immunocompetent individuals, MC presents on the trunk and anogenital area where it undergoes spontaneous resolution within a year (Ramdial, 2000:114).

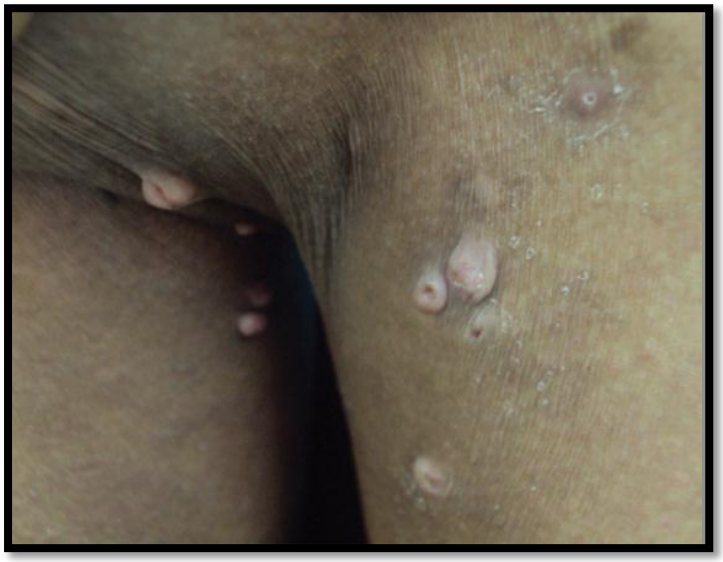


Figure 1.11: *Giant molluscum contagiosa (Vusadevan et al., 2012:23).*

1.2.3.5 Human papillomavirus

The human papillomavirus (HPV) is the most often occurring viral infection of the skin during HIV/AIDS. This comes with an increased incidence of ordinary and genital warts, and squamous carcinoma on the cervix. Different clinical lesions are seen with different HPV types (Ramdial, 2000:115). Skin lesions vary from verruca vulgaris, which can become enlarged and confluent, to patterns of extensive verruca plana and versicolour-like warts (figure 1.12). These

lesions are often unresponsive to treatment and require surgical excision (Dlova & Mosam, 2005:16). HPV infections are subclinical and difficult to detect. The highest prevalence is found in sexually active women above the age of 25 years (Koutsky, 1997:3).

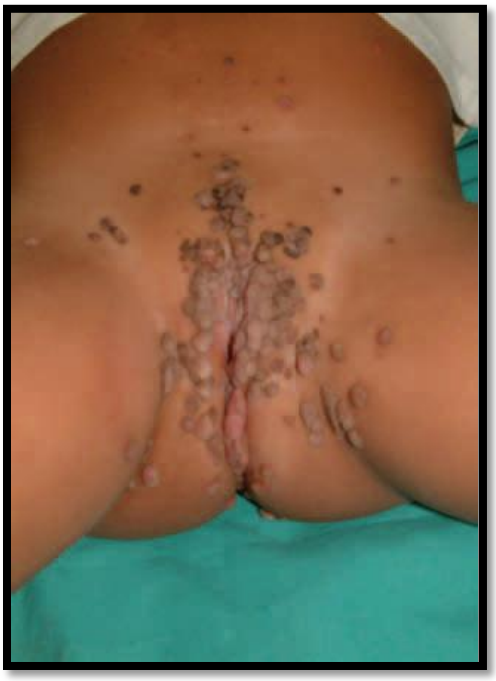


Figure 1.12: *Condyloma acuminatum caused by HPV (Dlova & Mosam, 2005:16).*

1.3 CUTANEOUS MANIFESTATIONS AS MARKERS IN THE DISEASE PROGRESSION OF HIV

Dermatological manifestations are often the first and only sign of HIV/AIDS, and should indicate treatment after proper diagnosis. Benefits from early treatment are decrease in disease progression and less transmission. In developing countries skin lesions can be used as a marker in HIV/AIDS progression (Vusadevan *et al.,* 2012:20). One of the most striking and consistent immunological characteristics of HIV/AIDS is the reduction of CD4 cells. Thus, the CD4 cell count is important in the clinical evaluation of HIV infections to determine the stage of disease and treatment. In HIV, the CD4 cell count serves as a marker to assess progression to AIDS as seen in table 1.1 (Sharma *et al*., 2004:239). Guidelines suggest that HAART therapy should be initiated with CD4 cell counts of between 200 and 350 cells/µl (Rigopoulos *et al*., 2004:493).

Table 1.1: *Relation between HIV infection and CD4 counts (Vusadevan et al., 2012:21).*

Type of illness	Cutaneous manifestation	Average CD4 count
Fungal	Onychomycosis	291
	Tinea corporis	296
	Oral candidiasis	233
	Genital candidiasis	226
Viral	*Herpes zoster*	208
	Molluscum contagiosum	211
	Herpes labialis	218

In early immunosuppression (CD4 count ≤ 450 cell/µL) fungal infections are frequently seen. The decreasing incidence of oral candidiasis can be due to more awareness about the condition and early treatment. Bacterial infections are reducing due to antibiotic treatment from early onset of the HIV/AIDS disease and occur in CD4 count > 200 cells/µL (Vusadevan *et al.,* 2012:21).

1.4 TREATMENT OF SKIN LESIONS ASSOCIATED WITH HIV/AIDS

1.4.1 Antiviral treatment

Viruses are parasites consisting of either double- or single-stranded DNA or ribonucleic acid (RNA). Some of these DNA viruses include *herpes* viruses (oral and genital *herpes*) and papillomaviruses (warts). Different antiviral agents are developed depending on their mechanism of action against viruses, including uncoating, transcription and cell entry. Acyclovir inhibits viral DNA synthesis in a process requiring viral thymide in kinase and is well-tolerated topically (Beers, 2006:1061; Hayden, 2006:1243). Anti-viral activity from acyclovir is seen against HSV-1, HSV-2 and VZV. However, acyclovir exhibits minimal activity against CMV (King, 1988:176; Beers, 2006:1061). There are several compounds that have activity against viruses for example the herpesvirus, including acyclovir, famciclovir, ganciclovir, trifluridine,

valaciclovir and valganciclovir (Beers, 2006:1061). Table 1.2 lists commonly used antiviral agents in South Africa.

Table 1.2: *Nomenclature of commonly used antiviral agents as adapted from Hayden, (2006:1246).*

Generic name	Acronyms or other names	Trade names (SA)	Dosage forms*
Acyclovir	ACV, acycloguanosine	Zovirax®, Acitop®, Lovire®, Acitab®, Cyclivex®, Activir®	IV, O, T, ophth
Famciclovir	FCV	Famvir®	O
Ganciclovir	GCV, DHPG	Cymevene®	IV
Trifluridine	TFT, trifluorothymidine	TFT Ophtiole	Ophth
Valacyclovir		Zelitrex®, Zelivire®	O
Valganciclovir		Valcyte®	O
Imiquimod		Aldara®	T

*IV- Intravenous

O-Oral

T- Topical

Ophth- Ophthalmological

1.4.2 Antifungal treatment

Antifungal agents are used for systemic or topical treatment. Azole antifungal agents dominate the antifungal compound use and development. Two broad classes of azoles with the same spectrum and working mechanism exist, namely imidazoles and triazoles. The imidazole agents are used either systemically or orally. Azoles have shown activity against *Candida spp*, *Cryptococcus neoformans* and *Histoplasma capsulatum*. *Sporothrix schenckii*, however, is less susceptible to azole therapy. Amphoterin B possesses activity against the same species as azoles and is often used in many mycoses treatments (Bennet, 2006:1225). In HIV, dermatophytic diseases are best treated with oral antifungal agents such as terbinafine, itraconazole or fluconazole as seen in table 1.3. Topical preparations may be helpful, especially in cases where there is a low response to oral antifungal agents, but does not serve as a cure (Johnson, 2000:413).

Table 1.3: *Treatment of fungal skin manifestations as adapted from Bennet (2006:1226).*

Mycoses	Compounds
Cryptococcosis	*Intravenous*
Non-AIDS and initial AIDS	Amphotericin B
Maintenance AIDS	*Oral*
	Fluconazole
Histoplasmosis	*Intravenous*
Rapidly progressing	Amphotericin B
Maintenance AIDS	*Oral*
	Itraconazole
Sporotrichosis	*Oral*
Cutaneous	Itraconazole
Extracutaneous	*Intravenous*
	Amphotericin B
Candidiasis	*Intravenous*
Deeply invasive or esophageal	Amphotericin B, Caspofungin
Vulvovaginal	*Topical*
	Clotrimazole, Miconazole, Nystatin, Tioconazole.
	Oral
	Fluconazole
	Topical
Cutaneous	Amphotericin B, Clotrimazole, Econazole, Ketoconazole, Miconazole, Nystatin
Ringworm	*Topical*
	Clotrimazole, Econazole, Ketoconazole, Miconazole, Terbinafine and Tolnaftate
	Systemic
	Griseofulvin, Itraconazole, Terbinafine.

Most bacterial skin infections seen in HIV/AIDS are caused by *streptococcus spp*. and treated with antibiotics, taking resistance into consideration. Agents used in bacterial skin infections include penicillinase-resistant penicillins, first generation cephalosporins, azithromycin, amoxicillin, clarithromycin or fluoroquinolone (Stulberg *et al*., 2002:123). Bacterial infections

such as folliculitis are treated with clindamycin 1% lotion or gel, alternatively benzoyl peroxide 5% is used to wash with. Mupirocin is the treatment of choice in impetigo therapy (Beers, 2006:983; Fox *et al*., 2006:1690). Antiseptic products also present bactericidal activity and are used in preventing secondary bacterial infections (Long, 2002:56).

1.5 SPECIFIC AGENTS

1.5.1 Acyclovir

The discovery of acyclovir in 1974 came as a result from a screening program for antiviral compounds in Burroughs, Wellcome, and the first topical dosage form was available to physicians in 1982 (King, 1988:176; Hayden, 2006:1246). Acyclovir has remarkable physical and chemical properties, as well as pharmacokinetic and chemotherapeutic characteristics (King, 1988:176).

1.5.1.1 Pharmacology and classification

Acyclovir is a synthetic purine nucleoside analogue and structurally related to guanine. Its main uses are for the treatment and prophylaxis of viral infections due to the herpesviruses. These infections include *herpes keratitis, herpes labialis* and genital *herpes*. Treatment may be conducted by means of intravenous, oral or topical route and should commence as soon as the appearance of any symptoms are noted (Sweetman, 2011).

1.5.1.2 Absorption, metabolism and excretion

Poor bioavailability (10 - 20%) is seen for oral acyclovir dosage due to poor absorption from the gastrointestinal tract (King, 1988:176; Sweetman, 2011). Prodrugs such as valaciclovir have been developed in order to overcome the reduced absorption (Sweetman, 2011).

Acyclovir is excreted in the urine in an unchanged form by glomular filtration in the kidneys. The half-life of acyclovir in a patient with normal renal function is 2 – 3 hrs (King, 1988:178; Sweetman 2011). Chronic renal failure may increase this value up to 19.5 hrs. Only 2% of the dose is excreted in faeces. Acyclovir can cross the placenta and is distributed into breast milk (Sweetman, 2011).

Absorption of acyclovir is low after topical delivery onto intact skin, though formulation changes can have a marked effect on absorption. Higher concentrations, on the other hand, are absorbed from ophthalmic ointments (Sweetman, 2011).

1.5.1.3 Adverse reactions

Overall, acyclovir is well-tolerated, with reactions of nausea, vomiting, headache and diarrhea experienced when treatment commenced for 6 months or longer. These effects are also noted in placebo therapy. Inflammation and phlebitis are most commonly noted at injection sites after intravenous acyclovir treatment. However, topical acyclovir preparations show little adverse effects on local or systemic levels, and overdose (toxic effect) is highly unlikely (Arndt, 1988:188).

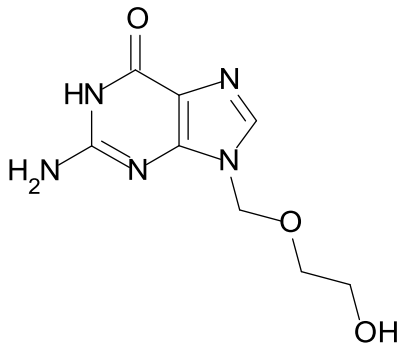
1.5.1.4 Uses

Topical acyclovir is used in a 5% cream for recurrent *herpes* labialis treatment, including perioral *herpes*, cold sores, and fever blisters. Acyclovir ointment may be used for the management of initial genital *herpes*; however, it is not recommended for the treatment of genital *herpes* since it offers limited benefit (Bethesda, 2010).

1.5.1.5 Physicochemical properties of acyclovir

The physicochemical properties of acyclovir are summarised in table 1.4.

Table 1.4: *Physicochemical properties of acyclovir (Drug bank, 2012a).*

Chemical structure 	
Chemical Formula	$C_8H_{11}N_5O_3$
IUPAC Name	2-amino-9-[(2-hydroxyethoxy)methyl]-6,9-dihydro-3H-purin-6-one
Description	A guanosine analog that acts as an antimetabolite. Viruses are especially susceptible. Used especially against *herpes*.
Appearance	An almost white crystalline powder
State	Solid
Molecular weight	Average: 225.2046 g/mol
Melting point	256.5 - 257 ºC
Solubility	Slightly soluble in water; very slightly soluble in alcohol; freely soluble in dimethyl sulfoxide; soluble in dilute solutions of alkali hydroxides and mineral acids
pKa	2.27 and 9.25
Log P	-1.56

1.5.2 Ketoconazole

Ketoconazole was the first compound available for the oral treatment of systemically based fungal infections and was released to the public in 1980. Until the year 1940 there were relatively few agents available for systemic treatment of fungal infections. The treatment of

choice for non-life-threatening mycoses has been ketoconazole for almost a decade (Maertens 2004:1).

1.5.2.1 Pharmacology and classification

Ketoconazole is a synthetic imidazole derivate (Bethesda, 2010). The major effect caused by azoles is the interference of ergosterol synthesis by the inhibition of 14-α-sterol demethylase, a microsomal cytochrome P450 enzyme. This alters the permeability of the cell membrane of specific fungi and leads to the accumulation of 14-α-methyl sterols (Bennet, 2006:1230; Sheppard & Lampiris, 2007:784; Sweetman, 2011). Methyl sterols may lead to disruption of phospholipid chains, impairing membrane functions and thus cause inhibition of fungi growth (Bennet, 2006:1230).

1.5.2.2 Absorption, metabolism and excretion

Absorption of ketoconazole from the gastrointestinal tract is erratic and an increase in absorption can be seen with a decrease in stomach pH. Systemic absorption is sub-therapeutic in healthy patients after topical or vaginal application of ketoconazole. More than 90% of ketoconazole is mainly bound to albumin and is widely distributed. Traces of ketoconazole can be seen in breast milk, but poorly diffuses into cerebrospinal fluid. Metabolism of ketoconazole into inactive metabolites takes place in the liver and excretion of unchanged compound is found in the faeces or to a smaller extend in the urine (Sweetman, 2011).

1.5.2.3 Adverse reactions

After oral use, gastrointestinal disturbances are often reported (Sheppard & Lampiris, 2007:785; Sweetman, 2011). Side-effects, for example nausea and vomiting, are mostly dose -related and can be lowered by administering food with ketoconazole treatment. Interference with steroid biosynthesis and endocrine effects are associated with ketoconazole treatment, especially in high doses (Sweetman, 2011). This can lead to gynaecomastia, oligospermia, menstrual irregularities, infertility and suppression of the adrenal cortex (Trevor *et al*., 2005:406; Sweetman, 2011). Other adverse effects reported are urticaria, angioedema, anaphylaxis after first dose, pruritus, rash, paraesthesia, raised intracranial pressure, photophobia and photosensitivity (Sweetman, 2011). With the inhibition of the hepatic cytochrome P450 enzyme caused by ketoconazole, the plasma levels of other compounds such as phenytoin, cyclosporin and warfarin can increase (Trevor *et al*., 2005:406).

With topical use of ketoconazole, effects such as irritation, dermatitis or burning sensation may be noted (Sweetman, 2011).

1.5.2.4 Uses

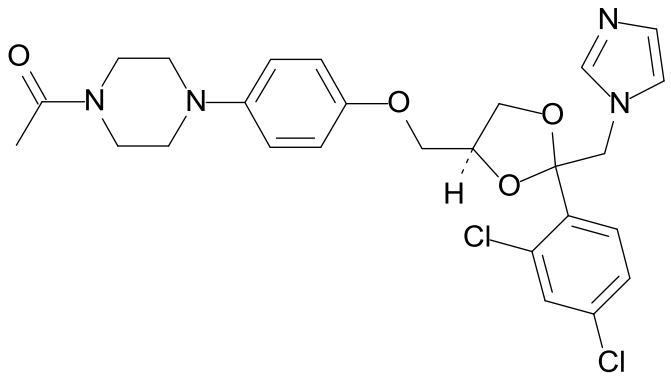
Ketoconazole is administerd orally to treat chronic mucocutaneous candidiasis; fungal infections found in the gastrointestinal tract; dermatophyte infections found in both skin and nails; and other systemic infections, including histoplasmosis (Trevor *et al*., 2005:406; Sweetman, 2011). In immune compromised patients, ketoconazole can be given as prophylaxis for fungal infections. Due to hepatic risks associated with ketoconazole treatment, it is advised that oral use is limited to the treatment of serious infections presenting with resistance to other treatment (Sweetman, 2011).

Ketoconazole can be topically applied in a 2% cream for the treatment of candidiasis or dermatophyte infections on the skin. Treatment consists of a twice daily application for a few days before symptoms will improve. Other topical preparations containing ketoconazole include a foam and a shampoo (Sweetman, 2011).

1.5.2.5 Physicochemical properties of ketoconazole

The physicochemical properties of ketoconazole are summarised in table 1.5

Table 1.5: *Physicochemical properties of ketoconazole (Drug bank, 2012b).*

Chemical structure 	
Chemical Formula	$C_{26}H_{28}Cl_2N_4O_4$
IUPAC Name	1-[4-(4-{[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy}phenyl)piperazin-1-yl]ethan-1-one
Description	Broad spectrum antifungal agent used for long periods at high doses, especially in immunosuppressed patients
Appearance	Almost white powder
State	Solid
Molecular weight	Average: 531.431 g/mol
Melting point	146 ºC
Solubility	Practically insoluble in water; sparingly soluble in alcohol; freely soluble in dichloromethane; soluble in methyl alcohol
pKa	2.94 and 6.51
Log P	4.4
Degradation	Light sensitive and degradation takes place within 24 hrs.

1.5.3 Synergy caused by combination of acyclovir and ketoconazole

Ketoconazole seems to show a synergistic anti-viral activity when used in combination with acyclovir (Bickers 1994:89). A study was done in 1986 to investigate the possibility of antiviral activity for ketoconazole. The effects of ketoconazole alone, and also in combination with acyclovir were examined with HSV-1 and -2 infected human lung (HL) cells with a yield reduction assay. Table 1.6 shows that treatment with ketoconazole presented a dose dependent reduction in viral titer (Pottage *et al*., 1986:215).

Table 1.6: *Yield reduction caused by ketoconazole and acyclovir in HL cells (Pottage et al., 1986:216).*

Compound	Concentration (µg/ml)	HSV-1 (F-strain)		HSV-2 (G-strain)	
		Titer	% of control	Titer	% of control
Control	0	4.1×10^6	100	1.5×10^6	100
Ketoconazole	1	3.6×10^6	87	1.1×10^6	79
	5	1.3×10^6	33	4.2×10^5	28
	10	2.4×10^6	5	7.5×10^4	5
Acyclovir	1	1.4×10^6	33	6.4×10^5	43
	5	1.0×10^5	2.5	3.0×10^4	2.3

All of the concentrations of ketoconazole and acyclovir combinations resulted in synergy against both HSV-1 and -2 (table 1.7). Results were more prevalent as the concentrations increased for both ketoconazole and acyclovir. Pottage *et al.,* (1986:216) stated that the mechanism of action against viruses for ketoconazole was unknown, but through their experiments they concluded that it did not have a direct effect on virus particles.

Table 1.7: *Yield reduction assay of ketoconazole/acyclovir in combination in HL cells (Pottage et al., 1986:217).*

Compound	Concentration (µg/ml)	HSV-1 (F-strain)	HSV-2 (G-strain)
		Titer	Titer
Ketoconazole/acyclovir	5/1	$3.9x10^5$	1.4×10^5
	10/1	$4.6x\ 10^4$	7.5×10^6
	5/5	$1.2x\ 10^4$	1.5×10^3
	10/5	1.3×10^3	4.3×10^2

In vitro inhibitory activity against microorganisms such as *Staphylococcus aureus* has also been found for ketoconazole. The exact mechanism is not known; however, it could be related to alterations in organism lipid metabolism (Pottage *et al*., 1986:217). Thus, topical treatment of localised skin lesions found in HIV/AIDS could possibly benefit from treatment with ketoconazole, especially when used in combination with acyclovir. This may present a synergistic mechanism of action against a broad spectrum of microbes on the skin during HIV/AIDS infection. The possibility of treating these lesions with a topical product is a motivation for this study, as studies have only been done on these compounds in a solution form. Many of the dermatological manifestations present on mucosal membranes and can serve as another motivation to formulate products that will be safe to use for mucosal delivery.

1.6 TOPICAL DELIVERY

Application of substances to the skin can be dated back to when ancient Greeks applied mixtures of water, oil and lead oxide as a balm to their skin. Until the 20^{th} century, skin preparations were used only for skin diseases. Nitroglycerin ointment was introduced for the management of angina in 1954, and was the first commercial topical preparation intended for systemic use (Morrow *et al*., 2007:36). Transdermal delivery is a term that is used to describe a situation where a compound is diffused through the different layers of the skin into the systemic circulation for a therapeutic effect. Dermal or topical delivery is used to target specific pathological sites on the skin, by penetrating the layers, and requires minimal systemic effect. Compounds are delivered to the skin by conventional preparations, i.e. ointments, creams and

gels (Dayan, 2005:67; Brown, 2006:175). Very few compounds deliver transdermally at levels that are clinically effective due to low permeability of the skin. However, topical preparations are often used (Prausnitz *et al*., 1993:10504).

1.6.1 Structure of the human skin

Human skin is a very complex tissue, which consists of several distinct layers that differ in morphology, as well as molecular composition or structure (Caspers *et al*., 1998:31). Traditionally, the skin is divided into three main layers: dermis, epidermis and hypodermis (Ho 2004:49). It forms a barrier against the environment and is the only organ in contact with the outside world. Furthermore, the skin also has aesthetic appeal and beauty (Majeed & Prakash, 2005:160). The many functions of the skin include homeostasis, protection, thermoregulation, sensation and metabolism. The skin contains active metabolising cells that facilitate transport to underlying tissues and organs in the rest of the body and excretes metabolic waste at the same time through perspiration. Several highly organized and heterogeneous layers are found in the skin and include sweat glands, sebaceous glands and hair follicles (Poet & Mcdougal, 2002:22). The skin surface contains on average 40 - 70 hair follicles and 200 - 250 sweat ducts in every square centimeter. The highest density is found on the head and shoulder area, and least on palms and plantar surfaces (Wang *et al.,* 2005:179).

The skin structures vary over different sites of the body and the stratum corneum (SC), which provides the barrier function of the skin, will be thicker in some areas (table 1.8) due to load-bearing. The site that is most permeable for transdermal compound delivery is the genital area and has the greatest prospects to deliver clinical levels of compounds administered. Other very permeable sites include the head and neck. The sites that are harder to permeate are the arms and legs. The trunk of the body has intermediate permeability (Williams 2003:16).

The SC found on the epidermis layer of the skin is approximately 10 - 20 µm thick (depending on state of hydration). This is the protective layer preventing water loss from the skin and limits the influx of chemicals from the environment. The SC is comprised of approximately 20% water and is a highly lipophilic membrane (Wang *et al.,* 2005:179). When observing the structure of the SC it can be compared to that of “bricks and mortar”; 10 - 15 layers of flattened cornified cells representing the bricks and the lipid-rich intercellular matrix represents the mortar. For a compound to pass through the skin, it has to traverse this barrier (Berti & Lipinsky 1995:581).

Table 1.8: *Comparison of the quantity of cell layers of the SC at different anatomical sites. As adapted from Ya-Xian et al., (1999:557).*

Location	Number of cell layers (mean ±SD)
Forehead	9 ± 2
Lip	10
Neck	10 ± 2
Abdomen	14 ±4
Genitals	6 ± 2
Upper arm	13 ± 4
Leg	18 ± 5

1.6.2 Physicochemical factors

Important physicochemical parameters in transdermal delivery are partitioning, diffusion and solubility. The optimum partitioning properties for compounds include a log [octanol-water partitioning coefficient] (log P) value of between 1 and 3. Diffusion is relative to molecular size, as small molecules move faster than larger ones. Compounds that penetrate the skin slowly are compounds with functional groups that can form hydrogen bonds. The solubility of a compound is related to its melting point and generally, compounds with a low melting point permeate the skin well (Hadgraft & Guy 2004:185). Important physicochemical properties needed for optimal transdermal permeation are listed in table 1.9.

Table 1.9: *Ideal limits for passive transdermal delivery (Naik et al., 2000:319).*

Aqueous solubility	>1 mg ml^{-1}
Lipophilicity	$10 < K_{o/w} < 1000$
Molecular weight	<500 Da
Melting point	<200ºC
pH of saturated aqueous solutions	pH 5-9
Dose deliverable	<10 mg day^{-1}

1.7 ADVANCES IN TRANSMUCOSAL COMPOUND DELIVERY

Oral administration of compounds can be challenging due to difficulty in keeping the medicament at the desired location for effective absorption, distribution and metabolism. Therapeutic effects can only be achieved when effective exposure time is reached. Due to excellent permeability and rich blood supply, the sublingual route is often used for compounds requiring a rapid onset of action. This stimulates considerable interest in using the mucosal lining of cavities for compound delivery. The cavities include nasal, rectal, vaginal, ocular and oral cavities (Madhav *et al.,* 2009:3; Patel *et al.,* 2011:107; Shakya *et al.,* 2011:3).

Compounds can be delivered across epithelium membranes by passive diffusion, transport-mediated active transport or specialised diffusion mechanisms. Studies have shown that passive diffusion is mostly seen in buccal absorption via either paracellular or transcellular pathways (figure 1.13). The paracellular spaces provide a hydrophilic barrier to lipophilic compounds, but is favourable to hydrophilic compounds. However, in transcellular diffusion, the compound will penetrate one cell and the next until the systemic circulation is reached. Compounds can transverse via both pathways simultaneously, with a predominant pathway depending on the physicochemical properties of the compound (Patel *et al*., 2011:107).

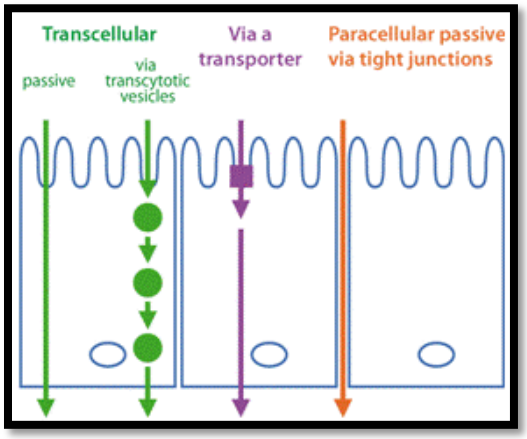


Figure 1.13: *Presentation of administration routes in transmucosal delivery (*Patel *et al*., 2011:107*).*

The oral mucosa includes buccal, sublingual, gingival, palatal and labial mucosa. Oral mucosa varies at different sites as does the composition of the epithelium. Transmucosal delivery is considered for either local effect or systemic treatment. (Patel *et al.,* 2011:107).

The vaginal cavity consists of a dense network of blood supply, which should be ideal for local or systemic effect (Hussain & Ahsan 2004:302). However, vaginal treatment is almost limited to localised infections, exclusively, due to low absorption found in this cavity. One of the considerations for topical vaginal use is to determine if the compound applied will remain on the area for a long enough period to be effective (Ranade & Hollinger, 2004:318). The release of a compound applied from a vaginal or mucosal formulation can be assessed by means of diffusion studies (Vermani & Garg, 2000:359; Madhav Satheesh & Yadav, 2011:1061).

1.7.1 Differences between mucosa and skin

Mucosa is characterised as non-keratinised epithelium covering different sites on the body, depending on the organ system involved (Elsner, 2011:1). The principle barrier function of the skin is removed by the absence of keratinised SC. Non-keratinised mucosal tissue is therefore more permeable compared to normal skin. A structure with less resistance to paracellular movement is created due to larger and more loosely packed cells in the mucosa. Tissue of the mucosa consists of a less structured lipid barrier which creates a lower resistance to molecular diffusion. Some parts of the vulva even show thinner epithelial tissues (figure 1.14) which results in faster diffusion of a component due to a shorter path length (Farage & Scheffler, 2011:117).

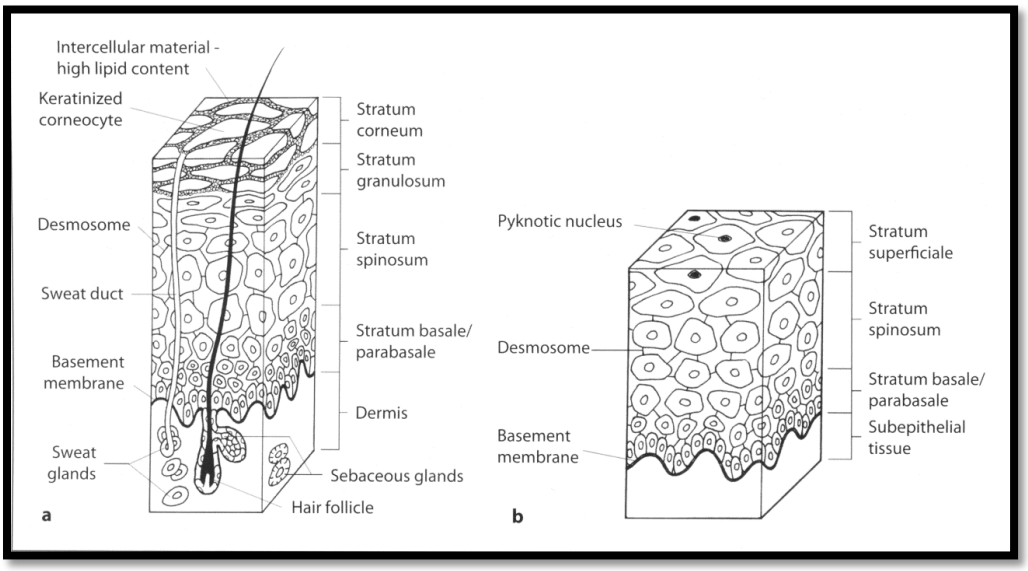


Figure 1.14: *Structural difference of (a) vulvar skin and (b) mucosa (Farage & Maibach 2011:12).*

1.7.2 The structure and properties of oral mucosa

The oral cavity is viewed as the lips, cheek, tongue, palate and floor of mouth. Epithelium in the oral cavity serves to protect against harmful effects and fluid loss. Beneath the epithelium is a basement membrane, lamina propia and submucosa in the innermost layer (figure 1.15). This epithelium is relatively permeable to water. Oral mucosa is seen to be 4 - 4000 times more permeable compared to other parts of skin. Parameters that influence permeability of compounds in the oral cavity include pH, fluid volume and enzyme activity (Patel *et al*., 2011:107).

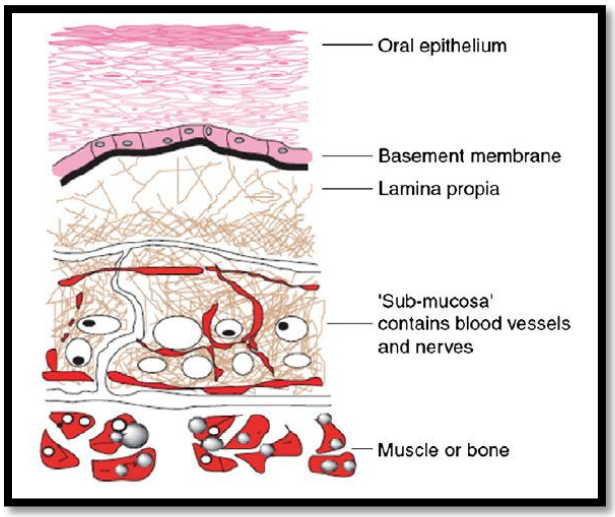


Figure 1.15: *Histological diagram of buccal mucosa (Patel et al., 2011:107).*

Three types of oral mucosa are found in this cavity: (i) lining mucosa, (ii) specialised mucosa and (iii) masticatory mucosa (figure 1.16). The lining mucosa comprises 60% of the total surface area of the oral mucosa and is seen as the buccal mucosa (lips and cheeks). This mucosa is a non-keratinised epithelium consisting of a thin lamina propia and submucosa (Patel *et al*., 2011:107).

Oral epithelium has a thickness ranging from 40 - 300 µm and is surrounded by substance called mucus acting as protective barrier over membranes. Most mucus is comprised of water containing mucin (1 – 5%), which is a glycoprotein. Saliva, consisting of water, mucus and enzymes, is secreted by major buccal glands to a volume of 0.5 - 2.0 ℓ daily. The functions of saliva are to keep the cavity moist, aid swallowing and digestion of food and to minimize acidic changes in the mouth. This secretion in the oral cavity is a weak buffer with a pH of 5.5 – 7.0 (Patel *et al*., 2011:107).

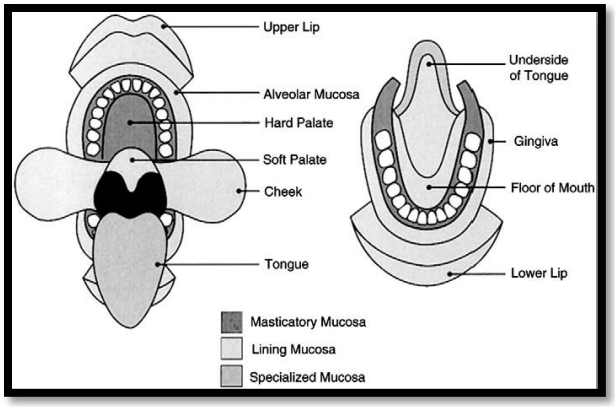


Figure 1.16: *Representation of mucosal differences in the mouth (Patel et al., 2011:107).*

1.7.3 The structure and properties of the lips

The vermilion border found on the lips is an outstanding structure due to its features differing from surrounding facial skin. It is at the vermilion border that the lip skin ends and where no hair follicles or sweat glands can be found. Gradually, the vermilion border transforms to a true mucosal membrane. Mucosa is seen to be five times more permeable to water than normal skin tissue. The red colour change is as a result of a decrease in keratin density, as well as constant hydration. The location of the lips causes constant exposure to air, water, cosmetics, dental materials, food, sunlight and other environmental factors (Kobayashi & Tagami, 2004:563). Lip anatomy is divided into three zones namely, (i) anterior, covered by a thin layer of facial skin, (ii) vermilion, as the junction between the skin and the mucosa and (iii) transition, between the vermilion and mucosa of the oral cavity (Rogers & Bekic, 1997:328). The lips are also comprised of the normal three layers found in the skin, with the exception that the SC is thinner (figure 1.17) than in all other sites of the human body. The only source of moisture on the lips is saliva (Blistex, 2009). In order for a dosage form to be delivered via the lips, it has to possess a few properties. These dosage forms should

- be economical;
- be able to deliver effective concentrations of the compound;
- be non-toxic and non-irritant;
- be easy to apply or remove;
- contain mucosal or bio adhesive properties;
- not increase drying of the lip; and
- be able to spread easily (Madhav Satheesh & Yadav, 2011:1061).

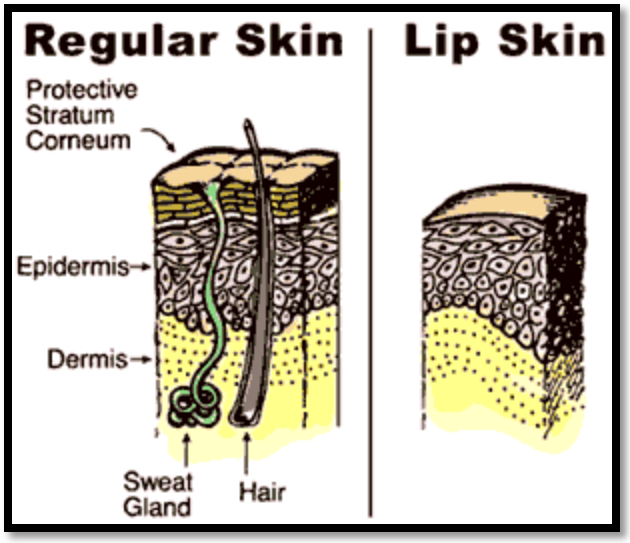


Figure 1.17: *Histological comparison of regular skin and lip skin (Blistex, 2009).*

Transmucosal delivery on the lips may provide attractive advantages for delivery of compounds on this area and pharmaceutical research and formulators have shown increased interest. The aim would be to design a dosage form of a compound by using the guidelines as listed in section 1.7.3 (Madhav Satheesh & Yadav, 2011:1061). Advantages and limitations for selecting feasible lip dosage forms are listed in table 1.10.

Table 1.10: *Advantages and limitations of using the lips as a site for delivering compounds (Madhav Satheesh & Yadav, 2011:1061).*

Advantages	Limitations
• Possesses long duration of action • Increase bioavailability • Is more comfortable to use (higher patient compliance) • Used as a painless, simple application • Presents with easy removal • Minimizes systemic adverse effects • Avoids the first-pass hepatic metabolism • Provides rapid absorption through thin SC and rich blood supply	• Limited use for local irritants, including excipients of formulation • The amount of dose is dependent on the small area of application • Very little amount of the compound will reach site of action

1.7.4 The structure and properties of vulvar skin

The human vagina is often described as slighty S-shaped fibromuscular collapsible tubes of approximately 6 - 10 cm long, from the cervix to the uterus. The wall of the vagina consists of three layers namely the epithelial, muscular coat and tunica adventia. The thickness of the epithelial layer and secretions from this area changes with the menstrual cycle. The surface of the vaginal wall is called rugae and contains many folds, causing an increased surface area. Elasticity is excellent in this area due to the smooth elastic fibres found in the muscular coat and also the loose connective tissue found in the tunica adventia. Compounds administered via the vaginal route do not undergo first-pass metabolism because the blood from the vaginal area flows into the peripheral circulation through blood rich veins (Hussain & Ahsan 2004:302). No glands are found in this area, but large amounts of fluids are excreted via Bartholin ducts, located behind the labia minora and their ducts opening into the posterolateral vestibules on each side (Lopez *et al.,* 2005:653). These fluids may contain enzymes, inhibitors of enzymes, proteins, carbohydrates, amino acids, alcohols, hydroxylketones and aromatic compounds. The high activity of enzymes found on the vaginal epithelium could affect the stability of products intended for vaginal use (Hussain & Ahsan 2004:302). Products developed for vaginal administration should be convenient for patients to use and should possess the following characteristics:

- No severe effect on sexual activity.
- Odourless and colourless.
- No leakage or messiness.
- No irritation, itching, burning or swelling.
- Convenient application (Vermani & Garg, 2000:362).

The vulvar area is a more permeable site for water than other sites on the body. It also seems to have a more proclivity to irritant reactions due to impaired barrier function (Elsner, 2011:1). The vulva area has high blood flow and a pH of approximately 4 - 5 which changes with the menstrual cycle, infections and sexual arousal (Vermani & Garg, 2000:359; Elsner, 2011:1). Advantages and limitations associated to utilising this route of administrations are listed in table 1.11.

Table 1.11: *Advantages and limitations of vaginal route administration (Vermani & Garg,2000:360; Hussain & Ahsan 2004:302).*

Advantages	Limitations
• Avoidance of first-pass metabolism by liver • Reduced incidence and severity of gastrointestinal effects • Self application and removal of dosage form • High permeability for low molecular weight compounds • Overcome pain with administration in comparison to parenteral routes	• Gender specific for patients • Personal hygiene of each individual • Less preferable or inconvenient for some patients • Influence of estrogen concentration on permeability

1.7.4.1 Dosage forms for vaginal use

Conventional dosage forms used for compound delivery to vaginal mucosa are lotions, gels, suppositories, tablets, aerosols and creams. These systems are seen as more comfortable and convenient to administer for local treatment. The mucus membrane folds (rugae) found in the vaginal mucosa inhibit the spread of compound in located mucosa and can cause irritation due to higher compound concentration in certain areas. An ideal system for vaginal compound delivery should contain: (i) a retention time long enough for maximum release, (ii) proper spreading over the epithelium for absorption in local treatment and (iii) easy application without discomfort (Wei-Ze *et al*., 2012:162).

1.7.4.2 Creams and gels

Topical gels and creams are used for anti-bacterial compound delivery on vaginal mucosa. Overall, vaginal applications are messy to apply, uncomfortable and can leak into undergarments. Non-uniform spreading can be experienced but desirable properties are found in vaginal microbial treatment. These formulations should be non-toxic and non-irritating to the mucus membranes as discussed in section 1.6.4. The principle of emulsion or hydrogels is the basis for vaginal compound delivery systems (Hussain & Ahsan 2004:302).

1.8 SUMMARY

Patients infected with HIV/AIDS present with a compromised immune system which causes them to be more susceptible to infections. These infections include dermatological manifestations which are almost inevitable when a patient is infected with HIV/AIDS. Although HIV/AIDS is not cured by modern medicine, the treatment of symptoms is possible. This study is focused on the treatment of fungal, viral and some bacterial skin manifestation occurring on mucosal membranes of HIV/AIDS patients. By treating skin lesions of these patients, an improvement of their physical wellbeing and state of mind can be expected. This will furthermore improve their quality of life, as well as increase their life expectancy.

Most patients infected with HIV/AIDS are already using large amounts of medication by means of the oral route. It is therefore beneficial to make use of topical treatment, as it may reduce the amount of interactions between compounds and adverse reactions. Acyclovir and ketoconazole are used as a possible combination product in order to treat various cutaneous manifestations associated with HIV/AIDS. Ketoconazole is seen as a broad spectrum antifungal agent, which demonstrates a synergistic anti-viral activity when used in combination with acyclovir. The physicochemical properties of acyclovir and ketoconazole indicate that acyclovir will permeate the skin more readily than compared to ketoconazole (Sweetman, 2011).

CHAPTER 2: EXPERIMENTAL METHODS

2.1 VALIDATED METHOD FOR ANALYSING ACYCLOVIR AND KETOCONAZOLE

2.1.1 Introduction

High-performance liquid chromatography (HPLC) is seen as an integral analytical tool in the pharmaceutical industry. Chromatography is considered as a physicochemical method used to separate complex mixtures and was discovered by Micheal S. Tswett. The actual name for HPLC was introduced in 1970 by Prof C. Horvath at the Twenty-first Pittsburg Conference in Cleveland (Kazakevich & Lubrutto, 2007:3).

Two immiscible phases are present and consequently an interface occurs between them. The one phase provides analyte transport (mobile phase) and the other phase is called the stationary or immobile phase. Space discrimination of the different components in mixtures is found in the high surface area between the two phases. Molecules of the analytes undergo phase transitions between the mobile phase and the adsorbent surface (stationary). The environment of the two phases, the mode of transport through the column and the partition coefficient of the analyte, are the foundation of HPLC (Kazakevich & Lubrutto, 2007:4).

Separation is based on the differences in the analyte's affinity for the stationary phase surface and is performed in a device called a "column". This column is a stainless steel tube packed with small particles (1 – 5 µm) of rigid porous material. Inside the column, material is retained with end-fittings equipped with porous frits, which allows the mobile phase to transverse to the column by forced transport. Differences in interaction of the analytes with the surface of the porous media will result in different migration times for components of the mixture (Kazakevich & Lubrutto, 2007:9).

Fractional absorption is explained as a process where molecules of different analytes have different affinities for the adsorbent surface; and analytes with weaker interactions are less retained (Kazakevich & Lubrutto, 2007:8).

A typical HPLC system consists of the following main components:

- *Solvent reservoir:* HPLC solvent storage for continuous operation of the system;
- *Pump:* Supply of continuous flow of mobile phase through the system;
- *Injector:* The analytes are introduced by the injector to the stream of the mobile phase before it enters the column;
- *Column:* A column acts as the heart of the HPLC system by providing the separation of the analytes in the mixture. The column acts as the position of contact between the mobile and stationary phase;
- *Detector:* A detector registers specific physical properties of the column effluent. Most commonly used, is the analysis with UV (ultraviolet) which allows registration of UV-absorbance at specific wavelengths;
- *Data acquisition and control system:* The HPLC-instrument is controlled by a computer based system (Kazakevich & Lubrutto, 2007:9).

2.1.2 Method development in preparative HPLC

Method development begins with selecting the most appropriate stationary and mobile phase composition. Parameters for example are:

- the solubility of a sample in the mobile phase,
- stability of the analyte and the mobile phase,
- stationary phase capacity and
- work-up of the product which has to be determined (Kuesters, 2007:940).

The method for this study was developed and validated specifically for transdermal membrane and diffusion studies at the Analytical Technology Laboratory (ATL) at the North-West University Potchefstroom Campus.

2.1.3 Chromatographic apparatus and conditions

The following chromatographic apparatus was used and the following conditions were applied in order to determine the acyclovir and ketoconazole concentrations in the different samples:

Analytical instrument:	An Agilent® 1100 Series HPLC system in a controlled laboratory environment at 25 ºC was used. This system was equipped with an Agilent® 1100 pump, UV detector and autosampler. The HPLC system was equipped with Chemstation Rev. A 10.03 software for data acquisition and analysis (Agilent Technologies, Palo Alto, CA).
Column:	A luna (5 µm, 250 x 4.60 mm), C-18 reversed phase column was used for the HPLC analysis.
Mobile phase:	The mobile phase consisted of 300 ml analytical grade methanol and 700 ml of an octanesulfonic acid aqueous solution (0.1% v/v). The methanol concentration was increased from 30% to 100% over 8 min. Octanesulfonic acid solution was prepared by accurately weighing 1.0 g octane-1-sulfonic acid sodium salt. It was dissolved in 950 ml HPLC water and the pH was adjusted to 3.5 ± 0.01 with a 10% orthophosphoric acid solution. The volume (1000 ml) was made up with HPLC-water.
Flow rate:	1.0 ml/min.
Injection volume:	5 µl.
Detection:	The UV detector was set at 252.4 nm for the detection of acyclovir and at 243.2 nm for ketoconazole.
Run time:	30 min.
Solvent:	Methanol, obtained from Merck™ was employed.
Retention time:	Acyclovir depicted a retention time of 3.6 – 3.7 min, whereas ketoconazole was retained for 9.6 - 9.7 min.

2.1.4 Preparation of the phosphate buffer

Buffer was prepared by weighing 6.81 g potassium dihydrogen orthophosphate and dissolving it in 250 ml Milli-Q® water. Sodium hydroxide (1.571 g) was weighed and dissolved in 393.4 ml Milli-Q® water. The two solutions were mixed and the pH of the subsequent solution was adjusted to 7.4 with 10% v/v orthophosphoric acid.

2.1.5 Standard preparation

A standard solution containing both acyclovir and ketoconazole was prepared by accurately weighing 50 mg acyclovir and 20 mg ketoconazole. Both the acyclovir and ketoconazole were transferred into a 100 ml volumetric flask and dissolved in approximately 20 ml methanol and a few drops of a 0.1 M sodium hydroxide solution. The flask was filled to volume with phosphate buffer solution and diluted by transferring 10 ml into a 50 ml volumetric flask and filling it to volume with phosphate buffer, providing a concentration of 50 µg/ml acyclovir and 20 µg/ml ketoconazole.

2.1.6 Validation parameters

2.1.6.1 Linearity

The purpose of measuring linearity is to illustrate that the analytical system shows a linear response, which is directly proportional to the amount (relevant concentrations) of analytes in the sample. Acceptability of linearity data can be judged by investigating the correlation coefficient and the y-intercept of the regression line for the response area through the comparison of concentration plot and residual standard deviation (Lubrutto & Patel, 2007:472).

Linear regression is defined as the range determined at the lowest and highest concentration between which the response will remain linear and accepted precision will be observed (Du Preez, 2010:4). A regression coefficient (r^2) of ≥ 0.99 is necessary to describe the regression analysis as linear.

Linearity of acyclovir and ketoconazole was performed by linear regression analysis on the graph of the peak area *versus* concentration (µg/ml). The peak areas (y-axis) were plotted against the concentrations of the active ingredients (x-axis) derived from integrating the peaks on the chromatogram. The data was best described by the linear equation (Equation 2.1):

$$y = mx + c \qquad \textbf{[2.1]}$$

Where:

- y = peak area of each analyte
- m = slope
- x = concentration of each analyte
- c = y-intercept

Standard solutions were prepared as described in section 2.1.5. A concentration range of 0.10 – 100 µg/ml for acyclovir and 0.40 – 320 µg/ml for ketoconazole was obtained by using different injection volumes. These samples were injected in duplicate onto the HPLC system.

2.1.6.2 Accuracy and precision

Accuracy is a crucial part of ensuring the quality, efficacy and safety of analytical results. The precision of an analytical method demonstrates the closeness of agreement (degree of scatter) between a series of measurements obtained from multi-sampling of the same homogenous sample under the specific conditions (Ermer, 2005a:3-4; Ermer 2005b:21). Results are expressed as percentage recovery (%), which serves as an indicator of accuracy of the system used. The acceptance criteria for accuracy are between 98 – 102% (Du Preez, 2010:4).

2.1.6.2.1 Accuracy

Three samples were prepared from the standard solution by diluting with phosphate buffer solution (pH 7.4) as described in section 2.1.5 in order to obtain the following concentrations:

- Sample 1 containing 10.06 µg/ml acyclovir and 4.00 µg/ml ketoconazole.
- Sample 2 containing 50.32 µg/ml acyclovir and 20.10 µg/ml ketoconazole.
- Sample 3 containing 100.60 µg/ml acyclovir and 40.10 µg/ml ketoconazole.

The samples were analysed in triplicate on the same day.

2.1.6.2.2 Inter-day precision

Inter-day precision is determined when samples are analysed by different analysts on different days, using a different set of equipment (ICH, 2005:5). The three samples used to determine accuracy were used to determine inter-day precision. These samples were injected into the HPLC in triplicate for 3 consecutive days. The acceptance criteria for inter-day precision are stipulated as a % relative standard deviation (RSD) ≤ 5.0 (Du Preez, 2010:5).

2.1.6.3 Ruggedness

Ruggedness is described as the measure in which test results can be reproduced while using different laboratories, under the same conditions.

2.1.6.3.1 Stability of sample solution

A sample was prepared as described in section 2.1.5. In order to determine the stability of the sample, it was injected into the HPLC-system and analysed at hourly intervals for a period of 24 hrs.

A sample should not be used for a period exceeding the time it takes to degrade by 2%. In cases where rapid degradation occurs, special precautions should be allowed to compensate for degradation (Du Preez, 2010:5).

2.1.6.3.2 System repeatability

The repeatability of the peak area and retention time of acyclovir and ketoconazole were determined. An HPLC-analysis was performed by injecting a standard sample (section 2.1.5) containing acyclovir (50.32 µg/ml) and ketoconazole (20.10 µg/ml) 8 times. According to the acceptance criteria, the peak area and the retention times should have a %RSD of less than 2% (Du Preez, 2010:6).

2.1.6.4 Specificity

This is the ability to assess unequivocally whether the analyte in the presence of the components may cause interference with the analyte detection (ICH 2005:4). The method is selected when no interference in peaks with the same retention time as the actives are found.

A standard solution was prepared, where after 1 ml thereof was placed in 4 different test tubes. The standard solution was diluted 1:1 with milli-Q® water, 0.1 M hydrochloric acid, 0.1 M sodium hydroxide and 10% hydrogen peroxide, respectively. These solutions were stored in closed test tubes overnight at room temperature to degrade. The following day the samples were injected in duplicate into the HPLC and examined to determine whether any additional peaks formed.

2.2 COMPATIBILITY OF COMPOUNDS

A study to determine the compatibility of acyclovir and ketoconazole was performed using microcalorimetry. This is a very important tool to detect whether incompatibility and/or instability exists between the pharmaceutical compounds. Microcalorimetry is a reliable way to detect the incompatibilities mostly because all physical and chemical processes are accompanied by the exchange of heat. This method is highly sensitive and makes it possible to carry out measurements at the temperatures that are close to the real conditions and can, therefore, detect very slow reactions (Phipps & Mackin, 2000:9).

During this compatibility study, the heat flow was measured for single components as well as for mixtures of the two compounds. The experimental heat flow curve for the mixture was compared to a calculated hypothetical curve for the same mixture with no physical or chemical interactions.

Compatibility of ketoconazole and acyclovir was determined with a 2277 Thermal Activity Monitor (TAM III) (Thermometric AB, Sweden) equipped with an oil bath with a stability of ± 100 µK over a 24 hr period (figure 2.1). A reference ampoule (control), as well as four samples were prepared. In table 2.1 the outline of the samples are shown. All samples were sealed with rubber stoppers and aluminium crimp seals.

Table 2.1: *Sample preparation.*

Sample	Mass weighed (g)
Acyclovir raw material	0.4981 g
Ketoconazole raw material	0.1921 g
Acyclovir:Ketoconazole (sample 1)	0.5056 g : 0.1986 g
Acyclovir:Ketoconazole (sample 2)	0.4968 g : 0.1941 g
Reference ampoule	Sealed empty

The heat flow data was recorded incessantly with an isothermal temperature program. A temperature of 80 ºC was set and the program was fixed to run for a three day period. This program consisted of isothermal and heating steps. It comprised of a total of 11 steps. The temperature was adjusted from 30 ºC to 80 ºC in increments of 10 ºC. Thereafter, it was lowered from 80 ºC to 30 ºC in increments of 10 ºC. Each of these steps lasted for approximately 12 hrs.

Figure 2.1: *Thermal Activity Monitor (TAM III).*

2.3 FORMULATION OF A PRODUCT FOR TOPICAL DELIVERY

Stable products can be developed due to advances made during the past few decades in the understanding of physicochemical properties of formulations and their ingredients. Properties of the skin and permeation factors are also well known. In modern-day pharmaceutical practice, dermatological products are developed, which can be applied for local or systemic delivery. A prerequisite for any formulation is to cross the outermost layer of the skin, the SC (Walters & Brian, 2002:320). Most topical formulations comprise of one or more of the following main components; aqueous solvents, powder and oil. This is then combined with thickening and emulsifying agents, buffers, preservatives, fragrances and colours (Barry, 2007:593).

2.3.1 Formulation of a cream

Creams are described as an emulsion (where two liquids do not mix), comprised of a water phase and an oil phase. A stable emulsion is formulated by dispersing one phase (internal

phase) in the other (external phase) to act as a dispersion medium. The semi-solid product that is formed is proven to be more stable than lotions and oil formulations (Mitsui, 1997:342). A dispersed phase can either be hydrophobic (oil in water) or it can be hydrophilic (water in oil) in an emulsion (Walters & Brian, 2002:326). An aqueous based cream is mostly preferred as it spreads more easily and is less greasy (Barry, 2007:594). Stable cream formulations can be developed from four simple ingredients: oil, water, surfactant and a fatty amphiphile (Walters & Brian, 2002:332). The raw materials used to formulate a cream in this study are listed in table 2.2

Table 2.2: *Formula of acyclovir and ketoconazole cream.*

Raw material	Amount	Activity
Acyclovir	5.00 g	Active ingredient
Ketoconazole	2.00 g	Active ingredient
Cetyl stearyl alcohol	7.00g	Thickening agent / emulsifier
Cremophor® A6	1.50 g	Emulsifier
Cremophor® A25	1.50 g	Emulsifier
Liquid paraffin	12.00 g	Oil phase of emulsion
Propyl paraben	0.20 g	Preservative
Water purified	62.80 g	Solvent
Propylene glycol	8.00	Co-solvent

The following procedure was conducted to prepare the cream formulation:

1. All ingredients listed in table 2.2 were accurately weighed or measured.
2. Cetyl stearyl alcohol; Cremophor® A6; Cremophor® A25; and liquid paraffin were mixed (oily phase).
3. The mixture was heated to 80 ºC.
4. Propyl paraben was added as preservative.
5. Purified water was heated to 80 ºC.
6. The acyclovir was added to the heated water (water phase).

7. Both the water- and oily phases were mixed by vigorously stirring the two phases in order to create an emulsion.
8. Ketoconazole was added to the propylene glycol and it was heated until the ketoconazole was dissolved.
9. The ketoconazole and propylene glycol mixture (step 8) was added to the formed emulsion (step 7) to form the cream.
10. This cream was homogenised at 200 rpm while cooling till 25 ºC.

2.3.2 Formulation of a gel

One characteristic feature present in all gels is the continuous structures providing solid like properties (Walters & Brian, 2002:325; Barry, 2007:593). Gels consist of a uniform external appearance ranging from transparent to semitransparent, leaving a light and moist feeling (Mitsui, 1997:351). A simple gel is comprised of water thickened with a natural gum (e.g., xanthan), synthetic materials (e.g., carboxymethylcellulose) or clays (e.g., silicates). The viscosity of a gel is a function of the weight and amount of the thickener added. It may be enhanced by adding inorganic suspending agents, such as magnesium aluminum silicate (Walters & Brian, 2002:325). Table 2.3 lists all the raw materials used to formulate the gel used in this study.

Table 2.3: *Formula of acyclovir and ketoconazole gel.*

Raw material	Amount	Activity
Acyclovir	2.50 g	Active ingredient
Ketoconazole	1.00 g	Active ingredient
Sodium carboxymethyl-cellulose (Na-CMC)	1.00 g	Thickening agent
Propylene glycol	10.00g	Emulsifier
Methyl hydroxybenzoate	0.05g	Preservative
Polyethylene glycol 400	2.00g	Solvent for ketoconazole
Water purified	34.50g	Solvent

The subsequent procedure was followed to prepare the gel formulation:

1. All ingredients listed in table 2.3 were accurately weighed or measured.
2. Water was heated to boiling point (± 80 ºC).
3. Acyclovir was added into the heated water.
4. Ketoconazole was mixed in the polyethylene glycol and heated until the ketoconazole was dissolved and a clear solution was seen.
5. Methyl hydroxybenzoate was dissolved in the propylene glycol.
6. Na-CMC was added and mixed vigorously.
7. The mixture formed in step 6 was added to heated water containing acyclovir (step 3) and mixed.
8. Ketoconazole and polyethylene glycol mixture was then added to the mixture above (step 7).
9. This was homogenised at 200 rpm until consistent and left to cool down.

2.3.3 Formulation of a lip balm

Lip balms are mainly oil based and formulated from waxes which are solids at room temperature (±25 ºC). The oils that are used are in a liquid form at room temperature or have a melting point of approximately 37 ºC, which is equal to body temperature (Mitsui, 1997:386). Materials used to formulate a lip balm for this study are listed in table 2.4.

Table 2.4: *Formula of acyclovir and ketoconazole lip balm.*

Raw material	Amount	Activity
Acyclovir	1200 mg	Active ingredient
Ketoconazole	480 mg	Active ingredient
Polyethylene glycol 4000	6.3 g	Solvent
Polyethylene glycol 400	15 ml	Co-solvent for ketoconazole
Spearmint flavour	Ad	Flavouring agent

The next procedure was followed to prepare the lip balm formulation:

1. All ingredients listed in table 2.4 were accurately weighed or measured.
2. Both the polyethylene glycols were heated as a mixture to 55 ºC.
3. Acyclovir and ketoconazole were added to the melted base as described in Step 2 and homogenised at 200 rpm until dissolved and homogenous.
4. Spearmint flavouring agent was added to the heated mixture.
5. The product was left to cool to just above the melting point (55 ºC) of the product as it started to thicken.
6. This was poured into suitable containers before it solidifies.

2.4 PERMEATION STUDIES BY MEANS OF FLOW-THROUGH DIFFUSION

Passive diffusion is mostly responsible for a compound to permeate from the formulation, through the tissue in the receptor phase. *In vitro* diffusion experiments are one of the important studies done in determining transdermal delivery of different formulations during the development of new preparations in industry and academia (Cordoba-Diaz *et al*., 2000:357). Advantages of *in vitro* testing include direct measuring of permeation immediately below the skin surface. This is in contrast to *in vivo* methods where systemic levels of the permeant are measured. Brain *et al*., (2002:198) stated that the most appropriate method to determine percutaneous compound delivery is *in vitro* diffusion for both, transdermal or topical absorption.

Franz-type diffusion cells are commonly used, but present with some inconveniences when compared to flow-through diffusion cells (figure 2.2). The design of flow-through receptor chambers allows for continuous flow throughout the experiment which is important in maintaining sink conditions, particularly with compounds containing large permeability coefficients through a membrane (Cordoba-Diaz *et al.*, 2000:357). Automated flow-through diffusion systems offer the advantage of unattended sampling (Brain *et al*., 2002:198).

Franz cell diffusion, on the other hand, comprises of glass chambers with round faces, which may be supported by metal clamps to ensure closure. A small well is in the side of the donor compartment to prevent spillage during air bubble replacement. The receptor phase which comprises of a 0.2 - 2 cm^2 exposed surface area and which can hold a volume of 2 – 10 ml

(containing phosphate buffer pH 7.4) is kept in water at 37 ºC and is continuously stirred with a bar magnet. One experimental difficulty in this process is accurate repetition of multi sampling and receptor replacement. This can be avoided by using flow-through diffusion cells, as the automatic fraction collector is responsible for collecting the samples. Sink conditions are easily kept in flow-through diffusion cells as a suitable pump maintains continual fluid replacement (phosphate buffer pH 7.4) in the receptor phase. Bubble traps are used to ensure that no air bubbles occur underneath the skin. Each of these cells, furthermore, has an approximate 0.1 cm^3 volume capacity in the donor phase and a 0.1 cm^2 membrane area (Barry, 1983:234; Brain *et al*., 2002:199).

In vitro diffusion conditions in flow-through diffusion systems can also be controlled in such a way that the only two variables will be the skin and the test material. One disadvantage of *in vitro* diffusion is that little information on metabolism, distribution and blood flow can be obtained (Brain *et al*., 2002:198).

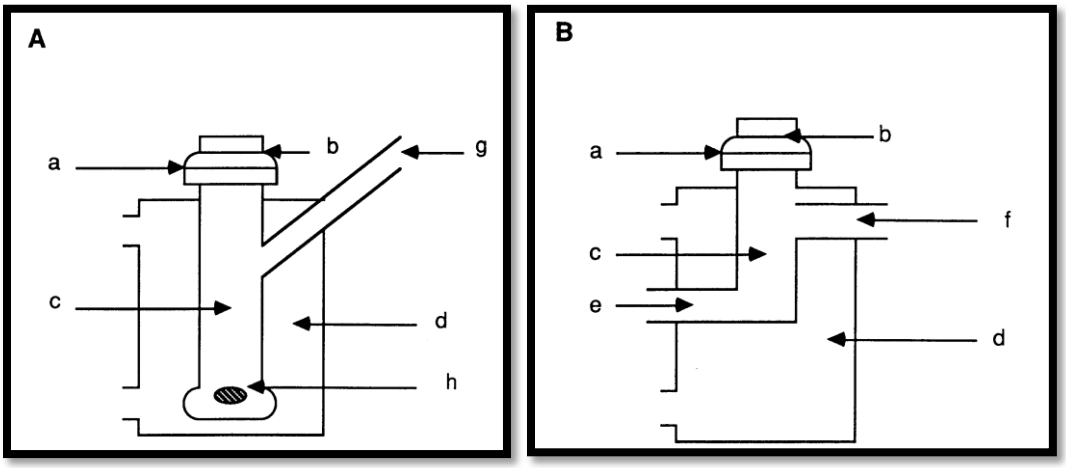


Figure 2.2: *Presentation of (A) Franz diffusion cell and (B) flow-through diffusion cell. Cell components presenting: (a) membrane; (b) donor compartment; (c) receiver compartment; (d) water jacket; (e) solvent inlet; (f) solvent outlet; (g) sampling port; (h) magnetic stir bar (Addicks et al., 1987:338).*

2.4.1 Collection of mucosal specimens

Vaginal porcine mucosal specimens (figure 2.3) were obtained from slaughtered pigs at the Faculty of Health Sciences of the University of Witwatersrand, Gauteng, South Africa. All permeation studies were conducted at the University of Witwatersrand. No ethical approval was required for this specific study as the animals were not kept at the university specifically for the

conduction of tissue experiments, but they were used in the training of students at the School of Medicine.

Porcine vaginal mucosa has been found to be similar to human vaginal mucosa in various ways. There are similarities in their lipid compositions and it also shows comparable histological characteristics (Kremer *et al*., 2001:851; Thompson *et al*., 2001:1091; Davis *et al*., 2003:1785).

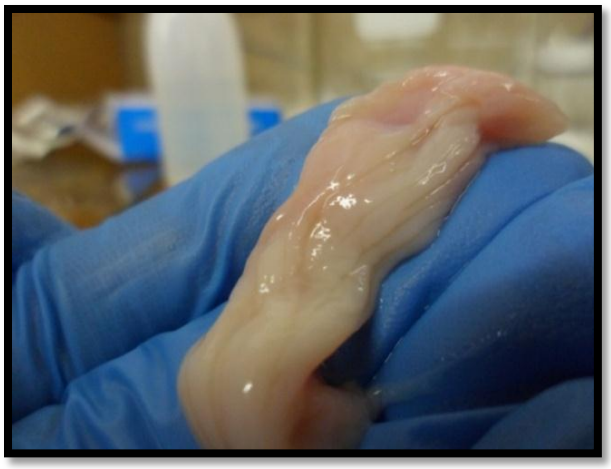


Figure 2.3: *Porcine vaginal mucosa.*

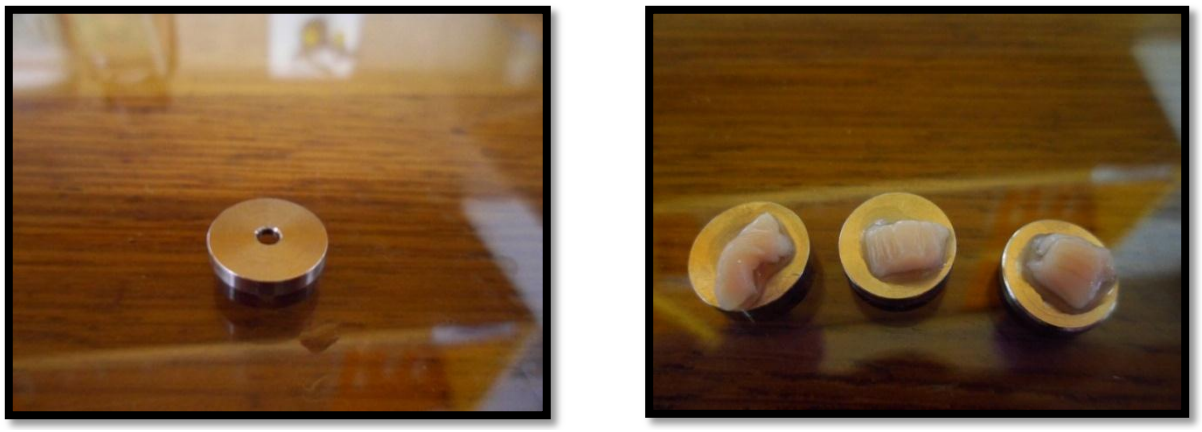
All the specimens were transported to the laboratory within 1 hour and were placed in transport fluid consisting of phosphate buffer saline (pH 7.4) or Eagle's Minimum Essential Medium (MEM) without L-glutamine and $NaHCO_3$. Excess connective and adipose tissue were trimmed away. Specimens were snap-frozen in liquid nitrogen and stored at –85 ºC. No specimens were used if any signs of disease were indicated that might influence the permeability characteristics of the vaginal mucosa.

2.4.2 Permeability experiments

For each diffusion study, an individual mucosa specimen was removed from the snap freezing compartment. Specimens were thawed to room temperature (25 ºC) in phosphate buffer (pH 7.4) within 10 min, and cut into 4 mm diameter pieces. These specimens were mounted onto metal tissue disks (figure 2.4) with exposed areas of 0.039 cm^2 and placed in the flow-through diffusion cells.

Flow-through diffusion cells comprised of a donor compartment and a receptor compartment. The formulation was placed in the donor compartment and the tissue specimens on the tissue disks were placed in the receptor compartment. Phosphate buffer (pH 7.4) was circulated

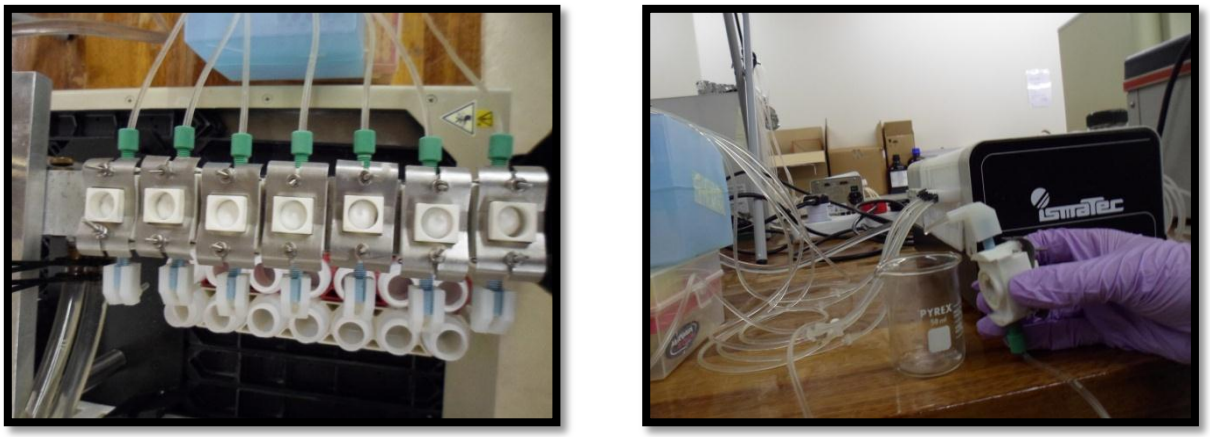
through the receptor phase by means of a pump. Prior to commencement of each experiment, the tissue disks were equilibrated for 10 min with a phosphate buffer solution (pH 7.4) in both the donor (approximately 10 ml volume) and the receiver compartment of the cells.



A B

Figure 2.4: *Tissue disks (a) without skin and (b) with skin.*

The donor compartment of each cell was filled with 1 ml formulation using a syringe. The filled donor compartment was placed on top of the tissue disks. Metal clamps were placed on the cells and used to keep each cell in place (figure 2.5). Each cell was visually inspected for air bubbles through the glass window on the bottom of the cell. Air bubbles were manually removed by means of a water pump (Ismatec, Wertheim-Mondfeld, Germany). The system was sealed using Parafilm® and a plastic lid to prevent contamination or possible evaporation.



A B

Figure 2.5: *Preparation of cells for permeation studies by (a) clamping and (b) removing air bubbles.*

Permeation studies were performed on seven tissue replicates for each formulation. Phosphate buffer solution (pH 7.4) at 25 ºC was pumped through the receiving chambers of the receptor phase at a rate of 1.5 ml/h. The phosphate buffer was used to dissolve the permeants which penetrated though the skin into the receptor compartment. Samples were collected in a fraction collector (Retriever IV, Teledyne Isco, Lincoln, USA) at 2 hr intervals for a 24 hr period (figure 2.6).

Figure 2.6: *Apparatus used for permeation studies.*

The permeation studies were performed under sink conditions and the chambers were kept at 37 ºC by means of a circulating water bath. Scintillation vials with a volume of 20 ml (Ready Protein+™, Beckman Instruments, Fullerton, CA, USA) were used as collectors of samples on the scintillation counter. Quenching of each sample was automatically corrected in the counter. After completion of the 24 hr diffusion studies, the scintillation vials were removed from the fraction collector and 1.5 ml of each sample was transferred (figure 2.7) into an HPLC vial by means of an Eppendorf® pipette (Hamburg, Germany). HPLC analyses were used to determine the concentration of compounds as discussed in section 2.1.3.

Figure 2.7: *Transfer of samples to HPLC vials.*

Flux can be described as the total amount of compound that diffuses through a membrane. The surface area of the membrane of diffusion and the contact time of the delivery vehicle are parameters for diffusion. With *in vitro* diffusion this is very important as the contact area available for diffusion is finite. Furthermore, the contact time is not always adequate for sufficient compound delivery. HPLC is mostly used to determine the flux of permeants (Smith & Surber, 2000:23). The flux of the compounds across mucusal membranes was calculated by using the following equation (Van Eyk & Van der Bijl, 2005:107):

$$J = \frac{Q}{A} t\left(dmp.cm^{-2}.\min^{-1}\right) \qquad \textbf{[2.2]}$$

Where:

- Q = quantity of substance crossing the membrane (dpm)
- A = membrane area that is exposed (cm^2)
- t = time (min) of exposure.

2.5 STABILITY TESTING

In this study a single master batch of 2000 ml was manufactured for both the cream and gel formulations. A lip balm formulation master batch was prepared by manufacturing a 600 ml batch. Small glass containers with sealed lids were used to divide the different formulations into smaller batches for stability testing at different temperatures and humidities. Foil was wrapped around each container to protect from light.

During this study, the conditions under which the formulations were stored, were determined according to climate changes in South Africa (MCC, 2011:1). Each formulation was stored at 25 ºC/60% relative humidity (RH), 30 ºC/60% RH and 40 ºC/75% RH for three months. The tests performed on each formulation are summarised in table 2.5. Due to the different characteristics of each formulation, only certain tests could be conducted on the gel and lip balm formulation. Viscosity and pH could not be conducted on the lip balm formulation as it was a solid. Due to the fact that a complete dispersion of the sample is needed to determine the zeta potential as well as the particle size, both the gel and lip balm formulations could not be used in these two stability tests.

Table 2.5: *Stability tests performed on each formulation.*

	Cream	Gel	Lip balm
Assay	√	√	√
pH	√	√	-
Mass variation	√	√	√
Viscosity	√	√	-
Visual appearance	√	√	√
Droplet size	√	-	-
Zeta-potential	√	-	-

2.5.1 Visual appearance

Photos were taken with a Samsung ES15 camera at months 0, 1, 2 and 3 to determine whether any visual changes occurred in the physical appearance of the products. These photos were used to compare colour changes by using a colour chart obtained from a local paint store. The smell, texture and overall appearance were manually inspected.

2.5.2 Mass variation

Each of the individual containers stored at the various stability conditions was initially weighed prior to storage on a calibrated Shimadzu AUW 120 D balance (figure 2.8) and marked. At months 1, 2 and 3 the foil wrapping was removed from each container and the container weighed for a total of three times to ensure accuracy.

Figure 2.8: *The Shimadzu AUW 120 D balance.*

2.5.3 Assay

All the tests were done under Good Laboratory Practice (GLP) conditions to ensure that the results were accurate. An HPLC analysis was conducted in order to determine the concentrations of both acyclovir and ketoconazole in the various formulations; and to establish if any change in concentration occurred during the exposure time. The validation of the HPLC analyses and the chromatographic conditions are discussed in section 2.1.3.

2.5.3.1 Standard preparation

Acyclovir (50.0 mg) and ketoconazole (20.0 mg) were weighed, dissolved in methanol, and the solution made to volume with a 100 ml flask. The standard solution was injected into the HPLC in duplicate. This procedure was repeated every month before the concentrations were determined.

2.5.3.2 Sample preparation

Approximately 1 g from each formulation was weighed in triplicate and transferred to a 100 ml flask using syringes with tubing equipped at the tip. Methanol was added to the samples and placed in the ultrasonic bath until maximum could be dispersed. The samples were also shaken by hand to ensure best dispersion in the methanol. Samples were left to cool down to room temperature and filled to volume in the various flasks. They were transferred to HPLC vials and analysed by means of HPLC (figure 2.9).

Figure 2.9: *Agilent 1100 Series HPLC system.*

2.5.4 pH

In order to measure the pH of each formulation a Mettler® Toledo pH meter (Schwerzenbach, Switzerland) with a glass Mettler Toledo Inlab® 410 electrode (figure 2.10) was used.

Calibration of the apparatus was conducted prior to each test. The pH of the cream and gel formulations stored at the different conditions was measured in triplicate.

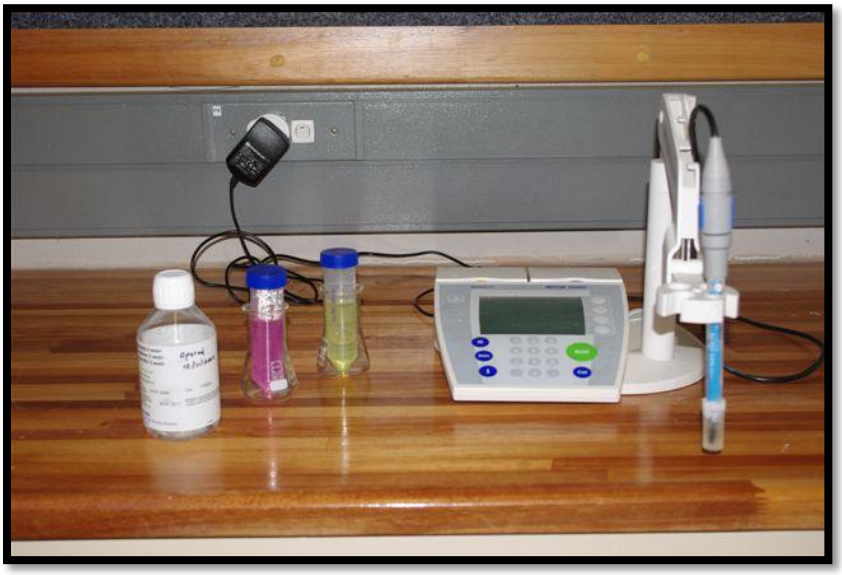


Figure 2.10: *Mettler® Toledo Multi pH meter.*

2.5.5 Viscosity

Viscosity of a fluid is defined as the resistance to flow or movement. The importance of understanding rheology in pharmaceutical products is essential for the development and evaluation of dosage forms. This serves an important role in the characterisation and classification of fluids and semi-solids. The rheology of pharmaceutical products is essential for preparation, development and performance of specific dosage forms (Marriotte, 2007:42).

Viscosity of the cream and gel formulations was determined with a Brookfield Model DV – II+ viscometer (Massachusetts, USA) (figure 2.11). The temperatures of the samples were controlled by the circulating water bath of the viscometer, set at 25 ºC. A Helipath spindle set was used to measure the viscosity and the Helipath D20733 (Massachusetts, USA) was used to move the viscometer up and down in the formulations. Table 2.6 depicts the individual temperatures and spindles used during the study.

Table 2.6: *Apparatus for the viscosity measurements.*

Formulation	Spindle	Rpm	Temperature	Entry code
Cream	T-F	0.3	25 ºC	96
Gel	T-F	0.3	25 ºC	96

The viscosity of each cream and gel sample stored at the three different temperatures and humidity conditions were tested each month, for three months. Viscosity readings were measured for 5 min, with readings taken every 10 s, rendering 32 measurements.

Figure 2.11: *The Brookfield Model DV - II+ viscometer, Brookfield circulating water bath with a temperature controller and Helipath 020733.*

2.5.6 Zeta potential

According to Jelvehgari *et al*. (2010:1240), zeta potential can be defined as the potential difference of the dispersion medium and the stationary layer of fluid attached to a dispersed particle. The degree of repulsion found between adjacent, similarly charged particles in a dispersion, is indicated by the zeta potential.

Samples were prepared by accurately weighing 500 mg of the cream formulation exposed to each of the stability conditions in a 50 ml volumetric flask in triplicate. The volume was made up

with 0.1 M potassium chloride solution and placed in ultrasonic bath to disperse. Each sample was injected into the Malvern® Zetasizer (Malvern® Instruments, Worcestershire, United Kingdom) as seen in figure 2.12 to determine the zeta potential. The zeta potential of each sample was measured in triplicate.

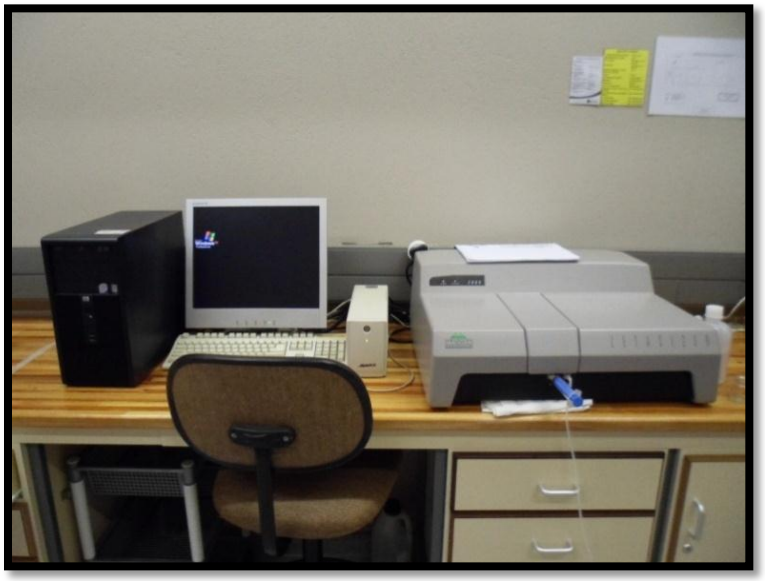


Figure 2.12: *Malvern® Zetasizer.*

2.5.7 Particle size

Approximately 0.5 g of the cream formulation stored under each stability condition was mixed with roughly 2 ml HPLC water in order to form a wet dispersion. These dispersions were made up with approximately 5 ml HPLC water, mixed and injected in triplicate into a Malvern® Mastersizer 2000 (Malvern® Instruments, Worcestershire, United Kingdom) using the wet cell, Hydro 2000 SM dispersion unit (figure 2.13).

Figure 2.13: *Malvern® Mastersizer 2000 with wet cell, Hydro 2000SM.*

2.6 STATISTICAL METHODS

The data was analysed in such a way that assumptions for all procedures were met. Due to the fact that the data was not normally distributed, non-parametric tests were performed, using Kruskal Wallis tests to determine if any statistical significant differences between the means of the test compounds and the standard compounds existed in general (Steyn *et al*., 1998:406). Dunn's post hoc multiple comparison tests were conducted to determine statistical significant differences between the test compounds. Bonferonni corrections on Dunn's p-values were calculated to determine which of the test compounds' means differed statistical significantly from the mean of the test compounds (Siegel & Castellan, 1988:213).

These procedures were done using the statistical data analysis software system Statistica (Statsoft, Inc. 2007). All tests were done at 0.05 significant level.

CHAPTER 3: VALIDATION AND FORMULATION ASSESSMENT

3.1 COMPATIBILITY

A requirement for all pharmaceutical products is to show acceptable chemical stability during the time of distribution and storage. Compatibility screening of compounds is recognised as an essential part of developing a product. Chemical and physical changes are always accompanied by an exchange of heat or enthalpy and can be studied by microcalorimetry. A sample is maintained under specific isothermal conditions and when a reaction occurs; a gradient in temperature is formed, which can be measured as a function of time. Microcalorimetry may be of some benefit in the study of complex, heterogeneous reactions. Thermal analytical techniques have been used to detect incompatibilities between compounds for over 30 years. The advantages of these methods include minimal compound requirements, rapid measurements and experimental simplicity (Willson *et al*., 1995:7108; Phipps & Mackin 2000:9; Schmitt *et al*., 2001:175).

In this study microcalorimetry was used to detect any incompatibilities and instabilities between acyclovir and ketoconazole. Figures 3.1 and 3.2 display the heat flow data (tables A.1 and A.2 annexure A) of acyclovir and ketoconazole samples. The spikes visible in these figures indicated the change in heat flow due to a change in temperature, whereas the areas between the spikes were the areas of heat flow. If there existed any form of incompatibility during the temperature elevation steps, it would have been visible through an increase or decrease in the normalised heat flow. No increase or decrease in the normalised heat flow was observed, and therefore, it could be concluded that acyclovir and ketoconazole will be compatible when used in combination.

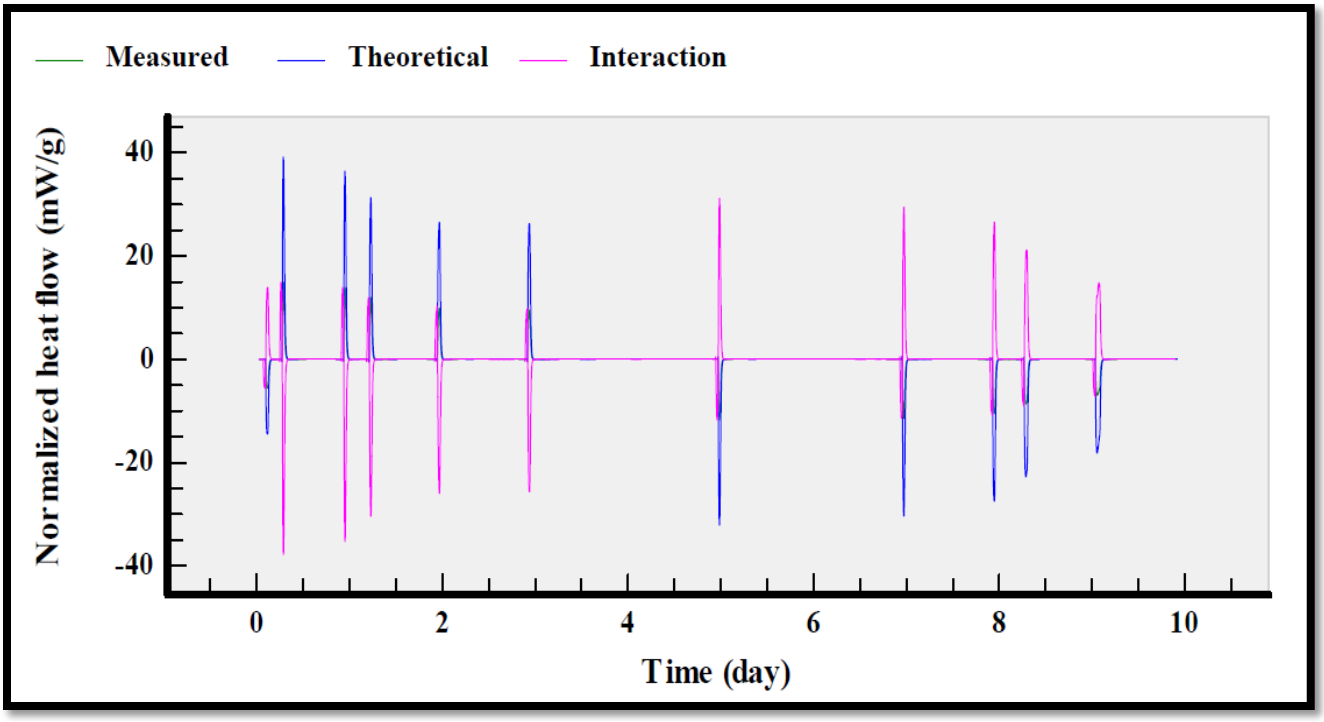


Figure 3.1: *Heat flow data of acyclovir and ketoconazole sample 1.*

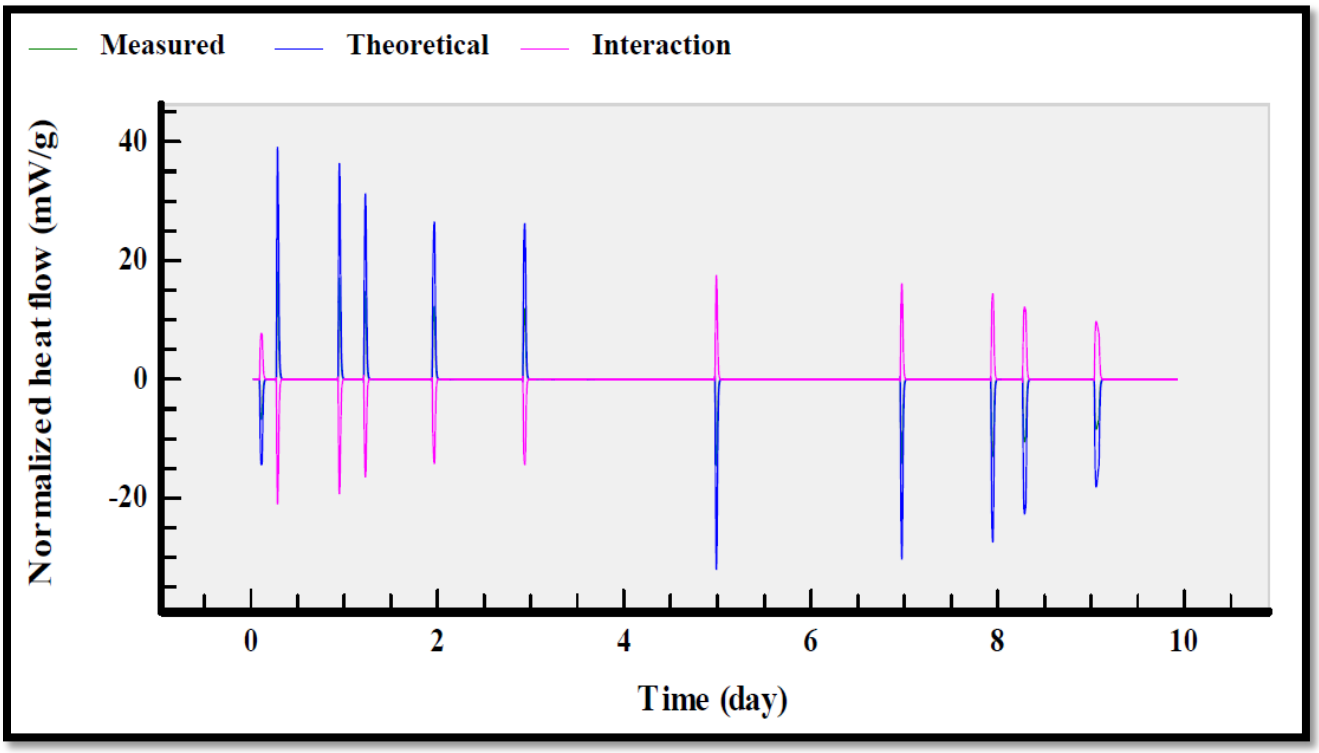


Figure 3.2: *Heat flow data of acyclovir and ketoconazole sample 2.*

A component graph (figure 3.3) depicts the heat flow data of the individual components. These heat flow signals were used to construct an interaction graph in order to compare the signals. Both curves were thus used to construct a hypothetical heat flow curve; which modelled a theoretical heat flow curve that would be obtained in the case where there were no physical or chemical interactions.

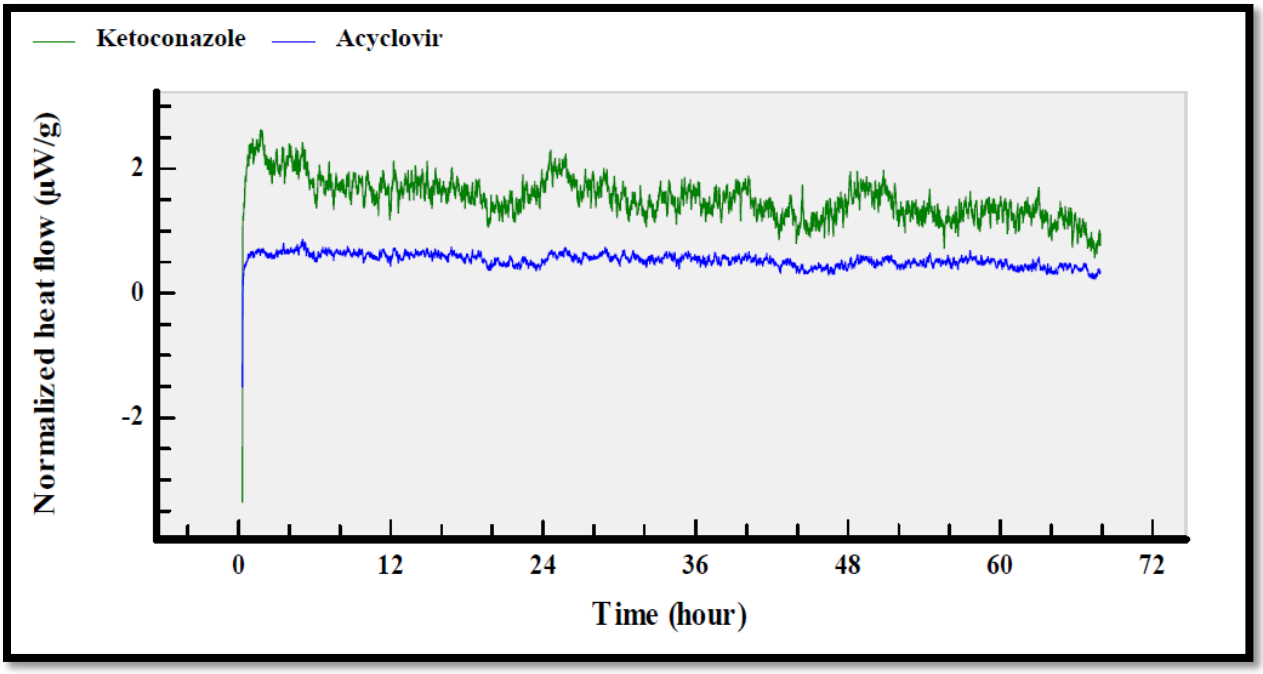


Figure 3.3: *Component graph depicting the individual heat flow signals for acyclovir and ketoconazole.*

Figure 3.4 depicts the interaction graph that was constructed from results obtained from figure 3.3. From the interaction graph it was clear that during an isothermal run of 80 ºC for a period of 3 days, no increase or decrease in heat flow of more than 1 µW/g occurred. Therefore, no incompatibility was observed between acyclovir and ketoconazole.

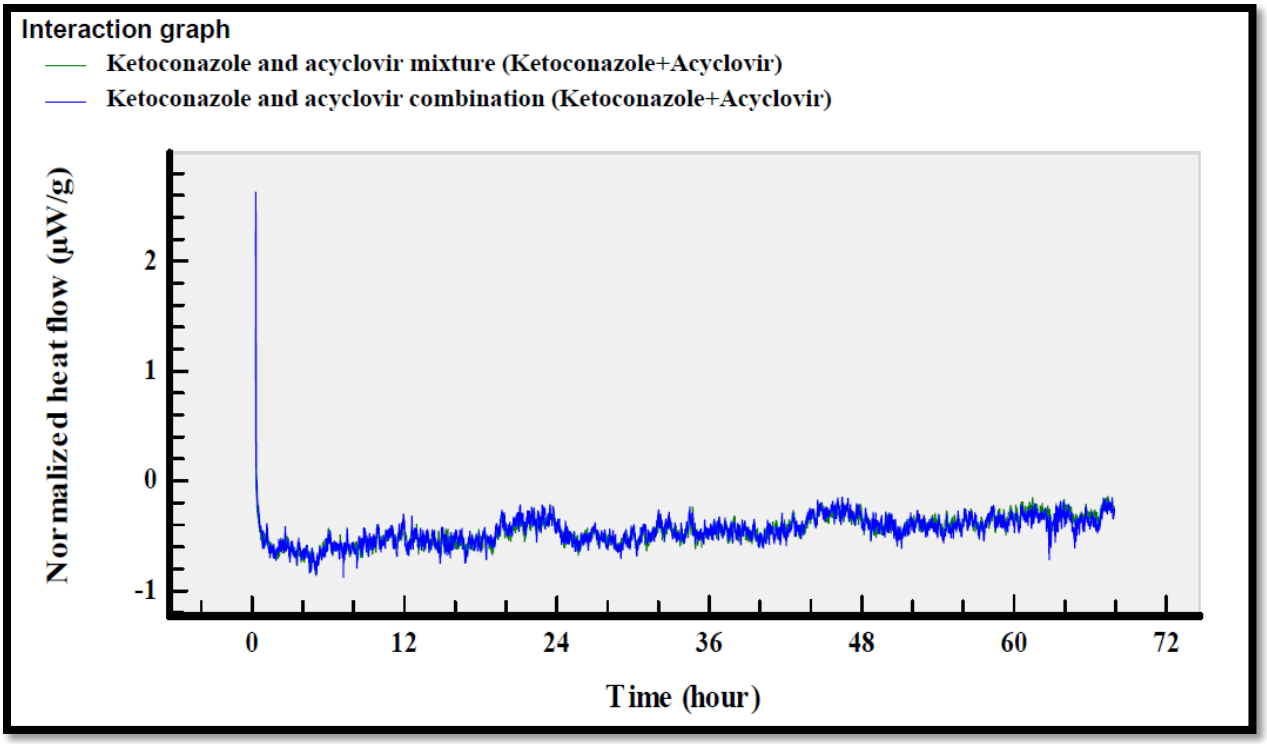


Figure 3.4: *Interaction graph for acyclovir and ketoconazole raw material.*

3.2 VALIDATION OF THE ANALYTICAL METHOD

3.2.1 Linearity

The linearity of an analytical method serves as an indication of how well a calibrated plot of peak area versus concentration will produce a straight line or how well the data will fit the linear equation (equation 2.1). A satisfactory value for the correlation coefficient (r^2) is > 0.999 (ICH 2005:8). Results obtained for the peak areas of acyclovir and ketoconazole are presented in annexure B, tables B.1 and B.2. The linear regression curves for acyclovir and ketoconazole are demonstrated in figures 3.5 and 3.6, respectively.

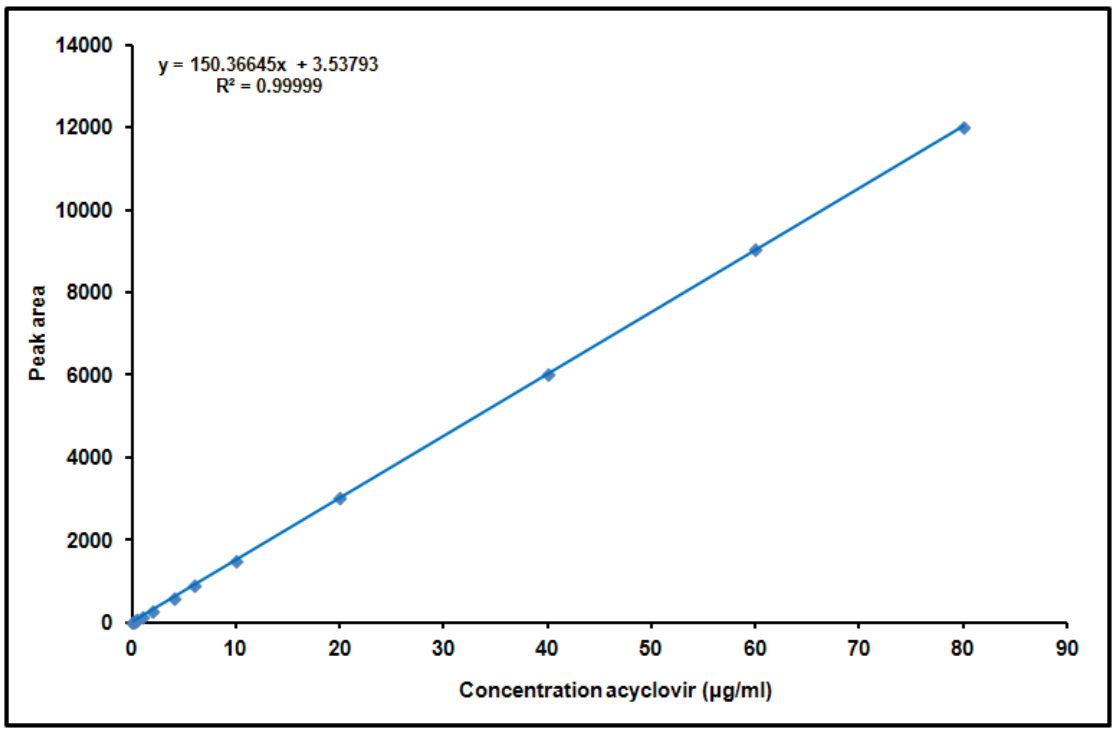


Figure 3.5: *Linear regression curve of acyclovir.*

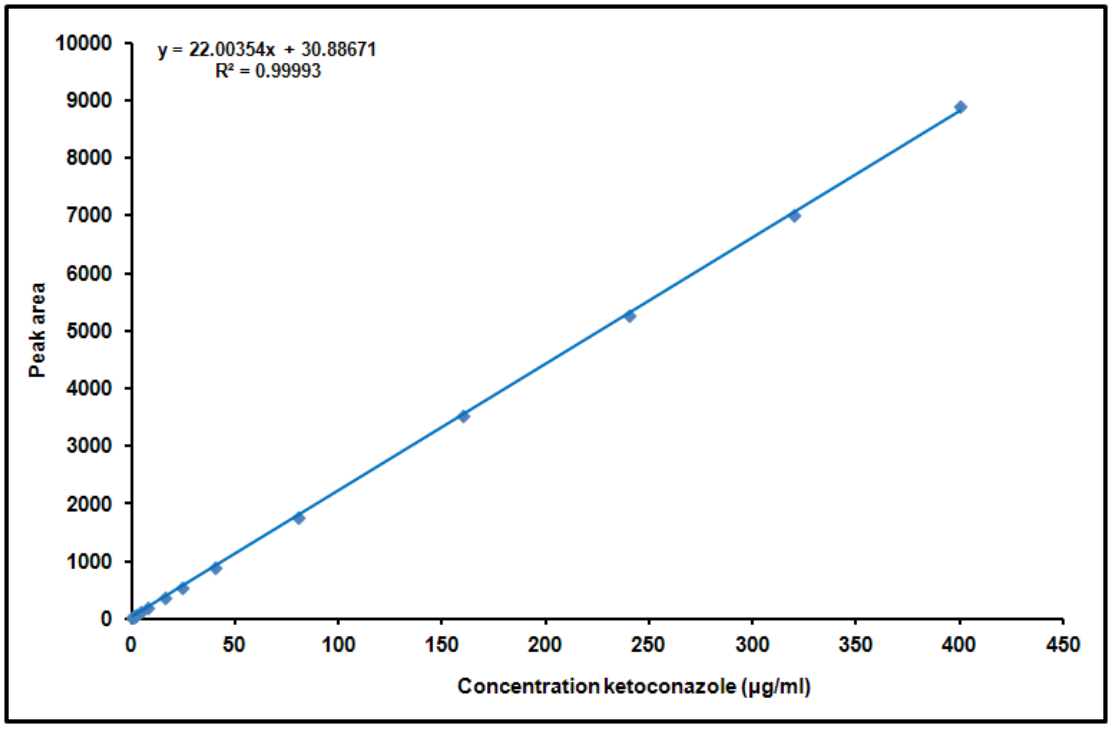


Figure 3.6: *Linear regression curve of ketoconazole.*

Both acyclovir and ketoconazole displayed acceptable r^2 values (0.99999 and 0.99993, respectively), which fell within the stipulated criteria. The closer the correlation is to 1, the stronger the positive linear relationship (Snyder, 1997:691). Therefore, these results indicated efficient stability of the analytical system as linear relationships were displayed for both acyclovir and ketoconazole within the concentration ranges tested.

3.2.2 Accuracy and precision

3.2.2.1 Accuracy

The accuracy of an analytical method demonstrates the closeness of agreement (degree of scatter) between a series of measurements obtained from multi-sampling of the same homogenous sample under the specific conditions (ICH, 2005:4).

Tables 3.1 and 3.2 present the mean, standard deviation (SD) and percentage relative standard deviation obtained for acyclovir and ketoconazole respectively. A mean recovery of 100.1% (1.3 SD) acyclovir and 99.7% (0.8 SD) ketoconazole were obtained which were within the acceptable range of 98 -102 % (Du Preez, 2010:4).

Table 3.1: *The accuracy of acyclovir.*

Conc.* spiked µg/ml	AUC 1**	AUC 2	Mean	Recovery µg/ml	%
10.06	1745.0	1746.0	1746	10.2	101.7
10.06	1748.0	1748.0	1748	10.2	101.8
10.06	1750.0	1750.0	1750	10.3	101.9
50.32	8350.0	8352.0	8351	49.7	98.7
50.32	8344.0	8362.0	8353	49.7	98.7
50.32	8352.0	8353.0	8353	49.7	98.7
100.6	16953.0	16877.0	16915	100.8	100.2
100.6	16873.0	16790.0	16832	100.3	99.7
100.6	16799.0	16833.0	16816	100.2	99.6

Statistical analysis	
Mean	100.1
SD ***	1.3
%RSD******	1.3

*Conc. refers to concentration (µg/ml)
**AUC refers to area under curve
***SD refers to standard deviation
****% RSD refers to relative standard deviation

Table 3.2: *The accuracy of ketoconazole.*

Conc.* spiked µg/ml	AUC 1**	AUC 2	Mean	Recovery µg/ml	%
4.0	322.7	328.1	325	4.0	99.0
4.0	332.9	326.6	330	4.0	100.3
4.0	327.3	325.9	327	4.0	99.4
20.1	1637.3	1627.5	1632	19.9	99.3
20.1	1625.8	1633.3	1630	19.9	99.1
20.1	1678.5	1665.2	1672	20.4	101.7
40.1	3255.2	3271.1	3263	39.8	99.3
40.1	3280.8	3274.9	3278	40.0	99.7
40.1	3245.3	3275.0	3260	39.8	99.2

Statistical analysis	
Mean	99.7
SD* **	0.8
%RSD** **	0.8

*Conc. refers to concentration (µg/ml)
**AUC refers to area under curve
***SD refers to standard deviation
****% RSD refers to relative standard deviation

3.2.2.2 Inter-day precision

Results as seen in tables 3.3 and 3.4 indicates that this method complied with the set criteria. Reproducibility was well within acceptable standards. Acyclovir depicted a %RSD of 1.14% and ketoconazole a %RSD of 0.91%. This was less than 5% (Du Preez, 2010:5).

Table 3.3: *Inter-day precision of acyclovir.*

Concentration %	Day 1	Day 2	Day 3	Between days
	98.7	101.3	101.3	
	98.7	101.3	100.7	
	98.7	101.3	100.7	
Mean	98.74	101.32	100.89	100.32
SD*	0.01	0.02	0.26	1.14
%RSD**	0.01	0.02	0.26	1.14

*SD refers to standard deviation
**% RSD refers to relative standard deviation

Table 3.4: *Inter-day precision of ketoconazole.*

Concentration %	Day 1	Day 2	Day 3	Between days
	99.3	98.4	99.1	
	99.1	99.9	98.9	
	101.7	98.8	99.0	
Mean	100.04	99.05	99.01	99.37
SD*	1.17	0.64	0.07	0.91
%RSD**	1.17	0.65	0.07	0.91

*SD refers to standard deviation
**% RSD refers to relative standard deviation

3.2.3 Ruggedness

Ruggedness refers to within-laboratory deviations for example differences in experimental periods, annalists, apparatus, columns, etc. An analytical method is conducted in the same laboratory, but all the other factors may vary (Snyder, 1997:701). This section is divided into two sections, namely stability and repeatability.

3.2.3.1 Stability of sample solution

A sample should not be used for a period exceeding the time it takes to degrade by more than 2%. In cases where rapid degradation occurs, special precautions should be taken to compensate for degradation (Du Preez, 2010:5). Tables 3.5 and 3.6 provide the mean SD and %RSD for acyclovir and ketoconazole respectively. Both acyclovir and ketoconazole showed degradation of less than 1.5% over the 24 hr period.

Table 3.5: *Percentage recovery of acyclovir over 24 hrs.*

Time (hours)	Peak Area	%
0	1713.7	100.0
1	1720.7	100.4
2	1723.8	100.6
3	1704.7	99.5
4	1719.8	100.4
5	1732.4	101.1
6	1776.1	103.6
7	1772.5	103.4
8	1780.1	103.9
9	1771.3	103.4
10	1769.9	103.3
11	1768.6	103.2
12	1767.1	103.1
13	1766.6	103.1
14	1770.9	103.3
15	1764.5	103.0
16	1767.6	103.1
17	1769.7	103.3
18	1766.0	103.1
19	1766.2	103.1
20	1764.3	103.0
21	1764.3	103.0
22	1757.4	102.5
23	1710.4	99.8
24	1764.3	103.0
Mean	1754.1	102.2
SD*	23.38	1.50
%RSD**	1.33	1.47

*SD refers to standard deviation
**% RSD refers to relative standard deviation

Table 3.6: *Percentage recovery of ketoconazole over 24 hrs.*

Time (hours)	Peak Area	%
0	799.7	100.0
1	821.9	102.8
2	826.6	103.4
3	809.1	101.2
4	829.1	103.7
5	812.8	101.6
6	812.5	101.6
7	820.9	102.7
8	797.8	99.8
9	794.4	99.3
10	794.9	99.4
11	809.9	101.3
12	812.4	101.6
13	811.6	101.5
14	821.8	102.8
15	823.8	103.0
16	804.4	100.6
17	813.4	101.7
18	802.0	100.3
19	813.6	101.7
20	819.9	102.5
21	821.8	102.8
22	815.4	102.0
23	804.3	100.6
24	799.7	100.0
Mean	811.7	101.6
SD*	9.84	1.36
%RSD**	1.21	1.33

*SD refers to standard deviation
**% RSD refers to relative standard deviation

3.2.3.2 System repeatability

Repeatability is the precision measured over a short time period under the same conditions (ICH 2005:5). According to the acceptance criteria, the peak area and the retention times should have a percentage relative standard deviation (%RSD) of less than 2% (Du Preez, 2010:6). Acyclovir (table 3.7) and ketoconazole (table 3.8) both depicted acceptable values, with a %RSD of 0.49 and 0.83, respectively.

Table 3.7: *System repeatability parameters for acyclovir.*

	Peak area	Retention times (minutes)
	8153.315	2.217
	8155.475	2.220
	8069.294	2.220
	8117.823	2.220
	8083.413	2.220
	8061.281	2.224
	8115.092	2.221
	8041.31	2.220
Mean	8099.63	2.220
SD*	39.73	0.002
%RSD**	0.49	0.080

*SD refers to standard deviation
**% RSD refers to relative standard deviation

Table 3.8: *System repeatability parameters for ketoconazole.*

	Peak area	Retention times (minutes)
	3735.699	4.725
	3775.468	4.694
	3772.744	4.657
	3766.136	4.661
	3776.982	4.664
	3743.18	4.669
	3703.525	4.669
	3814.408	4.671
Mean	3761.02	4.676
SD*	31.11	0.021
%RSD**	0.83	0.452

*SD refers to standard deviation
**% RSD refers to relative standard deviation

3.2.4 Specificity

This is the ability to assess unequivocally whether the compound in the presence of the components may cause interference with the specific compound detection (ICH 2005:4). Samples did not contain any peaks that could interfere with the determination of the compounds. Therefore, the method selected had no peaks appearing on the same retention time as the active compounds.

3.3 PRE-FORMULATION

During pre-formulation it seemed difficult to completely dissolve acyclovir in water. Acyclovir has a solubility of 1.3 mg/ml at 25 ºC according to Bethesda (2010). Poor solubility often causes crystal formation in products. Although it seems as if acyclovir dissolves partially in other solvents, water still proved the best solvent and was therefore used in both the cream and gel formulations. Acyclovir was dissolved in polyethylene glycol 400, as an alternative in the lip balm formulation.

Post formulation, all the formulations were inspected under a microscope (Nixon Optishot microscope) to determine if any crystals formed during formulation.

Crystal formation of acyclovir was present in all of the different formulations as seen in figure 3.7. The cream and lip balm depicted similar crystals which were not uniform in shape or size. In the gel formulation needle-like or rod shaped crystals were seen, which could present difficulty in acyclovir penetrating the skin.

A **B** **C**

Figure 3.7: *Crystals found in (a) cream, (b) gel and (c) lip balm formulations.*

Two commercial products on the market containing acyclovir were also inspected with the microscope (figure 3.8). Crystals were present in all the compared products, although they differed in form and quantity. A decision was made to reduce the particle size of acyclovir before formulation, in order to produce a smaller particle size of acyclovir powder. The benefit of smaller particle size distribution of acyclovir is that it may contribute to increase the solubility of, and therefore, the penetration through the skin of this compound. A smaller particle size distribution was obtained by grinding the acyclovir powder by means of a Retsck K- ZM1 type mill before the formulation process commenced (table 3.9).

Table 3.9: *Summary of particle size (µm) results of acyclovir prior to and after grinding. Values in brackets are the %RSD values.*

	d (0.1) (µm)	d (0.5) (µm)	d (0.9) (µm)
Prior to grinding	25.25 (6.36)	78.91 (23.07)	318.71 (0.45)
After grinding	13.196 (3.72)	31.95 (4.01)	85.14 (5.64)

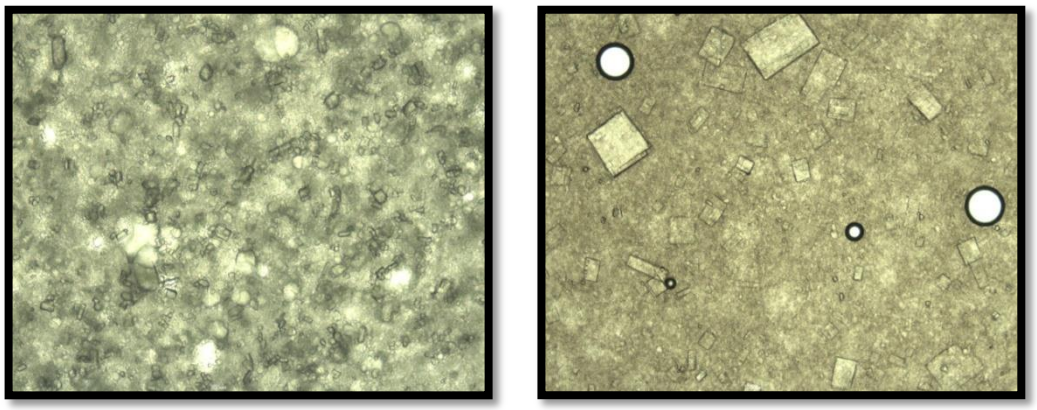


Figure 3.8: *Two products on the market (a) Acitop® and (b) Lovire® also presenting with crystals.*

The average particle size of acyclovir prior to grinding of the powder was 127.65 µm. Acyclovir depicted a 10.00% distribution of particles that were smaller than 25.25 µm, 50.00% that were smaller than 78.91 µm and 90.00% of the particles depicted a size less than 318.71 µm.

Following grinding of acyclovir powder, the average particle size was reduced to 51.20 µm. This was a pronounced decrease in particle size. Acyclovir depicted a distribution of 10.00% particles that were smaller than 13.20 µm, 50.00% that were smaller than 31.95 µm and 90.00% of the particles depicted a size less than 85.14 µm.

These results showed that the average size of acyclovir particles decreased with 76.45 µm after grinding of the powder.

3.4 MUCOSA PERMEATION STUDIES

Vaginal and buccal mucosa consists of similar histological characteristics and may be proposed as suitable models for *in vitro* permeation studies. The use of vaginal mucosa as *in vitro* permeation model has been proven to be very successful for various compounds according to Van Eyk and Van der Bijl (2004:387). The epithelia appeared thinner after the completion of permeability testing due to the compression of intermediate layers of cells and appear more flattened than usual. Diffusibility of compounds through mucosa depends on chemical nature, size and conformation, lipid/water partition coefficient, and degree of ionisation and not just the properties of the membranes involved (Van der Bijl *et al.*, 1998:395).

Mucosa specimens were used to conduct permeation studies over a period of 24 hrs. Table C.1 and C.2 in annexure C provide a summary of the average cumulative amount of acyclovir and ketoconazole respectively, released through the mucosa over 24 hrs. From these results figure 3.9 and figure 3.10 were constructed to illustrate the data obtained.

Considering results obtained for acyclovir released from the different formulations (figure 3.9), no statistical significant differences in the average acyclovir amount released were obtained ($p > 0.05$) between the formulations (table C.3 to C.6 in annexure C). However, relative differences could be observed from figure 3.9. The cream, gel and lip balm formulations depicted a decreased average cumulative acyclovir amount released through mucosa when compared to Acitop®. The following rank order could be established: Acitop®> gel > cream > lip balm. Furthermore, the gel formulation and Acitop® produced a relatively similar percentage acyclovir diffused (9.808% and 9.692%, respectively).

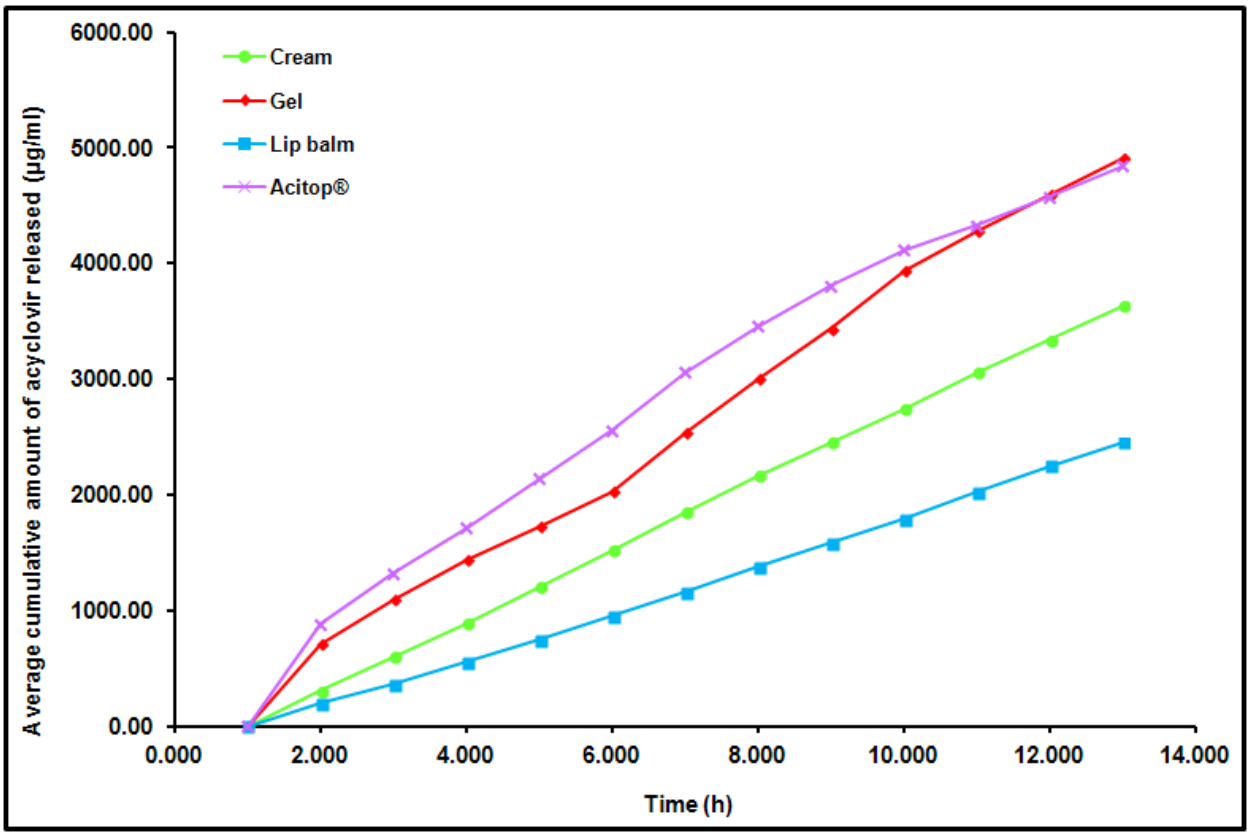


Figure 3.9: *Average cumulative amount of acyclovir permeation through the mucosa over 24 hrs.*

A relationship existed between the flux and the release rate of acyclovir from the different formulations (table C.11 in annexure C), which can be seen in figure 3.10. A linear relationship (r^2 = 0.9977) is evident from this figure. This indicated that as the acyclovir was released, the flux increased correspondingly.

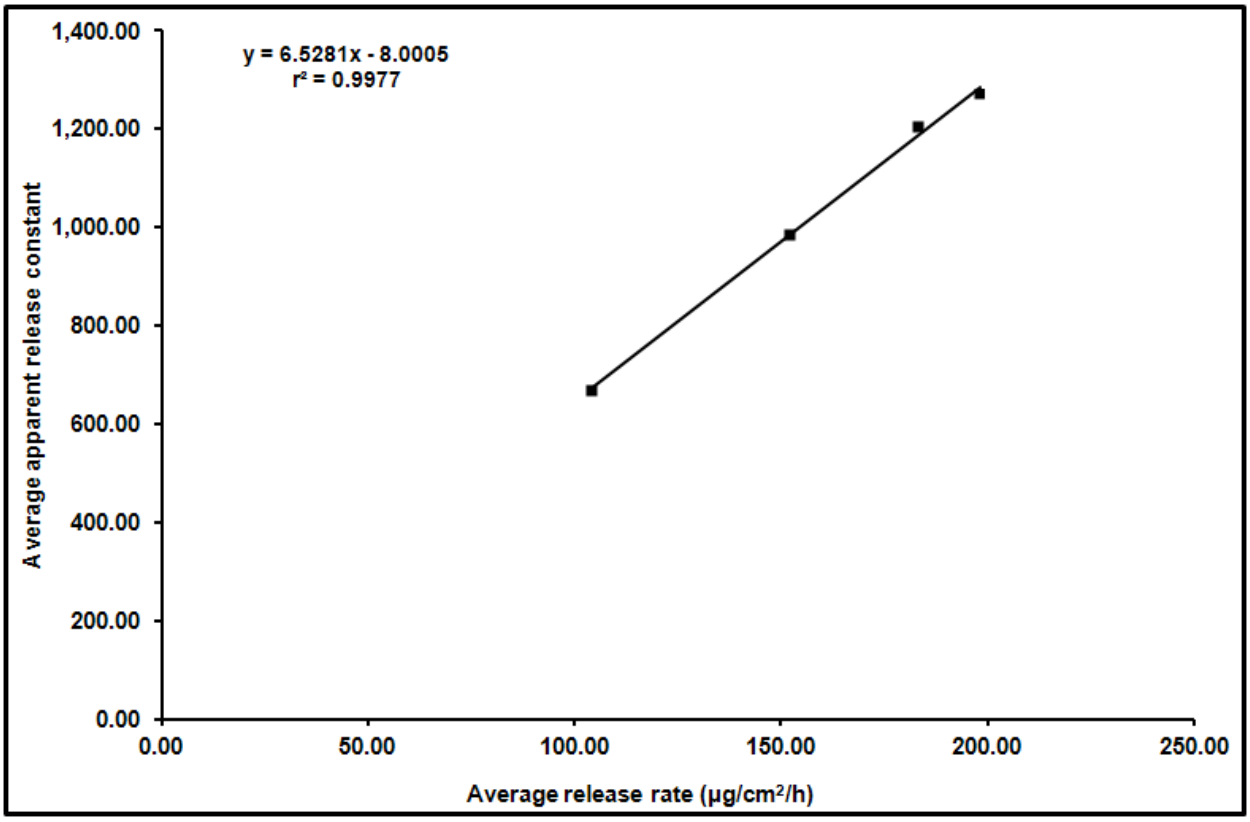


Figure 3.10: *Nature of the relationship between flux and release rate.*

Using the Higuchi model, the average cumulative amount of acyclovir released that permeated the mucosa per unit surface area was constructed against the square root of time ($h^{½}$). The Higuchi model is associated with some assumptions, namely: (i) only single compound molecules are assumed to diffuse; (ii) the compound is able to diffuse through the membrane; (iii) the compound is rapidly removed from the acceptor chamber. In order to maintain sink conditions, it is essential that less than 20% of compound introduced to the skin, would permeate into the receptor chamber. If the rate of compound released obeys this law, the amount of compound released is a linear function of the square root of time. The average apparent release rate (slopes) and lag times (*x*-axis intercepts) were calculated by means of the linear regression analysis of the plots constructed from ($\mu g/cm^2$) as a function of ($h^{½}$) by means of the Higuchi model as seen in equation 3.1 (Van der Bijl *et al.,* 2006:112).

$$ft = Q = A\sqrt{D(2C - C_s)C_s t} \qquad \textbf{[Eq 3.1]}$$

Where:

Q = the amount of compound released

t = time

A = unit area

C = initial compound concentration

C_s = compound solubility

D = diffusivity of the compound molecule (diffusion coefficient) in a matrix substance (Dash *et al.*, 2010:219).

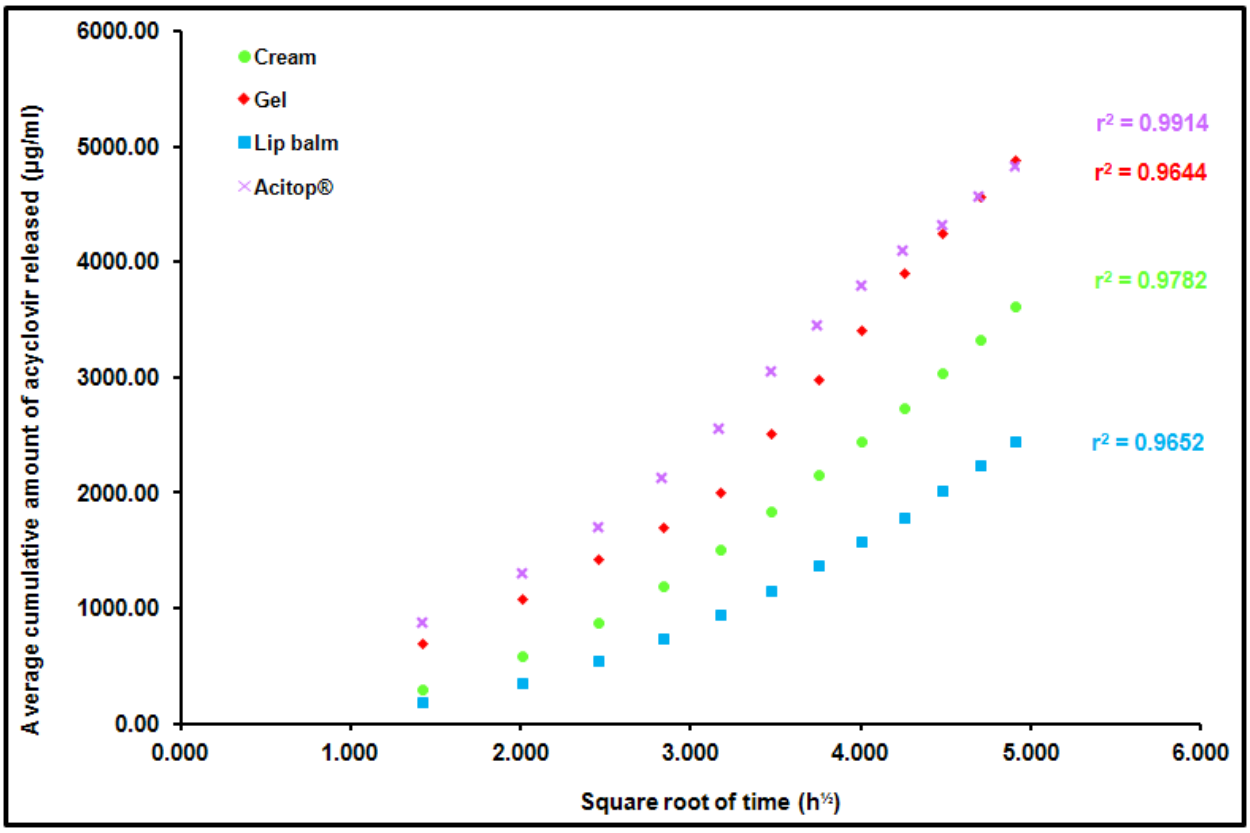


Figure 3.11: *Higuchi plot for acyclovir in different formulations by plotting the average cumulative amount released over the square root of time.*

All formulations depicted a correlation coefficient (r^2) of 0.9644 – 0.9914 for acyclovir (figure 3.11), indicating that the release of acyclovir from the different formulations could be described by the Higuchi model. No statistical significant differences could be obtained

between any of the formulations for % diffused, apparent release constant (ARC), release rate (RR) and lag times.

Considering the amount of ketoconazole that permeated the mucosa (figure 3.12), both the gel and cream formulations exhibited a smaller average cumulative amount that permeated through the mucosa when compared to Ketazol®. The lip balm was the only formulation that showed a statistically significant ($p < 0.05$) increase in permeation through the mucosa (table C.7 – C.10, annexure C) in comparison to Ketazol®. A rank order for the average cumulative amount of ketoconazole that permeated through the mucosa could be established namely: lip balm >>> Ketazol® > gel > cream.

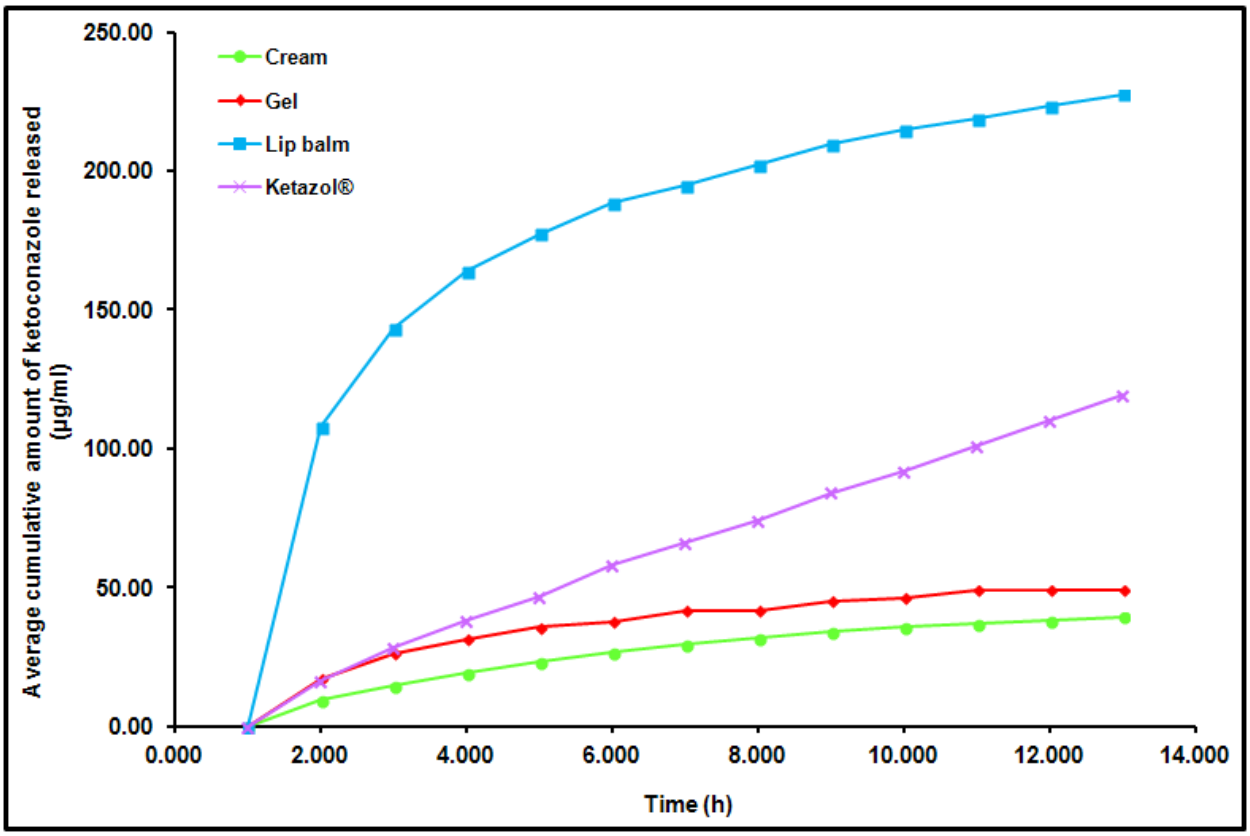


Figure 3.12: *Average cumulative amount of ketoconazole permeation through the mucosa over 24 hrs.*

Figure 3.13 depicts a linear relationship ($r^2 = 0.9991$) between the average release constant (flux) and the average release rate from each of the different formulations (table C.12, annexure C). This indicated that as the compound was released, the flux increased correspondingly which was in accordance with the acyclovir release tendency. The only statistically significant difference ($p < 0.05$) was seen for the release rate of ketoconazole from

the lip balm formulation compared to that of the cream and gel formulations. Release rate and flux of ketoconazole was the highest from the lip balm formulation.

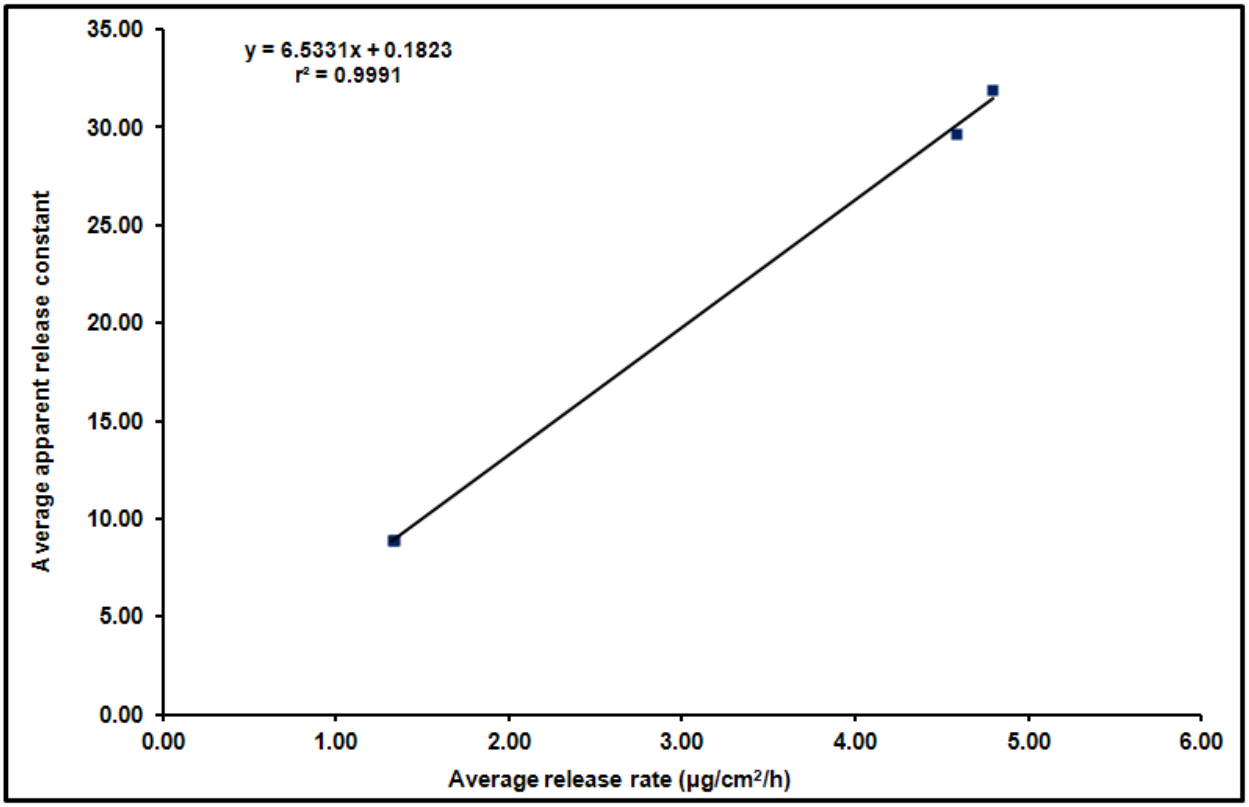


Figure 3.13: *Nature of the relationship between flux and release rate for ketoconazole.*

Figure 3.14 was obtained by constructing the average cumulative amount of ketoconazole released that permeated the mucosa per unit surface area against the square root of time ($h^{½}$). From this figure it was clear that the rate of ketoconazole released from all of the different formulations obeyed the Higuchi model as the amount of compound released from each formulation was a linear function of the square root of time (r^2 = 0.9584 - 0.9899).

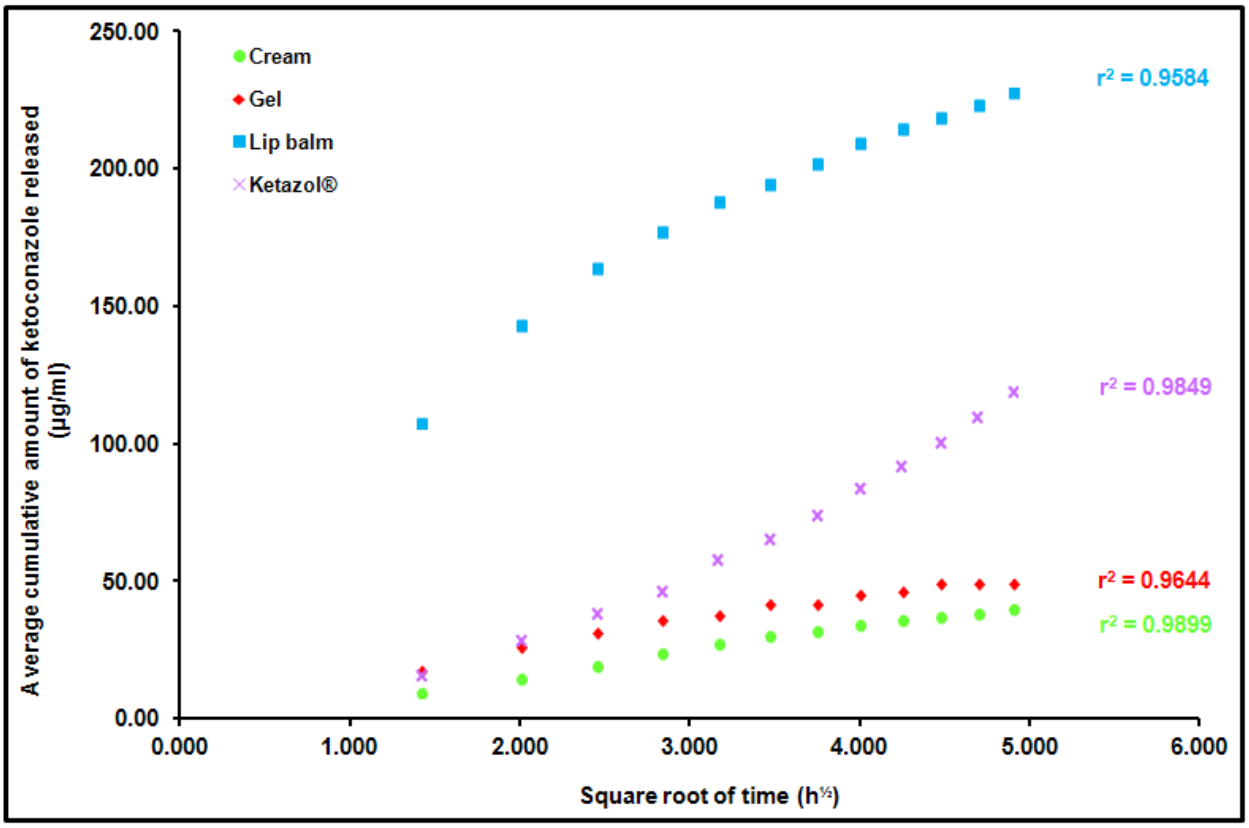


Figure 3.14: *Higuchi plot for ketoconazole in different formulations by plotting the average cumulative amount released over the square root of time.*

Statistically significant ($p < 0.05$) differences were furthermore noted between the lip balm and both the cream and gel formulations (table C.13, annexure C) when % diffused, ARC and RR were compared. The lip balm depicted the highest percentage diffused, the highest ARC as well as the fastest RR, however, no statistical differences were obtained between the cream and gel formulation even though the gel formulation performed slightly better. Considering the lag time, all the formulations presented with a relatively shorter initial time of release of less than an hour. Shorter lag time values indicate that the ketoconazole was preferentially released by the base of the formulations. Statistically significant differences ($p < 0.05$) between the lag times of Ketazol® and the lip balm formulation, as well as between the lip balm and the cream and gel formulations existed. These results might have been due to the type of formulation into which the ketoconazole was incorporated. Ketoconazole is practically insoluble in water and is relatively lipid soluble. The lip balm formulation did not comprise of any water as compared to the other formulations. Ketoconazole might therefore have been more soluble in the lip balm formulation and would therefore have been more permeable through the mucosa. Furthermore,

the permeation studies were conducted at body temperature (37 ºC) where the polyethylene glycol 4000 (solvent in the lip balm formulation) proceeded to liquefy causing the formulation to melt, which in turn contributed to increased dissolution of the compound.

3.5 SUMMARY

Microcalorimetry was used to determine whether any incompatibilities or instabilities occurred between acyclovir and ketoconazole in order to produce a product containing both these compounds. Chemical and physical changes are always accompanied by an exchange of heat or enthalpy. Spikes were used to indicate change in heat flow due to a change in temperature and the areas between the spikes were the areas of heat flow. If any instabilities or incompatibilities existed during the temperature elevation steps, it would have been visible through an increase or decrease in the normalised heat flow. No changes in the normalised heat flow were observed, and therefore, it could be concluded that acyclovir and ketoconazole will be compatible when used in combination.

Furthermore, results obtained during the validation process indicated that the HPLC method for the determination of both acyclovir and ketoconazole was sensitive and reliable enough in order to determine the compound concentration in the different formulations containing both acyclovir and ketoconazole.

The grinding of acyclovir powder before formulation indicated a resulted in a reduction of the average size of acyclovir particles in formulations.

The results from the permeation studies conducted, demonstrates that the permeation flux of acyclovir from any of the three formulations produced, did not indicate a statistically significant ($p > 0.05$) difference from the flux of Acitop® through mucosa. This was an indication that the formulated products can be well compared to the product on the market. In the lip balm formulation, ketoconazole depicted a statistically significant increase ($p < 0.05$) compared to Ketazol® in the flux through mucosa.

CHAPTER 4: STABILITY TESTING

4.1 INTRODUCTION

An essential consideration for pharmaceutical and cosmetic products is the stability of the product. The physical stability of these products is characterised by absence of coalescence or creaming, and the maintenance of appearance, odour, colour and other physical properties. Creaming of pharmaceutical products results due to a lack of uniformity in compound distribution and presents a problem for the formulator. Phase inversion and flocculation are other important factors influencing stability of pharmaceutical emulsions (Sheraz, 2009:39).

Chemical instability of a compound may lead to change in concentration by chemical reactions under stressed or even normal conditions. A change in potency can be seen in pharmaceutical products with varied concentrations. The degradation of a pharmaceutical compound may furthermore cause the product to be aesthetically unacceptable to the consumer if significant changes in colour or odour occur. Degradation of a product may even lead to it being toxic to the consumer (Sheraz, 2009:39).

Chemical degradation of a compound may involve hydrolysis; oxidation; dehydration; isomerisation and racemisation; decarboxylation and elimination; photo degradation, and compound interactions; depending on the structural characteristics of the compound. To determine the chemical stability of a compound many factors can be considered. Intrinsic factors include the molecular structure of the compound whereas environmental factors such as temperature, light, pH, moisture and excipients may also be considered (Sheraz, 2009:39).

The objective of stability testing is to ensure that a given product has an adequate shelf-life in its container, before it can be marketed and sold. Shelf-life can be described as the “period of time in which a given product remains in full compliance with its specifications, when it is held under the conditions of the market”. Every product is subjected to change as it ages up until the point where it is unsatisfactory and the content is no longer acceptable for its specific use (Cannell, 1985:292).

According to the Food and Drug Administration (FDA) semisolid preparations should be evaluated for appearance, colour, homogencity, odour, pH, viscosity, particle size distribution (when feasible), assay, degradation and weight variation (Sheraz, 2009:46).

Information is provided through stability testing about how the quality of bulk product varies over a specific period of time under the influence of environmental factors including, temperature, humidity and light. This serves as determination of recommended storage conditions for each product (Farmer *et al.,* 2002:28). Some of the parameters that are taken into account for pharmaceutical and cosmetic product stability are listed in table 4.1.

Table 4.1: *Parameters for the evaluation of stability (Knowlton & Pearce 1993:436).*

Physicochemical	**Product/package compatibility**
• Appearance • Colour • Odor • pH • Viscocity • Signs of phase separation • Light stability	• Incompatibility of product and pack • Mass variation • Moisture loss • Perfume loss • Pack degradation / softening / cracking

An intermediate stability study was conducted on formulated products as described by the International Conference on Harmonisation (ICH) Tripartite Guidelines (2003:12), with a testing frequency of minimum four time intervals, including the initial and final testing.

In this study a cream, gel and lip balm formulation were formulated in bulk, containing both acyclovir and ketoconazole (section 2.3). Each formulation was divided into smaller containers, sealed with lids and marked for the specific tests. These containers were stored at 25 ºC/60% relative humidity (RH), 30 ºC/60% RH and 40 ºC/75% RH, respectively. Stability tests were conducted at months 0, 1, 2 and 3. The tests performed on the cream, gel and lip balm are summarised in table 4.2.

Table 4.2: *Tests and apparatus used during stability testing (Knowlton & Pearce 1993:439).*

Product attribute	Test method
Assay	HPLC
pH	Mettler® Toledo pH meter
Viscosity	Brookfield viscometer
Mass loss	Shimadzu scale
Particle size	Malvern® Mastersizer 2000
Zeta-potential	Malvern® Zetasizer
Appearance	Visual

According to the Medicines Control Council (MCC) (2011:12) of South Africa, a significant change for a formulated product is described as:

- a 5.00% change in active ingredient assay from the initial value;
- any degradation of products exceeding its acceptance criterion;
- failure to meet acceptance criteria for appearance, physical attributes and functionality (e.g., colour, phase separation, caking, hardness); and
- failure to meet the accepted criterion for pH.

4.1.1 Visual appearance

Visual appearance was assessed each month for all the formulations stored at the various storage conditions over a 3 month period. Tables 4.3 – 4.5 present the photographs taken each month of each of the formulations.

A change in colour may be indicative of instability as it can be caused by chemical degradation or interactions in products. The appearance of the product can be compromised by separation of the formulation which can also be visually assessed as the oil phase and water phase will be

seen as two separate layers in the formulation. Other indicators of instability can be the development of smells or loss of moisturising effect (Sheraz, 2009:39).

Colour changes were noted by comparing the cream with a colour chart obtained from a local paint store (figure D.1, annexure D). The overall appearance of the cream changed slightly over the period of three months (table 4.3). After three months the cream depicted a change of 2 shades darker on the colour chart (OW 105-soap suds to OW 107-bridal bouquet). No smells, oiliness or other signs of degradation was found. Furthermore, the texture of the cream remained frothy through-out the 3 month period at all the storage conditions. Thus, the container was deemed suitable for storage of the cream formulation.

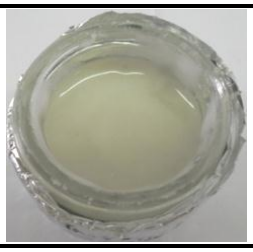
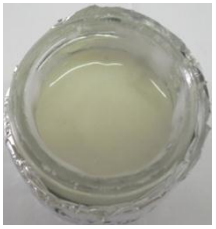
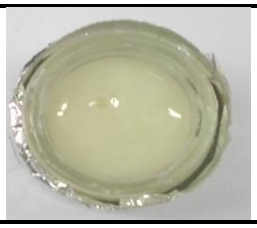
Table 4.3: *Change in colour of the cream formulation at different conditions after each time interval.*

Initial	Month 1	Month 2	Month 3
25 ºC 60% RH			
			
30 ºC 60% RH			
			
40 ºC 75% RH			
			

.

Considering the gel formulation (table 4.4), no significant colour changes occurred at any of the different stability storage conditions over the 3 month period. The gel remained an off-white colour (OW 131-lovely lace) and no change was indicated when the photographs were compared to the colour chart. This was an indication of stability. Furthermore, no changes in smell, texture or moisture content was noted and the gel continued to have a sticky feel through-out the test period. Thus, the container was considered suitable for storage of the gel formulation.

Table 4.4: *Change in colour of the gel formulation at different conditions after each time interval.*

Initial	Month 1	Month 2	Month 3
25 ºC 60% RH			
			
30 ºC 60% RH			
			
40 ºC 75% RH			
			

The lip balm formulation (table 4.5) was formulated with an off-white colour (OW 12 -amberling), a spearmint flavour and smell. No change in colour was observed for any of the storage conditions over the three month testing period. The flavour and smell did not change and remained unchanged after the exposure to the different storage conditions during the three months. Crystal formation was, however, visible at the surface of all of the formulation samples stored at the different storage conditions. Furthermore, at the 40 ºC/75% RH condition, an oily layer developed on the surface of the formulation at month 1. This was due to the low melting point of polyethylene glycol (PEG) 4000 (50-58 ºC) used during manufacturing. The oil layer was due to the PEG 4000 liquefying and therefore not an instability. A lip balm should have a melting point of approximately 37 ºC (body temperature) in order to produce effective application and to ensure efficient contact time on the surface (Mitsui, 1997:386).

Table 4.5: *Change in colour of the lip balm formulation at different conditions after each time interval.*

Initial	Month 1	Month 2	Month 3
25 ºC 60% RH			
			
30 ºC 60% RH			
			
40 ºC 75% RH			
			

4.1.2 Mass variation

Mass loss during stability testing can be an indicator of water evaporating from the formulation due to continued exposure to high temperatures (Arranberri *et al.,* 2004:2069). Mass variation was determined at months 0, 1, 2 and 3 for each of the formulations for each of the storage conditions (tables D.1 – D.3, annexure D). Figures 4.1 - 4.3 depict the mass variation of each of the formulations at the three different storage conditions.

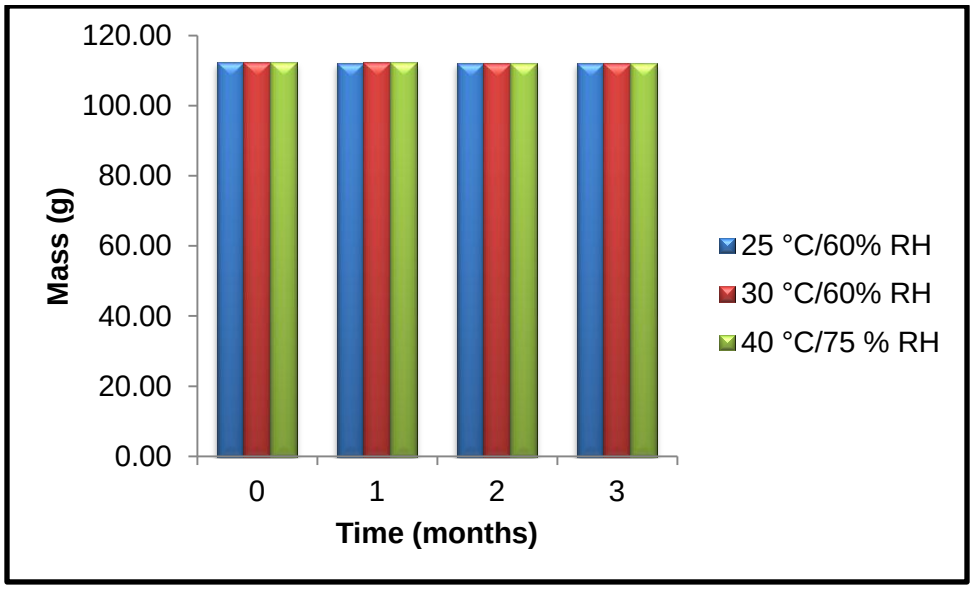


Figure 4.1: *Mass variation (g) for the cream formulation at the different storage conditions after each time interval.*

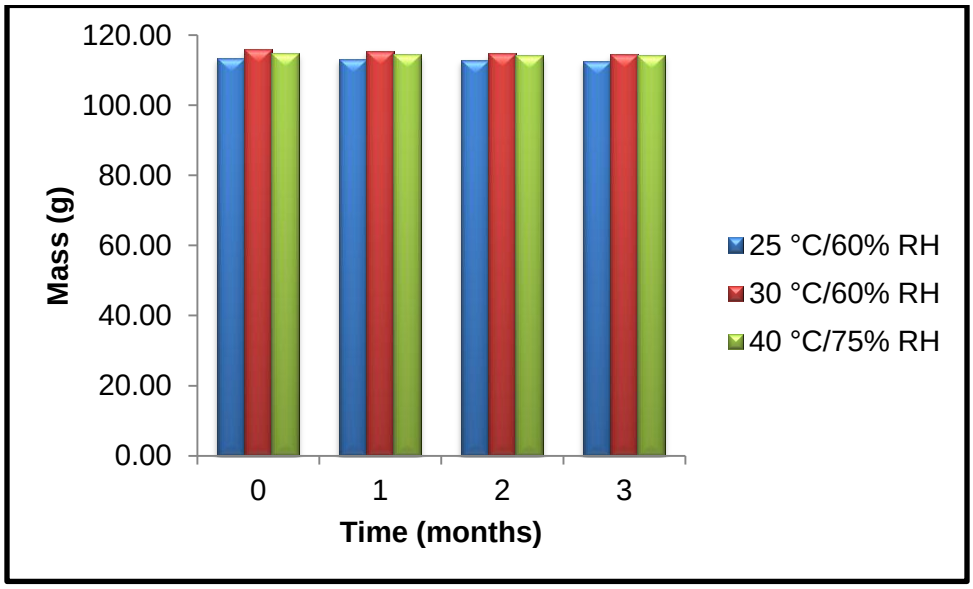


Figure 4.2: *Mass variation (g) for the gel formulation at the different storage conditions after each time interval.*

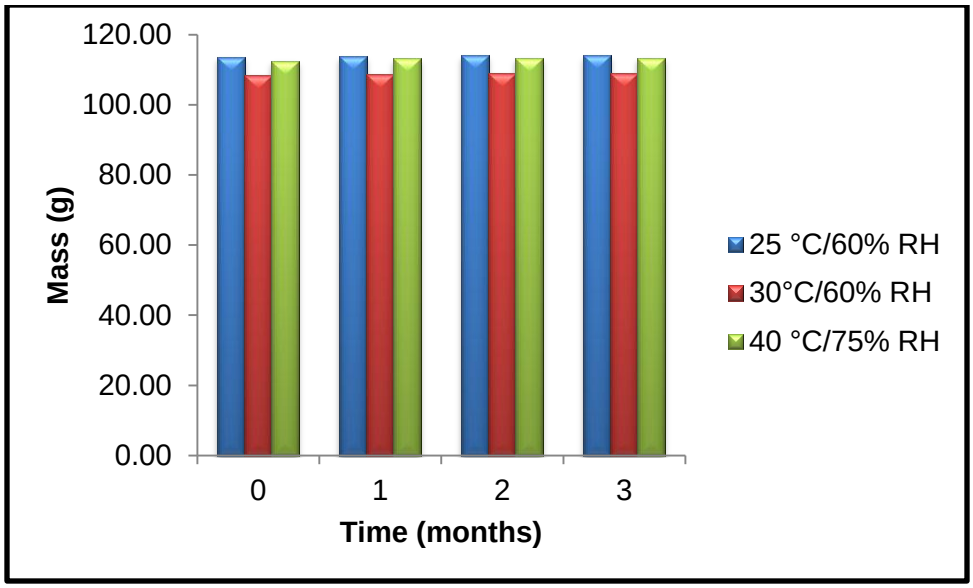


Figure 4.3: Mass variation *(g) for the lip balm formulation at the different storage conditions after each time interval.*

As observed from figures 4.1 - 4.3, no significant change in mass could be estimated for any of the formulations stored at the various storage conditions during the testing period. The highest decrease in mass was seen at the 40 ºC/75% RH condition for the gel formulation, which was only 0.40%. It could therefore be conducted that all the formulations were stable in their containers with respect to mass variation.

4.1.3 Assay

The assay of acyclovir and ketoconazole in all the formulations stored at the various storage conditions were determined by means of HPLC analysis (section 2.1.3) at months 0, 1, 2, and 3. These results are presented in tables D.4 – D.6 in annexure D. Figures 4.4 – 4.6 depict the percentage acyclovir present in the different formulations stored for 3 months at the various storage conditions.

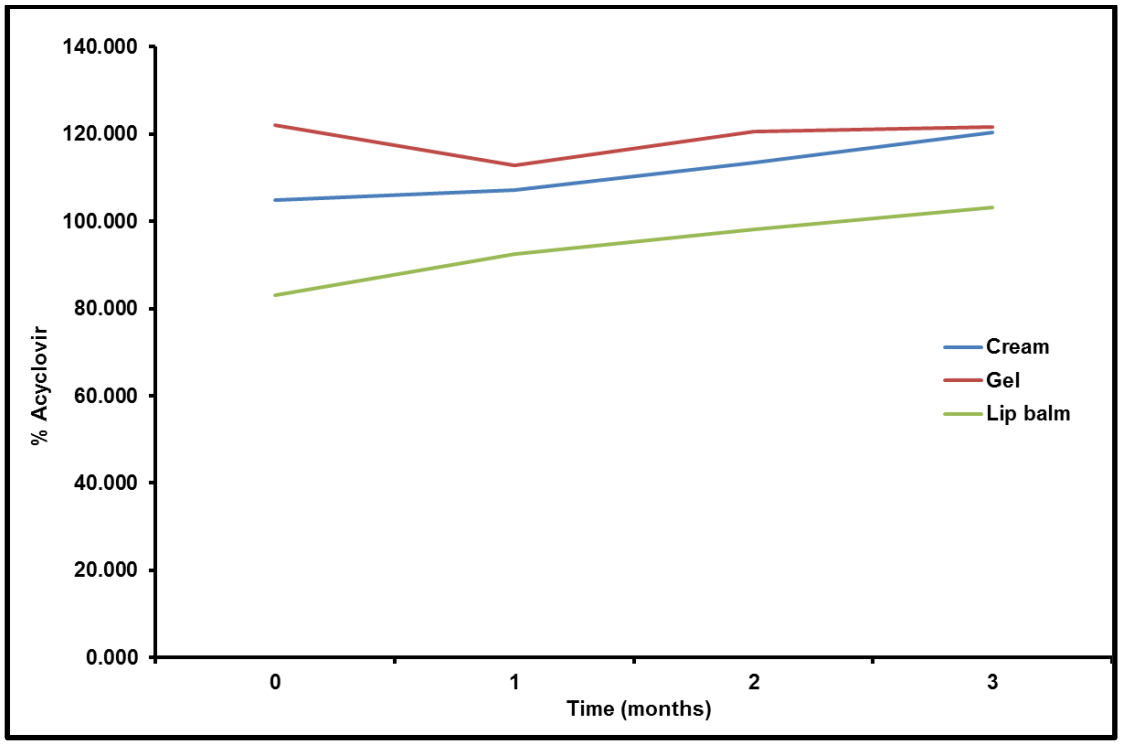


Figure 4.4: *Percentage acyclovir present in the different formulations at 25 ºC/60% RH after each time interval.*

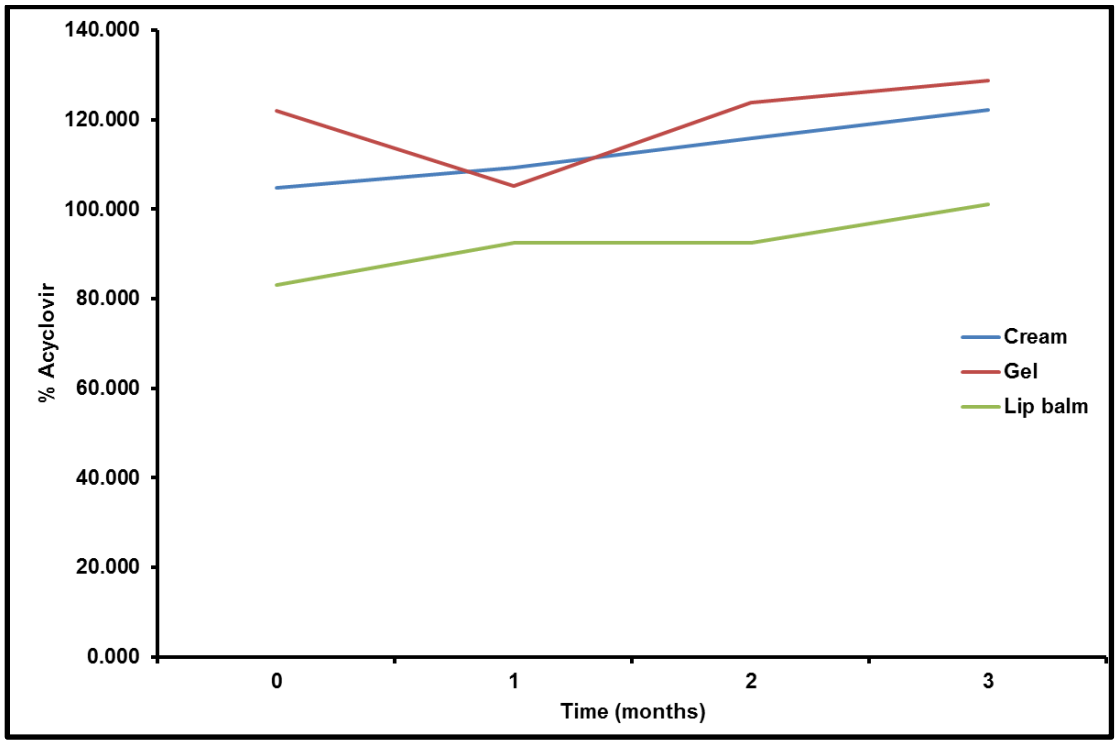


Figure 4.5: *Percentage acyclovir present in the different formulations at 30 ºC/60% RH after each time interval.*

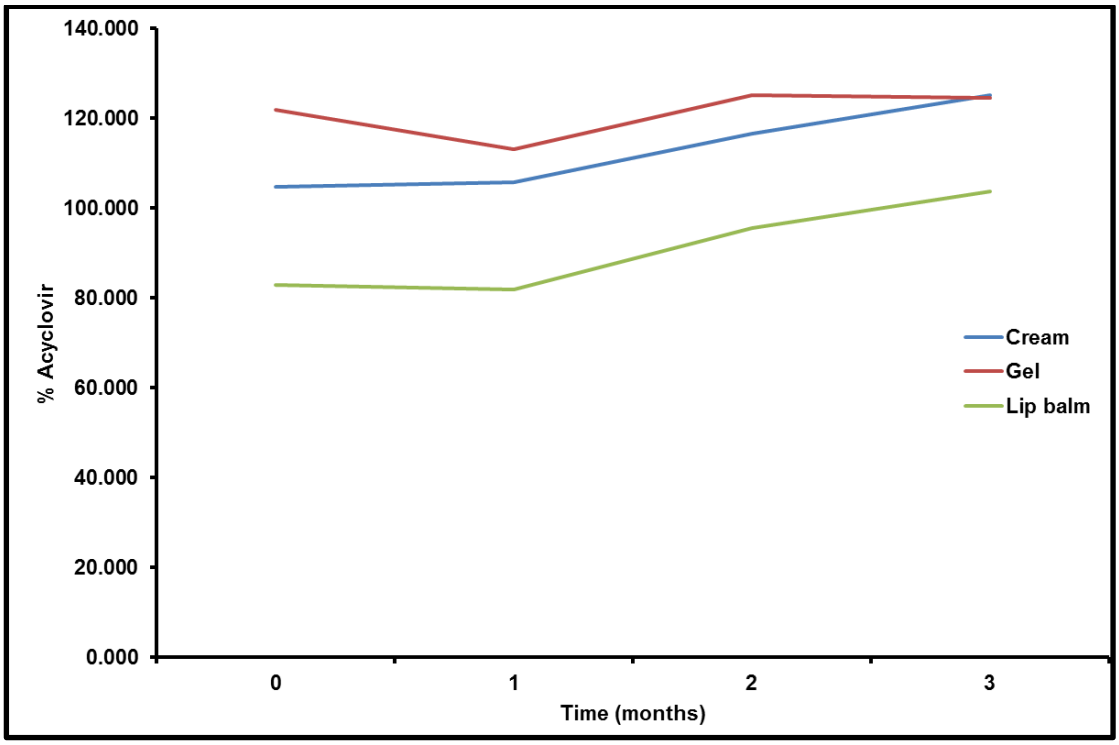


Figure 4.6: *Percentage acyclovir present in the different formulations at 40 ºC/75% RH after each time interval.*

Considering both the cream and lip balm formulation, the concentration of acyclovir, increased with more than 10.00% (cream formulation) and more than 15.00% (lip balm formulation), respectively at each of the temperatures over the three months. This marked change in concentration observed could be an indication of product instability. The gel formulation, on the other hand, depicted the least variation in assay over the 3 month period for all of the storage conditions compared to the other formulations. It could be considered stable as the overall change in acyclovir concentration was less than the stipulated value of 5% for all the storage conditions.

Acyclovir is classified as slightly soluble in water (Sweetman, 2011). The water phase of the cream and gel formulations was used to dissolve the acyclovir and this phase had to be heated in order to properly dissolve it. Even though the cream formulation was formulated at a pH of 8.9 where acyclovir is considered most stable (Skiba*, et al.,* 2000:1), this formulation still developed crystals directly after formulation as the cream was cooled to room temperature. However, compared to the commercial product, Acitop®, the crystals that precipitated in the cream formulation were relatively smaller. Both the cream formulation and Acitop® depicted

cubical crystal formation, whereas the gel formulation, which also presented with crystals, depicted significantly smaller, crystals that were more rod-formed or needle-like.

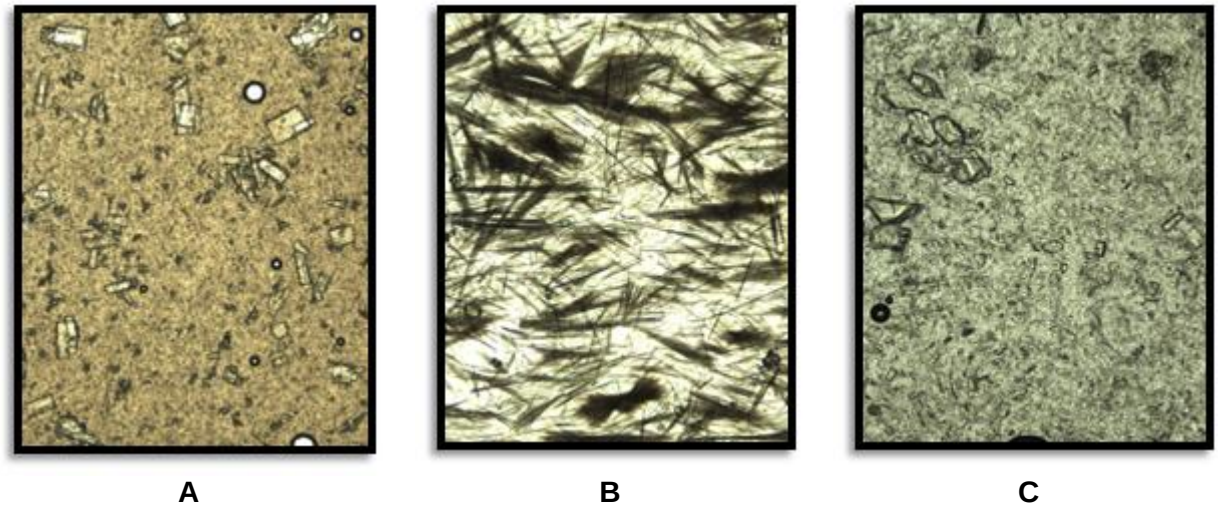


Figure 4.7: *Crystals found in (a) cream, (b) gel and (c) lip balm formulations.*

Nucleation (crystal growth) is described as an assembly process where a number of molecules are needed in order to achieve a phase change from solution or melt into a crystal. Once the nucleation process is achieved, the growth of crystals begins to dominate, which will lead to the development of embryonic crystals into a crystal form of distinct shape and size. The key components concerned with the shape of crystal growth are related to the crystal lattice of the compound and the effects of the chosen solvents on the process of crystal growth. According to Blagden *et al.* (2007:619*)* a number of studies have been noted in literature which demonstrates the effects of changing crystal morphology on *in vitro* dissolution of compounds. These studies concluded that dissolution of rod shaped particles was notably more rapid than needle shaped crystals. Although there were differences observed in the dissolution rates of varied morphology of crystals, these differences were primarily attributed to changes in surface areas rather than the improvement of wetting of polar surfaces. Increased dissolution rates are derived from a combination of changes in crystal habit, size or polymorphic form (Blagden *et al.,* 2007:619).

Dissolution rates can be optimised by techniques that influence the crystal habit and surface. One technique commonly used to increase the dissolution rates of crystals is micronisation. This method is used to obtain small particles through the disruption of large crystals of sparingly water-soluble compounds. However, due to the high energy input of micronisation, a disruption in the crystal lattice can be seen which may cause physical or chemical instability. A further

disadvantage of micronisation is a broad size distribution of particles (Rasenack *et al*., 2003:138).

Crystals were present in all the formulations (even in Acitop® and Lovire®), as seen in section 3.3, which was expected due to the low solubility of acyclovir. In order to produce the smallest possible crystals, the acyclovir was micronised in the pre-formulation phase. However, this did not evade the formation of crystals. A possible explanation for the change in assay values can be ascribed to non-homogenous sample preparation due to crystals in each formulation. The dissolution of crystals will have an influence on the permeability of the compound as described by Blagden *et al.* (2007:619).

Ketoconazole, on the other hand, did not depict any significant changes in concentration when considering the cream and gel formulations stored at the various conditions. This was an indication of stability regarding ketoconazole concentration in both of these formulated products. However, the lip balm formulation depicted the highest change in ketoconazole concentration compared to the cream and gel formulations. The lip balm was formulated to melt at body temperature (37 ºC) in order to ensure effective application and to ensure efficient contact time on the applied surface (Mitsui, 1997:386). Therefore, it was expected that this formulation would show the most pronounced change in assay. The solubility of ketoconazole increases with an increase in temperature which could have had an effect on the concentration measured. Furthermore, polyethylene glycol 4000 used in the formulation as solvent has a melting point of 50-58 ºC (Rowe *et al.*, 2006:545) that could also have had an influence on the ketoconazole solubility; and thus the concentration measured.

Figures 4.8 - 4.10 depict the percentage ketoconazole present in different formulations stored for 3 months at various storage conditions. The results are presented in tables D4 – D6, annexure D.

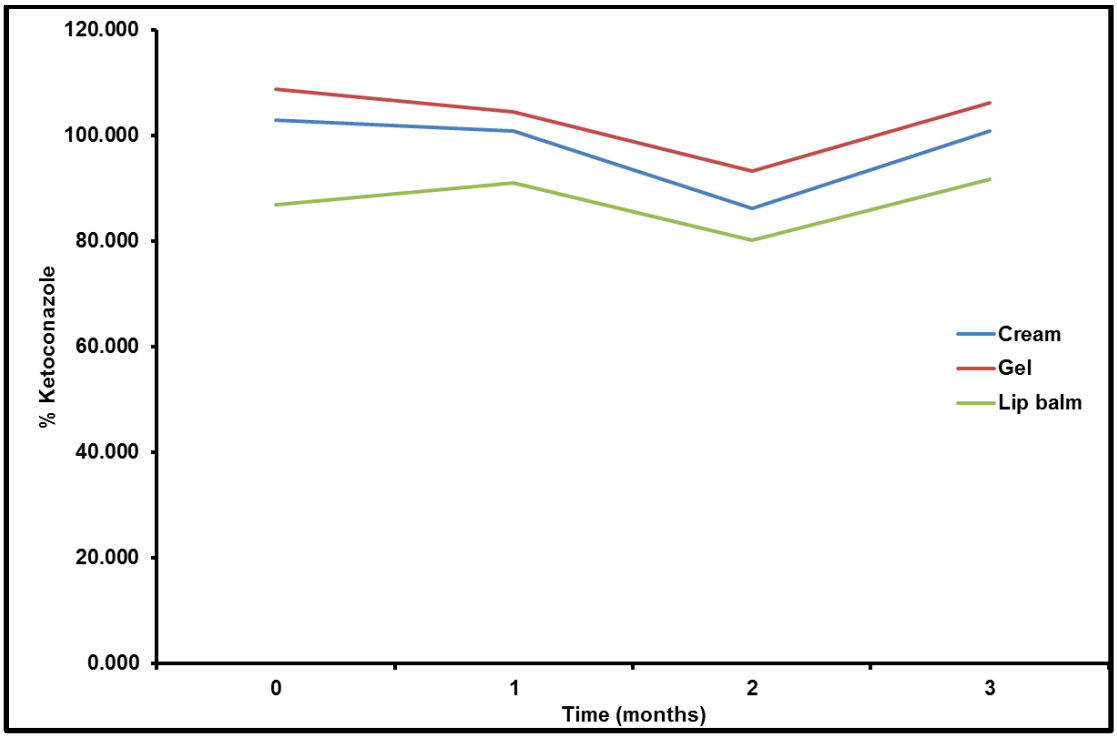


Figure 4.8: *Percentage ketoconazole present in the different formulations at 25 ºC/60% RH after each time interval.*

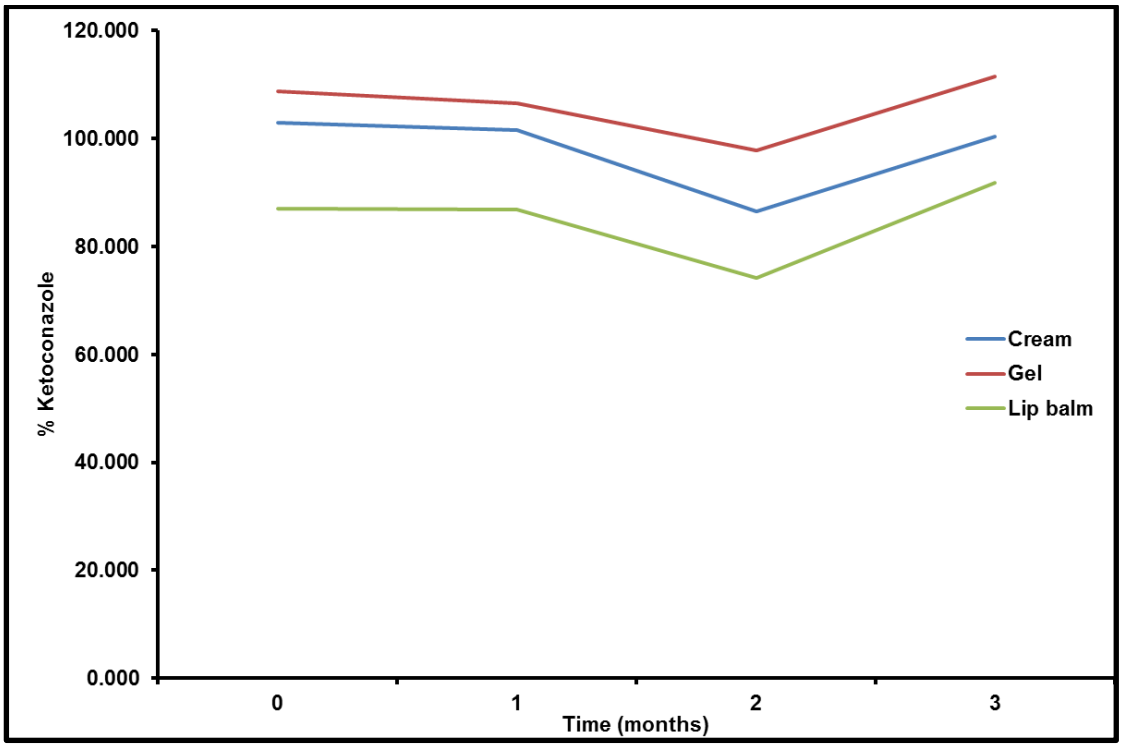


Figure 4.9: *Percentage ketoconazole present in the different formulations at 30 ºC/60% RH after each time interval.*

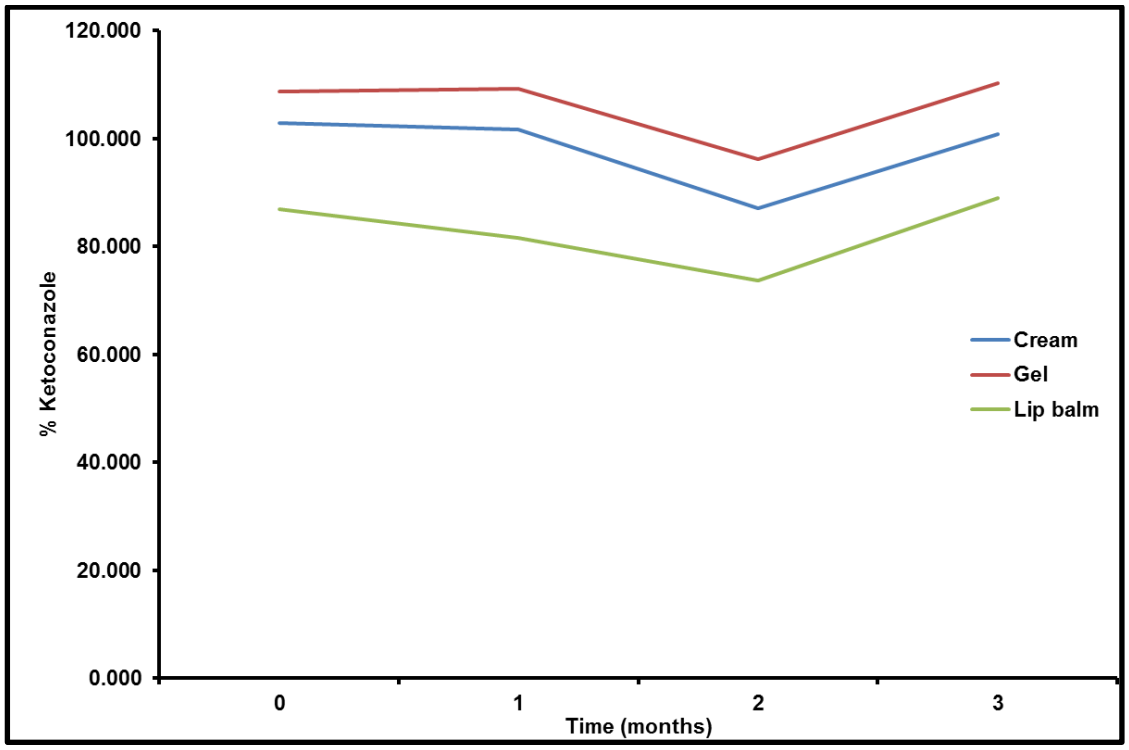


Figure 4.10: *Percentage ketoconazole present in the different formulations at 40 ºC/75% RH after each time interval.*

4.1.4 pH

The stability of a product can be severely influenced by the pH of the formulation. Acyclovir is stable in alkaline conditions and at a neutral pH. Ketoconazole is found to be least stable at a pH of 1 and most stable at a pH of 9 (Skiba *et al.,* 2000:1). According to Barry (2007:576) the skin has a high tolerance to pH changes and can tolerate a pH ranging between 3 – 9. Changes in pH were measured for the cream and gel formulations at the different storage conditions for three months and the results are depicted in figure 4.11 and figure 4.12. Due to the solid state of the lip balm formulation, the pH could not be measured with the electrode of the pH-meter which needs to be placed inside the required formulation.

A change in the pH values obtained (table D.7, annexure D) for the cream formulation over the three month stability testing of the 25 ºC/60% RH condition was 0.007 units. The smallest change was noted at this temperature. According to the ICH this change could be described as insignificant (<5%) and the cream could therefore be regarded as stable with regards to pH.

The gel depicted a decrease in pH of 0.009 units at 25 ºC/60% RH, over the three months of stability testing (table D.8, annexure D). These values are seen as insignificant according to the

ICH (<5%) and indicated that this product is stable with regards to pH. According to this study, this gel should be stored below 25 ºC to ensure maximum stability as the smallest difference was noted at this temperature.

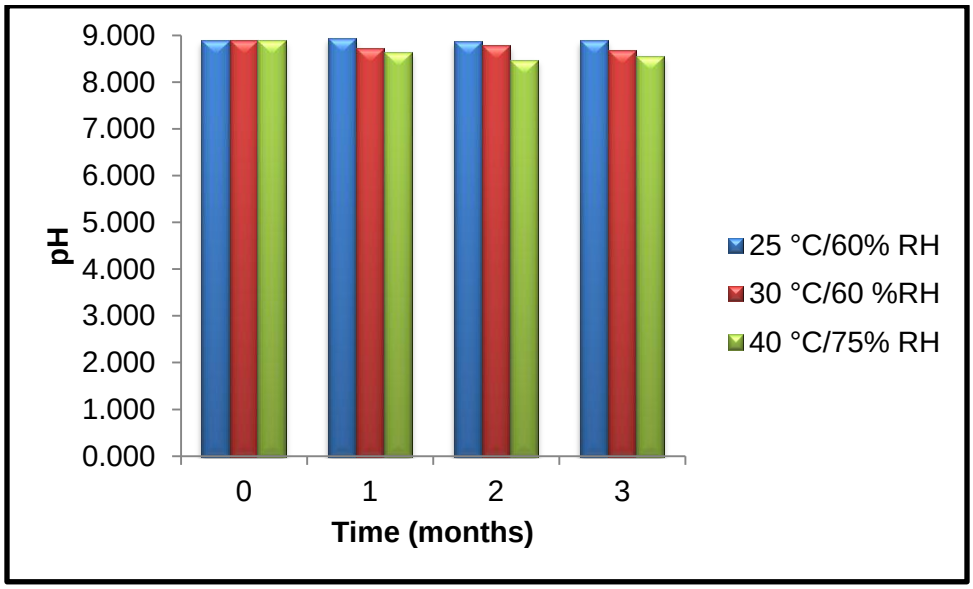


Figure 4.11: *pH measurements for the cream formulation at the different storage conditions after each time interval.*

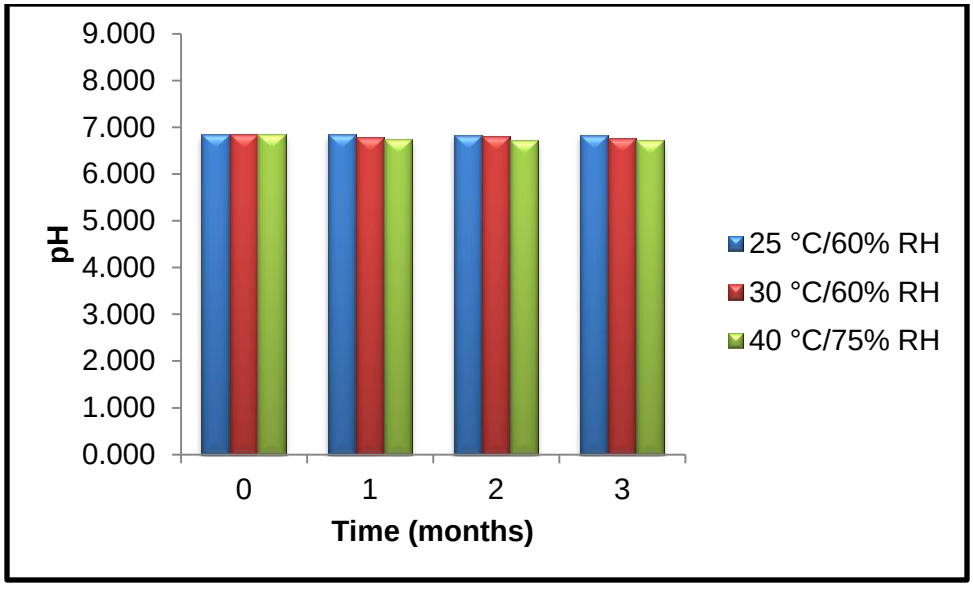


Figure 4.12: *pH measurements for the gel formulation at the different storage conditions after each time interval.*

4.1.5 Viscosity

The viscosity of the cream and the gel were determined at months 0, 1, 2 and 3 at each of the different stability storage conditions. Results are depicted in tables D.9 – D.10, annexure D, and figure 4.13 – figure 4.14.

Directly after the preparation, the cream exhibited a viscosity value of 1010526.6 centipose (cP). At the end of month 3 the viscosity values were 895400.1 cP, 825481.9 cP and 1313879.9 cP for the three storage conditions respectively. The effect of storage condition had a variable effect on the viscosity. No clear trends could be observed.

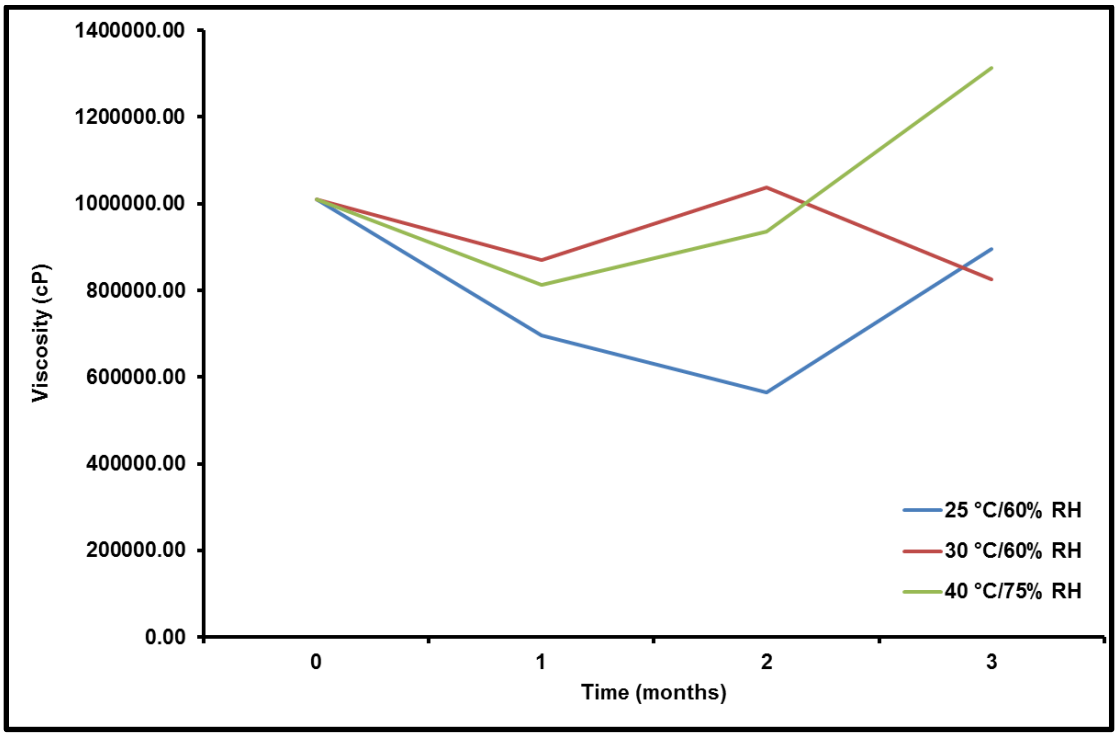


Figure 4.13: *Change in viscosity of cream at the different conditions after each time interval.*

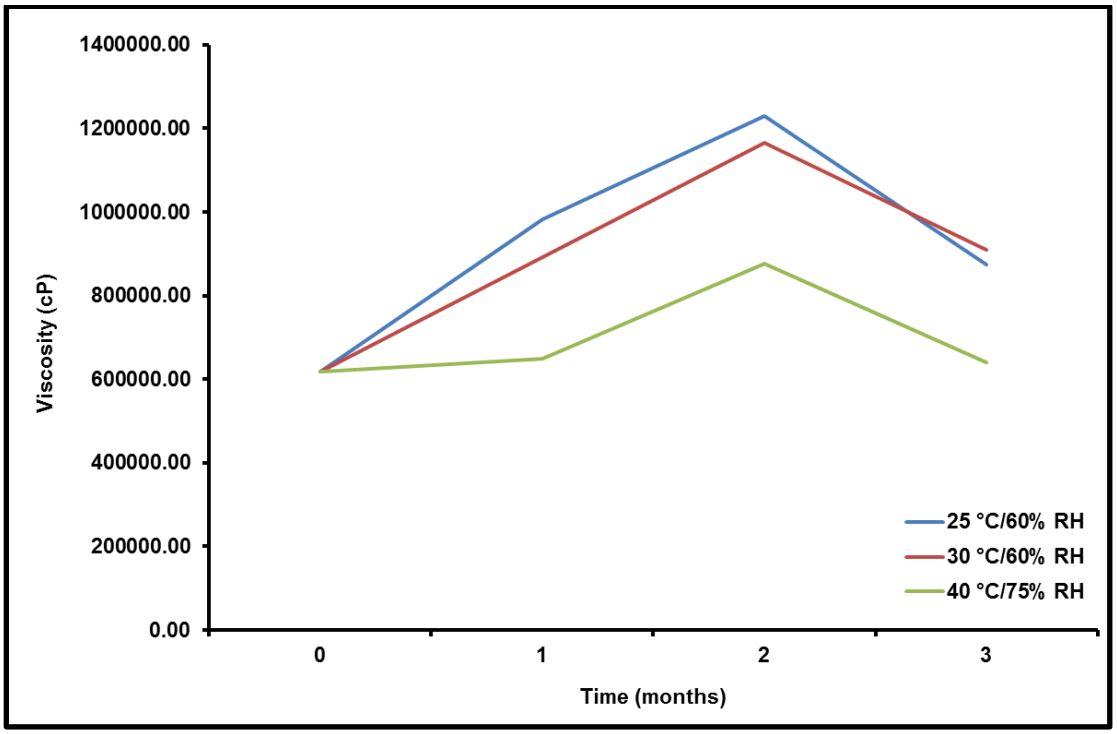


Figure 4.14: *Change in viscosity of gel at the different conditions after each time interval.*

Sodium- carboxymethylcellulose (Na-CMC) was used as thickening agent in the gel formulation, and might be responsible for changes in viscosity seen for this formulation. Gels behave like non-Newtonian liquids and can be pseudoplastic when cellulose is used as thickening agent. This means that the material of the gel will flow as soon as a shear force is applied. The slope of the curve will gradually decrease with an increased rate of shear force (Marriott, 2007:49).

Na-CMC is prone to absorb water if stored in humid conditions and is therefore shipped in moisture proof containers. A product containing Na-CMC will depict an increased viscosity as it dissolves. Progressive hydration takes place in Na-CMC products as dissolution progresses and can influence viscosity significantly. The delay of hydration is dependent on the pH of the formulation and can therefore be controlled by changing the pH value. A formulation containing Na-CMC in powder form will fully hydrate at a pH of 8.5. Furthermore, viscosities of formulations containing Na-CMC are dependent on the temperature. As the formulation is heated, a lower viscosity will initially be depicted. Afterwards a sharp increase will be seen (figure 4.15) as the formulation reaches a type-specific gel temperature and flocculation can occur. As the formulation is heated further, the viscosity will significantly decrease; however, this process is reversible as the viscosity re-asserts itself by cooling down the formulation (Aqualon, 1996:10).

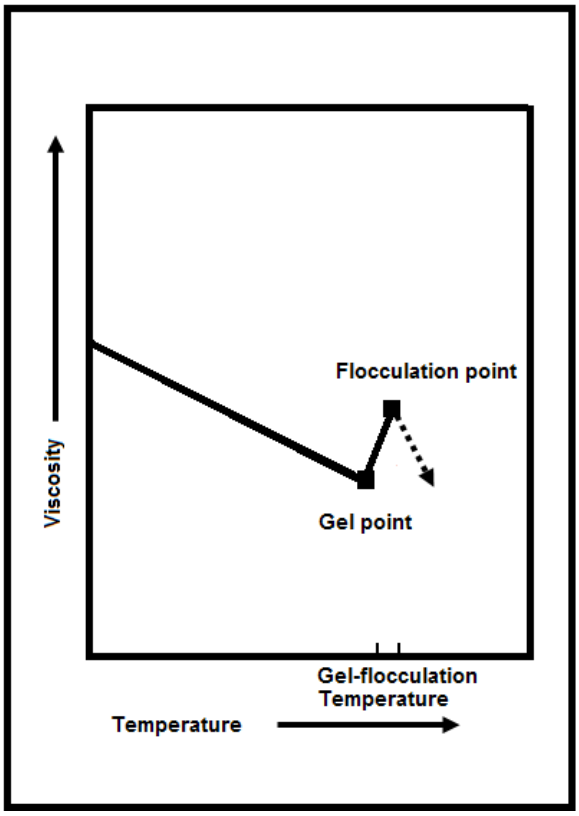


Figure 4.15: *Viscosity curve on increased temperature up to flocculation temperature (Aqualon, 1996:17)*.

Considering the gel formulation, a clear trend could be observed in the viscosity. An increase from the initial value could be seen for the first two months, followed by a decrease at all storage conditions. This was to be expected from the gel formulation due to the properties of temperature on Na-CMC described by Aqualon. (1996:17). As the gel is formulated at pH 6.8, the complete state of hydration for the cellulose was not yet reached, probably causing further fluctuations in the viscosity of the formulation.

4.1.6 Zeta potential

The zeta potential was determined for the initial cream at month 0, as well as at months 1, 2 and 3 for each of the different stability conditions. Table D.11, annexure D and figure 4.16 illustrates the changes that occurred from month 0 to month 3 of stability testing. A complete dispersion could not be formed from the gel or lip balm formulation and as a consequence, the zeta potential could not be determined.

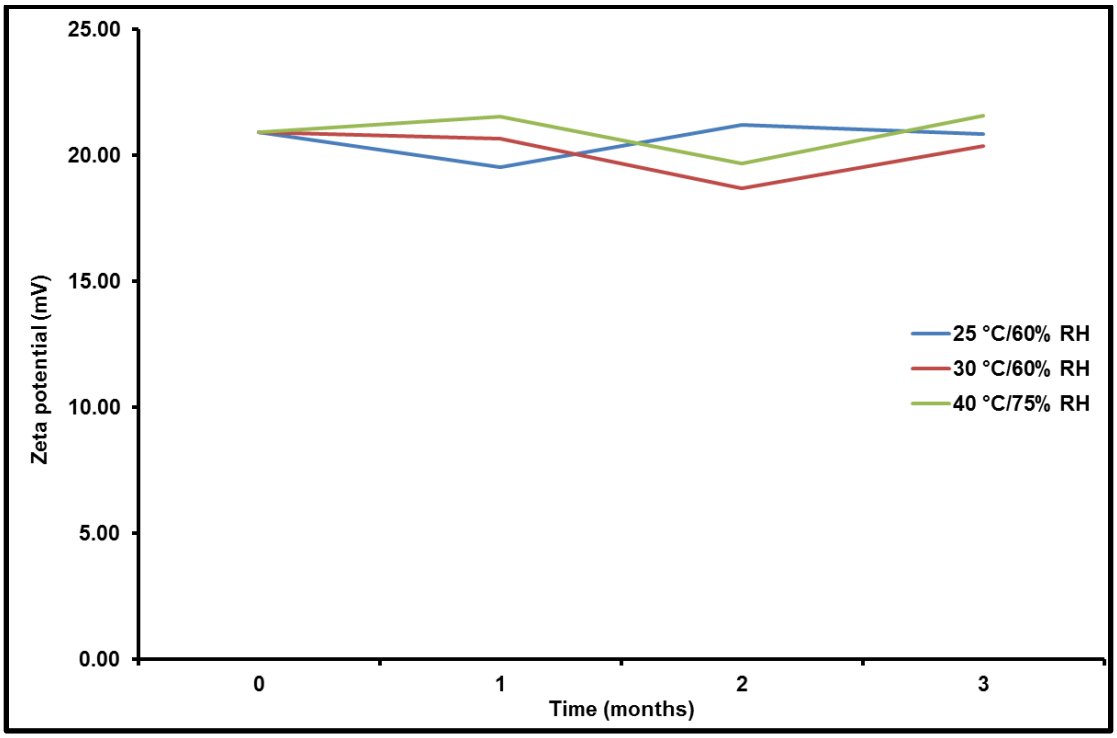


Figure 4.16: *The change in zeta potential (mV) for the cream at the different conditions after each time interval.*

The zeta potential for the cream ranged from 20 mV to 21.5 mV for all storage conditions respectively, indicating similar zeta potential values for the samples at all the different storage conditions. It therefore appears that the storage condition did not have a marked effect on the zeta potential.

4.1.7 Particle size

The particle size of the cream was determined at months 0, 1, 2 and 3 at each of the different stability conditions. Table D.12 in annexure D and figure 4.17 reflects the results, D (0.5) revering to the size in microns (µm) at which 50.00% of the sample is smaller and 50.00% larger. This value is known as the Mass Median Diameter (MMD) (Malvern, 2000:6.3). A fully dispersed sample was required from the formulation in order to conduct this test, which could not be obtained from the gel or lip balm formulation.

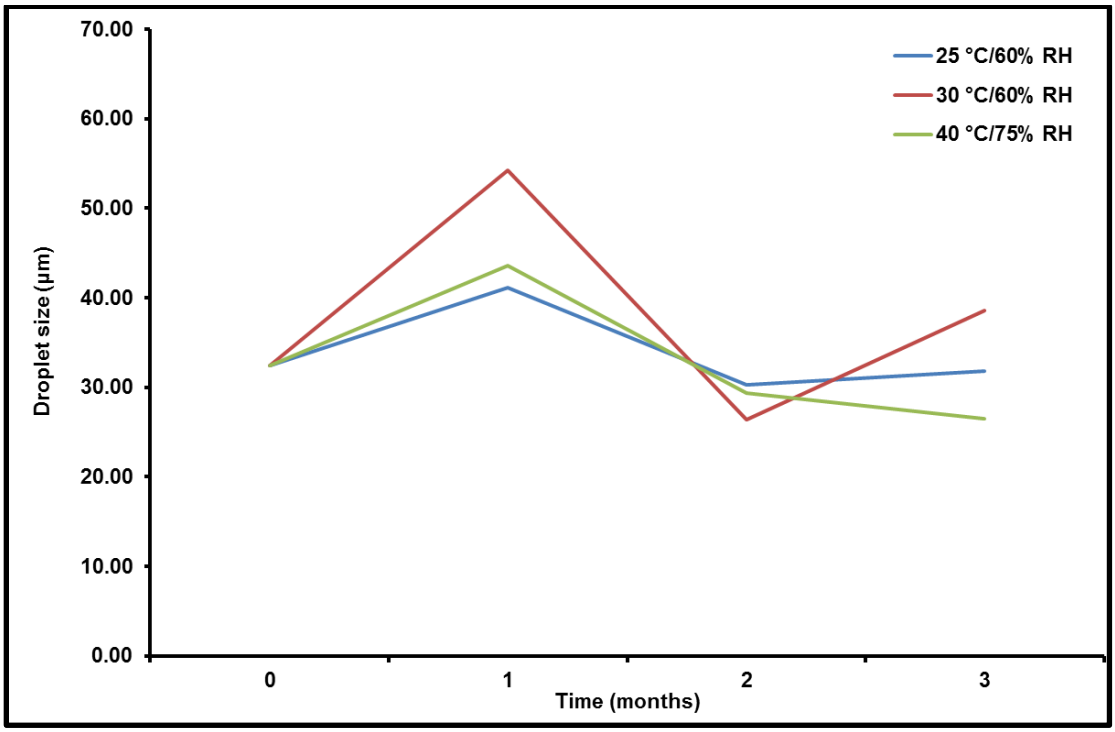


Figure 4.17: *The change in average particle size (µm) for the cream at the different conditions after each time interval.*

The average particle size of the cream depicted an increase over the three months for the all storage conditions. This was to be expected due to the low zeta potential found in this study for the cream formulation. A low zeta potential (below 25 mV) found in dispersions tends to lead to coagulation or coalescence (Jelvehgari *et al*., 2010:1240). The particles in this formulation seem to adhere to one another in the dispersion to form aggregates of significant increased size by means of coalescence.

4.2 SUMMARY

The formulated products were tested for stability over a period of 3 months. The outcomes of the stability tests showed that all three formulations presented acceptable results for the specific tests conducted. No significant changes were noted in the visual appearance, mass variation and pH values of all tested formulations at the specified storage conditions.

Results obtained from the assay indicated a significant increase in acyclovir concentration for both the cream and lip balm formulations. Acyclovir is slightly soluble in water and has a solubility of 1.3 mg/ml at 25 ºC according to Bethesda (2010). Low solubility often causes

crystal formation in products. All of the formulations developed crystals on the surface. The differences in assay concentration can be due to non-homogenous sample preparations caused by crystals in the formulations. Ketoconazole did, however, not depict any significant changes in concentration for any of the formulations at all storage conditions.

The viscosity of the cream depicted variable changes over the three months, showing no clear trend. A possible explanation for this tendency could have been the low zeta potential depicted during this study, which could have caused possible coalescence. Furthermore, the energy of attraction arises from van der Waals forces between the molecules of the cream. If these forces are disrupted for example by stirring, a decrease in viscosity is observed (Attwood 2007:80).

In the gel formulation a definite trend could be observed in the viscosity results depicting an increase in viscosity followed by a sharp decrease. The Na-CMC used as the thickening agent in this formulation was responsible for this trend obtained in the results, due to the effects of pH, hydration and temperature on this excipient (Aqualon, 1996:10).

Zeta potential was tested for the cream formulation which depicted no significant change and the values remained below 25 mV. When all particles of a dispersion present with larger zeta potentials (above 25 mV), they tend to repel each other and therefore are inclined not to coalescent (Jelvehgari *et al*., 2010:1240). In this stability study a zeta potential below 25 mV was observed which is an indication of possible coalescence.

Another factor to consider was the average size of the particles in dispersion. It is important to determine this factor as only particles smaller than 3 µm is able to penetrate the skin's follicles and stratum corneum. Particles larger than 10 µm remain on the skins surface (Barry, 2007:573). The cream depicted an increase in particle size over the three months stability testing. Due to the low zeta potential it was expected that coalescence would occur over time, which would explain the increase in particle size of the cream formulation.

From these results it was clear that manufacturing formulations containing both acyclovir and ketoconazole proved difficult due to the significant differences between their physicochemical properties. Furthermore, it was evident that formulation at specific pH values as well as the incorporation of certain excipients played a significant role in the stability of formulations.

CHAPTER 5: ARTICLE FOR PUBLISHING IN THE INTERNATIONAL JOURNAL OF PHARMACEUTICS

Evaluation of the topical delivery of different dosage forms containing acyclovir and ketoconazole

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ABSTRACT

The aim of this study was to formulate topical products containing both acyclovir (5% w/w) and ketoconazole (2% w/w) which can be applied to mucosal tissue. The rationale behind this combination lies in the synergy provided by ketoconazole in anti-viral activity when used together with acyclovir. Ketoconazole has a broad anti-fungal activity and acyclovir is the anti-viral agent used for most pathological viral infections. Dermatological manifestations often occur in immune compromised patients. *In vitro* permeation studies were conducted using porcine vaginal mucosa in a flow-through diffusion system over a 24 hour period. A cream, gel and lip balm formulation containing both acyclovir and ketoconazole were manufactured and compared to the commercial products Acitop® cream and Ketazol® cream. Stability tests were conducted over a three month period. A flux for acyclovir and ketoconazole was depicted from all of the formulations indicating effective release. No statistical significant differences were obtained for the release of acyclovir through the mucosa when compared to Acitop®. However, a significant increased release of ketoconazole was seen from the lip balm formulation in comparison with Ketazol® cream.

Keywords: acyclovir, ketoconazole, topical delivery, mucosa, stability, compatibility.

5.1 INTRODUCTION

Since the first reported cases of acquired immunodeficiency syndrome (AIDS) more than 25 years ago, occurred, we are still unable to fully grasp the complexity of this disease. Infection with the human immunodeficiency virus (HIV) may lead to a variety of clinical manifestations on the skin and can be very diverse (Dlova & Mosam, 2004:12; Cedeno-Laurent *et al.,* 2011:5). The clinical dermatological lesions seen in HIV/AIDS patients are caused by decreased mucocutaneous immunity (Uthayakumar *et al*., 1997:595). Severity of the dermatological condition increases as the cluster of differentiation 4 (CD4) lymphocyte cell count decreases. The CD4 cell count is important in HIV/AIDS as it serves as a marker to assess progression of the disease as well as the time to initiate treatment (Sharma *et al.,* 2004:239). According to Rigopoulos *et al*., (2004:493) treatment should commence when the CD4 cell count decreases below 350 cells/µl.

Numerous pathological manifestations develop on the skin during HIV/AIDS, including fungal, viral and bacterial infection, which can cause emotional distress as the skin is the most visible organ. In HIV/AIDS patients, dermatological disorders are almost inevitable and occur in approximately 90% of the patients (Dlova & Mosam, 2004:12; Cedeno-Laurent *et al.,* 2011:5). The most commonly found dermatological manifestations that present in HIV/AIDS patients are fungal infections including: candidiasis, dermatophytosis, cryptococcosis, histoplasmosis and sporotrichosis. The defective immune system of HIV/AIDS patients provides an ideal environment for fungi to invade the tissue of the skin and cause damage (Durden & Elewski, 1997:200; Dlova & Mosam, 2004:13). As a result from a damaged immune system in HIV/AIDS patients, opportunistic viral infections increase among these patients. The *herpes simplex* virus (HSV) has a high prevalence in low immunity patients, however, *molluscum contagiosum* virus (MCV) and human papillomavirus infection also increases in prevalence in HIV/AIDS patients (Dlova & Mosam, 2005:12).

Viruses are parasites consisting of either double- or single-stranded deoxyribonucleic acid (DNA) or ribonucleic acid (RNA). Some of these DNA viruses include *herpes* viruses (oral and genital *herpes*) and papillomaviruses (warts). Different antiviral agents are developed depending on their mechanism of action against these viruses including; uncoating of viral nucleic acid, transcription of viral RNA and cell entry of virus into host cell. There are several compounds that have activity against viruses for example the herpes virus including acyclovir, famciclovir, ganciclovir, trifluridine, valaciclovir and valganciclovir (Beers, 2006:1061). Acyclovir inhibits viral DNA synthesis in a process requiring viral thymidine kinase and is well tolerated

topically (Beers, 2006:1061; Hayden, 2006:1243). Anti-viral activity from acyclovir is seen against HSV-1, HSV-2 and the *varicella-zoster* virus (VZV). However, acyclovir exhibits minimal activity against the cytomegalovirus (CMV) (King, 1988:176; Beers, 2006:1061).

Antifungal agents are used for systemic or topical treatment. Azole anitifungal agents dominate the antifungal drug use and development. Two broad classes of azoles with the same spectrum and mechanism of action exist, namely imidazoles and triazoles. The imidazole agents are used either systemically or orally. Azoles have shown activity against *Candida spp*, *Cryptococcus neoformans* and *Histoplasma capsulatum*. *Sporothrix schenckii*, however, is less susceptible to azole therapy. In HIV patients, dermatophytic diseases are best treated with oral antifungal agents such as terbinafine, itraconazole or fluconazole. Amphoterin B posesses activity against the same species as azoles and is often used in many mycoses treatments (Bennet, 2006:1225). Topical preparations may be helpful, especially in cases where there is a low respons to oral antifungal agents, but does not serve as a cure (Johnson, 2000: 413).

The target site for acyclovir and ketoconazole (after topical application) is the basal epidermis. In order to inhibit the growth of viral and fungal pathogens in the skin, adequate concentrations of each compound should be delivered to this target site of the skin (Persing *et al.,* 1994; Jiang *et al.,* 1998). The physicochemical properties of acyclovir may lead to transdermal penetration as the aqueous solubility in water is 1.3 mg/ml at 25 ºC and molecular weight is 225.205 g/mol according to Bethesda (2010). However, the high melting point of 256.5 - 257 ºC and low log P value of -1.56 could present an obstacle in membrane penetration (Drug bank, 2012). Ketoconazole, on the other hand, presents a lower melting point (146 ºC), but has a very low aqueous solubility in water (40 mg/ml at 23 ºC), a higher molecular weight (531.431 g/mol) and a high log P value of 4.4 (Bethesda, 2010). According to these properties it can be predicted that ketoconazole will have poor penetration into the skin.

Passive diffusion is mostly responsible for a compound to permeate from the formulation, through the tissue and into the skin. *In vitro* diffusion experiments are one of the important studies done in determining transdermal delivery of different formulations in the development of new preparations in industry and academia (Cordoba-Diaz *et al.,* 2000:357). Advantages of *in vitro* testing include direct measuring of permeation immediately below the skin surface. This is in contrast to *in vivo* methods where systemic levels of the permeant are measured. Brain *et al*., (2002:198) stated that the most appropriate method to determine percutaneous compound delivery, is *in vitro* diffusion for both, transdermal or topical absorption.

Franz-type diffusion cells are commonly used, but present with some inconveniences when compared to flow-through diffusion cells. The design of flow-through receptor chambers allows for continuous flow throughout the experiment which is important in maintaining sink conditions, particularly with compounds containing large permeability coefficients through a membrane (Cordoba-Diaz *et al.,* 2000:357). Furthermore, automated flow-through diffusion systems offer the advantage of unattended sampling. *In vitro* diffusion conditions in flow-through diffusion systems can also be controlled in such a way that the only two variables will be the skin; and the test material. One disadvantage of *in vitro* diffusion is that little information on metabolism, distribution and blood flow can be obtained (Brain *et al*., 2002:198).

An essential consideration for pharmaceutical and cosmetic products is the stability of the product. The physical stability of these products is characterised by absence of coalescence or creaming, and the maintenance of appearance, odour, colour and other physical properties. The objective of stability testing is to ensure that a given product has an adequate shelf-life in its container, before it can be marketed and sold. According to the Food and Drug Administration (FDA) semisolid preparations should be evaluated for appearance, colour, homogeneity, odour, pH, viscosity, particle size distribution (when feasible), assay, degradation and weight variation (Sheraz, 2009:46).

This study was conducted to determine if a stable product can be formulated containing both acyclovir and ketoconazole that would provide an efficient flux for both compounds when applied to the mucosal membranes. *In vitro* permeation studies were performed to determine skin permeation of acyclovir and ketoconazole by using a flow through diffusion system. The formulated products were compared to Acitop® and Ketazol®, which are two products available on the South African market, containing acyclovir and ketoconazole, respectively. However, no product is yet available containing both acyclovir and ketoconazole.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Acyclovir: 2-amino-9-[(2-hydroxyethoxy)methyl]-1,9-dihydro-6*H*-purine-6-one (MW = 225.2 Da) and ketoconazole: 1-acetyl-4-[4-[[(2RS,4SR)-2-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)1,3-dioxolan-4-yl]methoxy]phenyl]piperazine (MW = 531.4 Da) were obtained from DB Fine chemicals (Pty) Ltd. (Rivonia, Johannesburg, South Africa). Potassium dihydrogen orthophosphate (Merck, Wadeville, South Africa) and sodium hydroxide (Merck, Wadeville, South Africa) were used in the preparation of phosphate buffer, used as the solvent for the active compounds during the *in vitro* permeation studies. Octane-1-sulfonic acid sodium salt (Merck, Darmstad, Germany) and methanol AR (Merck, Darmstad, Germany) were used in the mobile phase of the high-performance liquid chromatography (HPLC) analysis of the permeation studies.

The ingredients used in the formulation of the products were obtained as follow: liquid paraffin, methyl paraben, propyl paraben, propylene glycol cetylstearyl alcohol, polyethylene glycol 400 and polyethylene glycol 400 were obtained from Merck Laboratory Supplies (Midrand, South Africa). Cremaphor® A 6 and Cremaphor® A 25 were obtained from BASF Chemicals (Johannesburg, South Africa). Sodium carboxymethyl cellulose and methyl paraben were obtained from Sigma (St Louis, USA). Water used during all preparations and formulations was purified by a Milli-Q academic purification system (Millipore, Milford, USA).

5.2.2 Methods

5.2.2.1 Compatibility study

Compatibility of ketoconazole and acyclovir was determined with a Thermal Activity Monitor equipped with an oil bath with a stability of ± 100 µK over a 24 hr period. A reference ampoule (control), as well as four samples were prepared. All samples were sealed with rubber stoppers and aluminium crimp seals.

5.2.2.2 Formulation of cream, gel and lip balm

Both acyclovir and ketoconazole were incorporated into existing formulae (cream, gel and lip balm) obtained from literature and altered as necessary. The main ingredients of each formulation were analysed by means of an assay method developed. Products were prepared

in bulk for testing purposes and stored. A stability program was conducted according to the International Conference of Harmonisation (2003) Tripartite Guidelines at three different storage conditions for a time period of three months.

5.2.2.3 Permeation studies

5.2.2.3.1 Collection of mucosal specimens

Vaginal porcine mucosal specimens were obtained from slaughtered pigs at the Faculty of Health Sciences of the University of Witwatersrand, Gauteng, South Africa. All permeation studies were conducted at the University of Witwatersrand. No ethical approval was required for this specific study as the animals were not kept at the university specifically for the conduction of tissue experiments, but they were used in the training of students at the School of Medicine.

Porcine vaginal mucosa was found similar to human vaginal mucosa in various ways. There are similarities in their lipid compositions and it also shows comparable histological characteristics (Kremer *et al*., 2001:851; Thompson *et al*., 2001:1091; Davis *et al*., 2003:1785).

All the specimens were transported to the laboratory within 1 hour and were placed in transport fluid consisting of phosphate buffer saline (pH 7.4) or Eagle's Minimum Essential Medium (MEM) without L-glutamine and $NaHCO_3$. Excess connective and adipose tissue were trimmed away. Specimens were snap-frozen in liquid nitrogen and stored at –85 ºC. No specimens were used if any signs of disease were indicated that might influence the permeability characteristics of the vaginal mucosa.

5.2.2.3.2 Permeability experimental methods

For each diffusion study, an individual mucosa specimen was removed from the snap freezing compartment. Specimens were thawed to room temperature (25 ºC) in phosphate buffer (pH 7.4) within 10 min, and cut into 4 mm diameter pieces. These specimens were mounted onto metal tissue disks with exposed areas of 0.039 cm^2 and placed in the flow-through diffusion cells.

Flow-through diffusion cells with fraction collector (Retriever IV, Teledyne Isco, Lincoln, USA) was used for the permeation studies and comprised of a donor compartment and a receptor compartment. The formulation was placed in the donor compartment and the tissue specimens on the tissue disks were placed in the receptor compartment. Phosphate buffer (pH 7.4) was circulated through the receptor phase by means of a pump (Ismatec, Wertheim-Mondfeld,

Germany). Prior to commencement of each experiment, the tissue disks were equilibrated for 10 min with a phosphate buffer solution (pH 7.4) in both the donor (approximately 10 ml volume) and the receiver compartment of the cells.

Each cell was filled in the donor compartment with 1 ml formulation using a syringe. The filled donor compartment was placed on top of the tissue disks. Metal clamps were placed on the cells and used to keep each cell in place. Each cell was visually inspected for air bubbles through the glass window on the bottom of the cell. Air bubbles were manually removed by means of a water pump. The system was sealed using Parafilm® and a plastic lid to prevent contamination or possible evaporation.

Permeation studies were performed on seven tissue replicates for each formulation. Phosphate buffer solution (pH 7.4) at 25 ºC was pumped through the receiving chambers of the receptor phase at a rate of 1.5 ml/h. The phosphate buffer was used to dissolve the permeants which penetrated though the skin into the receptor compartment. Samples were collected in a fraction collector at 2 hr intervals for a 24 hrs period.

The permeation studies were performed under sink conditions and the chambers were kept at 37 ºC by means of a circulating water bath (Grant instruments, Cambridge, England). Scintillation vials (Ready Protein+™, Beckman Instruments, Fullerton, CA, USA) with a volume of 20 ml were used as collectors of samples on the scintillation counter. Quenching of each sample was automatically corrected in the counter. After completion of the 24 h diffusion studies, the scintillation vials were removed from the fraction collector and 1.5 ml of each sample was transferred into an HPLC vial by means of an Eppendorf® pipette (Hamburg, Germany). HPLC analyses were used to determine the concentration of compounds.

5.2.2.3.3 Preparation of the phosphate buffer solution

Buffer was prepared by weighing 6.81 g potassium dihydrogen orthophosphate and dissolving it in 250 ml Milli-Q® water. Sodium hydroxide (1.571 g) was weighed and dissolved in 393.4 ml Milli-Q® water. The two solutions were mixed and the pH of the subsequent solution was adjusted to 7.4 with 10% v/v orthophosphoric acid.

5.2.2.3.4 Preparation of mobile phase

Octanesulfonic acid solution was prepared by accurately weighing 1.0 g octane-1-sulfonic acid sodium salt. It was dissolved in 950 ml HPLC water and the pH was adjusted to 3.5 ± 0.01 with a 10% orthophosphoric acid solution. The volume (1000 ml) was made up with HPLC-water.

5.2.2.3.5 Analysis of permeation studies

HPLC analysis of acyclovir and ketoconazole was conducted by means of a method developed and validated at the North-West University, Potchefstroom Campus, South Africa. The samples collected from the *in vitro* permeation studies were analysed by means of HPLC. A luna (5 µm, 250 x 4.60 mm), C-18 reversed phase column was used for the HPLC analysis. The mobile phase consisted of 300 ml analytical grade methanol and 700 ml of an octanesulfonic acid aqueous solution (0.1% v/v). The methanol concentration was increased from 30% to 100% over 8 min. The flow rate was set to 1 ml/min and a temperature of 25 ºC was maintained. A total runtime was set to 30 min. The UV detector was set at 252.4 nm for the detection of acyclovir and at 243.2 nm for ketoconazole. Acyclovir depicted a retention time of 3.6 – 3.7 min, whereas ketoconazole was retained for 9.6 - 9.7 min.

5.2.2.3.6 Permeation and statistical data analysis

Data were analysed in such a way that assumptions for all procedures were met. As a result of the fact that the data was not normally distributed, non-parametric tests were performed, using Kruskal Wallis tests to determine if any statistical significant differences between the means of the test compounds and the standard compounds existed in general (Steyn *et al*., 1998:406). Dunn's post hoc multiple comparison tests were conducted to determine statistical significant differences between the test compounds. Bonferonni corrections on Dunn's p-values were calculated to determine which of the test compounds' means differed statistical significantly from the mean of the test compounds (Siegel & Castellan, 1988:213).

These procedures were done using the statistical data analysis software system Statistica (Statsoft, Inc. 2007). All tests were done at a 0.05 significance level.

5.2.2.4 Stability program

Three different semi-solid formulations (cream, gel and lip balm) were formulated and stored at 25 ºC/60% relative humidity (RH), 30 ºC/60% RH and 40 ºC/75% RH. An intermediate stability test study was conducted according to the ICH (2003). The formulated products were stored in Labcon® humidity chambers (Johannesburg, South Africa). The following stability tests were done at 0, 1, 2 and 3 months: visual appearance, assay, mass variation, pH, viscosity, zeta potential and particle size. Due to the different properties of the gel and lip balm formulations, some of the stability test could not be conducted.

5.2.2.4.1 Visual appearance

Photographs were taken with a Samsung ES15 camera at months 0, 1, 2 and 3 to determine whether any visual changes occurred in the physical appearance of the products. These photos were compared to a colour chart obtained from a local paint store in order to determine any colour changes that might have occurred. The smell, texture and overall appearance were manually inspected.

5.2.2.4.2 Mass variation

Each of the individual containers stored at the various stability conditions was initially weighed prior to storage on a calibrated Shimadzu AUW 120 D balance and marked. At months 1, 2 and 3 the foil wrapping was removed from each container and the container weighed for a total of three times to ensure accuracy.

5.2.2.4.3 Assay

Approximately 1 g from each formulation was weighed in triplicate and transferred to a 100 ml flask using syringes with tubing equipped at the tip. Methanol was added to the samples and placed in the ultrasonic bath until completely dispersed. The samples were shaken by hand to ensure complete dispersion in the methanol. Samples were left to cool down to room temperature and filled to volume in the various flasks. They were transferred to HPLC vials and analysed by means of HPLC. The assay determination for each of the formulated products was done with an Agilent 1100 Series HPLC system.

5.2.2.4.4 pH

In order to measure the pH of each formulation a Mettler® Toledo pH meter (Schwerzenbach, Switzerland) with a glass Mettler Toledo Inlab® 410 electrode was used. Calibration of the apparatus was conducted prior to each test. The pH of the cream and gel formulations stored at the different conditions was measured in triplicate.

5.2.2.4.5 Viscosity

Viscosity of the cream and gel formulations was determined with a Brookfield Model DV – II+ viscometer (Massachusetts, USA). The temperatures of the samples were controlled by the circulating water bath of the viscometer, set at 25 ºC. A Helipath spindle set was used to measure the viscosity and the Helipath D20733 (Massachusetts, USA) was used to move the viscometer up and down in the formulations. The viscosity readings were measured for 5 min,

with readings taken every 10 s, rendering 32 measurements. The temperatures of the samples were controlled by a circulating water bath at 25 ºC.

5.2.2.4.6 Zeta potential

Samples were prepared by accurately weighing 500 mg of the cream formulation stored at each storage condition in a 50 ml volumetric flask in triplicate. The volume was made up with 0.1 M potassium chloride and placed in an ultrasonic bath to disperse and injected into the Malvern® Zetasizer (Worcestershire, United Kingdom) and the zeta potential of each sample was measured in triplicate.

5.2.2.4.7 Particle size

Approximately 0.5 g of the cream formulation stored at each stability condition was mixed with roughly 2 ml HPLC water in order to form a dispersion. These dispersions were made up with approximately 5 ml HPLC water, mixed and injected in triplicate into a Malvern® Mastersizer 2000 (Worcestershire, United Kingdom) using the wet cell, Hydro 2000 SM dispersion unit.

5.3 RESULTS AND DISCUSSION

5.3.1 Incompatibility determination between acyclovir and ketoconazole

Microcalorimetry was used to detect any incompatibilities and instabilities between acyclovir and ketoconazole. The spikes visible in figure 5.1 indicated the change in heat flow due to a change in temperature, whereas the areas between the spikes were the areas of heat flow. If there existed any form of incompatibility during the temperature elevation steps, it would have been visible through an increase or decrease in the normalised heat flow. No increase or decrease in the normalised heat flow was observed, and therefore, it could be concluded that acyclovir and ketoconazole will be compatible when used in combination. From the data obtained, an interaction graph was constructed (figure 5.2) from this graph is was clear that during an isothermal run of 80 ºC for a period of 3 days, no increase or decrease in heat flow of more than 1 µW/g occurred. Therefore, no physicochemical interactions were observed between acyclovir and ketoconazole.

Figure 5.1: *Heat flow data of acyclovir and ketoconazole sample*.

Figure 5.2: *Interaction graph for acyclovir and ketoconazole raw material.*

5.3.2 Formulated cream, gel and lip balm

The cream formulation presented with a white colour and a frothy texture that was easy to apply. An off-white colour was seen in the gel formulation which presented with a sticky texture and it remained on the applied surface. The lip balm formulation was an off-white solid product which presented with crystals on the surface. A spearmint flavour and smell was added to the formulation.

5.3.3 Permeation studies

Considering results obtained for acyclovir released from the different formulations (figure 5.3), no statistical significant differences in the average acyclovir amount released were obtained ($p > 0.05$) between the formulations. However, relative differences could be observed from figure 5.3. The cream, gel and lip balm formulations depicted a decreased average cumulative acyclovir amount diffused through the mucosa when compared to Acitop®. The following rank order could be established: Acitop®> gel > cream > lip balm. Furthermore, the gel formulation and Acitop® produced a relatively similar percentage acyclovir diffused of 9.808% (5.91 SD) and 9.692% (4.24 SD), respectively.

Figure 5.3: *Average cumulative amount of acyclovir permeation through the mucosa over 24 hrs.*

Using the Higuchi model, the average cumulative amount of acyclovir released that permeated the mucosa per unit surface area was constructed against the square root of time ($h^{½}$). If the rate of compound released obeys this law, the amount of compound released is a linear function of the square root of time. The average apparent release rate (slopes) and lag times (*x*-axis intercepts) were calculated by means of the linear regression analysis of the plots constructed from ($\mu g/cm^2$) over ($h^{½}$) by means of the Higuchi model as seen in equation 5.1 (Van der Bijl *et al.,* 2006:112).

$$ft = Q = A\sqrt{D(2C - C_s)C_s t} \qquad \textbf{[Eq 5.1]}$$

Where Q is regarded as the amount per time t of unit area A, and C is seen as the initial compound concentration, C_s is the solubility of the compound and D is diffusivity of the compound molecule (diffusion coefficient) in a matrix substance (Dash *et al.*, 2010:219).

All formulations depicted a correlation coefficient (r^2) of 0.9644 – 0.9914 for acyclovir (figure 5.4), indicating that the release of acyclovir from the different formulations could be described by the Higuchi model. No statistical significant differences for acyclovir could be obtained between any of the formulations for % diffused, apparent release constant (ARC), release rate (RR) and lag times.

Figure 5.4: *Higuchi plot for acyclovir in different formulations by plotting the average cumulative amount released over the square root of time.*

Considering the amount of ketoconazole that permeated the mucosa (figure 5.5), both the gel and cream formulations exhibited a smaller average cumulative amount that permeated through the mucosa when compared to Ketazol®. The lip balm was the only formulation that showed a statistically significant ($p < 0.05$) increase in permeation through the mucosa in comparison with Ketazol®. A rank order for the average cumulative amount of ketoconazole that permeated through the mucosa could be established namely: lip balm >>> Ketazol® > gel > cream.

Figure 5.5: *Average cumulative amount of ketoconazole permeation through the mucosa over 24 hrs.*

Figure 5.6 was obtained by constructing the average cumulative amount of ketoconazole released that permeated the mucosa per unit surface area against the square root of time ($h^{½}$). From this figure it was clear that the rate of ketoconazole released from all of the different formulations obeyed the Higuchi model as the amount of compound released from each formulation was a linear function of the square root of time (r^2 = 0.9584 - 0.9899).

Figure 5.6: *Higuchi plot for ketoconazole in different formulations by plotting the average cumulative amount released over the square root of time.*

Statistically significant ($p < 0.05$) differences were furthermore noted between the lip balm and both the cream and gel formulations when % diffused, ARC and RR were compared. The lip balm depicted the highest percentage diffused, the highest ARC as well as the fastest RR, however, no statistical differences were obtained between the cream and gel formulation even though the gel formulation performed slightly better. Considering the lag time, all the formulations presented with a relatively shorter initial time of release of less than an hour. Shorter lag time values indicate that the ketoconazole was preferentially released by the base of the formulations. Statistically significant differences ($p < 0.05$) between the lag times of Ketazol® and the lip balm formulation, as well as between the lip balm and the cream and gel formulations were obtained. These results might have been due to the type of formulation in

which the ketoconazole was incorporated into. Ketoconazole is practically insoluble in water and is relatively lipid soluble. The lip balm formulation did not comprise of any water as compared to the other formulations. Ketoconazole might therefore have been more soluble in the lip balm formulation and would therefore have been more permeable through the mucosa. Furthermore, the permeation studies were conducted at body temperature (37 ºC) where the polyethylene glycol 4000 (solvent in the lip balm formulation) proceeded to liquefy, causing the formulation to melt, which in turn contributed to increased dissolution of the compound (Rowe *et al.,* 2006:545).

5.3.4 Visual appearance

Colour changes were noted by comparing the cream with a colour chart obtained from a local paint store. The overall appearance of the cream changed slightly over the period of three months. After three months the cream depicted a change of 2 shades darker on the colour chart (OW 105-soap suds to OW 107-bridal bouquet). No smells, oiliness or other signs of degradation was found for the cream formulation, which was an indication of stability. Furthermore, the texture of the cream remained frothy through-out the 3 month period at all the storage conditions. Thus, the container was deemed suitable for storage of the cream formulation.

Considering the gel formulation, no significant colour changes occurred at any of the different stability storage conditions over the 3 month period. The gel remained an off-white colour (OW 131-lovely lace) and no change was observed when the photographs were compared to the colour chart. This was an indication of stability. Furthermore, no changes in smell, texture or moisture content was noted and the gel continued to have a sticky feel through-out the test period. Thus, the container was considered suitable for storage of the gel formulation.

The lip balm formulation was formulated with an off-white colour (OW 12 -amberling), a spearmint flavour and smell. No change in colour was observed for any of the storage conditions over the three month testing period. The flavour and smell did not change and remained unchanged for each of the storage conditions during the three months. Crystal formation was, however, visible at the surface of all of the formulation samples stored at the different storage conditions. Furthermore, at the 40 ºC/75% RH condition, an oily layer developed on the surface of the formulation at month 1. This was probably due to the low melting point of polyethylene glycol (PEG) 4000 (50 - 58 ºC) used during manufacturing (Rowe

et al., 2006:545). The oil layer was due to the PEG 4000 liquefying, and therefore, not an instability (Mitsui, 1997:386).

5.3.5 Mass variation

No significant change in mass was observed for all the formulations stored at the various storage conditions during the testing period. The highest decrease in mass was seen at the 40 ºC/75% RH condition for the gel formulation, which was only 0.40%. It could therefore be said that all of the formulations were stable in their containers when considering mass variation.

5.3.6 Assay

Considering both the cream and lip balm formulations, the concentration of acyclovir, increased with more than 10.00% (cream formulation) and more than 15.00% (lip balm formulation), respectively, at each of the temperatures over the three months. This marked change in concentration observed could be an indication of product instability. The gel formulation, on the other hand, depicted the least variation in assay over the 3 month period for all of the storage conditions compared to the other formulations. It could be considered stable as the overall change in acyclovir concentration was less than the stipulated value of 5% for all the storage conditions.

Crystals were present in the all the formulations (even in Acitop® and Lovire®), which was expected due to the low solubility of acyclovir. In order to produce the smallest possible crystals, the acyclovir was micronised in the pre-formulation phase. However, this did not evade the formation of crystals.

Ketoconazole, on the other hand, did not depict any significant changes in concentration when considering the cream and gel formulations stored at the various conditions. This was an indication of stability regarding ketoconazole concentration in both of these formulated products. The solubility of ketoconazole increases with an increase in temperature which could have increased the homogeneity of the formulated products.

5.3.7 pH

A change in the pH value obtained for the cream formulation over the three month stability testing of the 25 ºC/60% RH condition was 0.007 units. The smallest change was noted at this

temperature. According to the ICH this change could be described as insignificant (< 5%) and the cream could therefore be regarded as stable with regards to pH.

The gel depicted a decrease in pH of 0.009 units at 25 ºC/60% RH, over three months. This was seen as insignificant according to the ICH (< 5%) and indicated that this product was stable.

5.3.8 Viscosity

Directly after the preparation, the cream exhibited a viscosity value of 1010526.6 cP. At the end of month 3 the viscosity values were 895400.1 cP, 825481.9 cP and 1313879.9 cP for the three storage conditions, respectively. The effect of storage condition had a variable effect on the viscosity. No clear trends could be observed.

Considering the gel formulation, a clear trend could be observed in the change in viscosity. An increase from the initial value could be seen for the first two months, followed by a decrease at all storage conditions at month 3. This was to be expected from the gel formulation due to the properties of temperature on sodium carboxymethylcellulose (Na-CMC) used as the thickening agent described by (Aqualon, 1996:10). As the gel is formulated at pH 6.8, the complete state of hydration for the Na-CMC was not yet reached, causing further fluctuations in the viscosity of the formulation.

5.3.9 Zeta potential

The zeta potential of the cream ranged from 20 mV to 21.5 mV for all storage conditions, respectively; indicating similar zeta potential values for the samples at all the different storage conditions. It therefore appeared that the storage conditions did not have a marked effect on the zeta potential.

5.3.10 Particle size

The average particle size of the cream depicted an increase over three months at all storage conditions. This was to be expected due to the low zeta potential obtained in this study. A low zeta potential (below 25 mV) found in dispersions tends to lead to coagulation or coalescence (Jelvehgari *et al*., 2010:1240). The particles in this formulation seem to adhere to one another in the dispersion to form aggregates of significant increased size (> 5%) by means of coalescence.

5.4 CONCLUSION

The aim of this study was to formulate a product containing both acyclovir (5% w/w) and ketoconazole (2% w/w) which could be applied to mucosa. The rationale behind this combination lied in the synergy provided by ketoconazole in anti-viral activity when used together with acyclovir (Bickers, 1994:89). Ketoconazole has a broad anti-fungal activity and also show activity against some bacterial infections which often occur in HIV/AIDS patients (Pottage *et al.*, 1986:217; Bennet, 2006:1225). Acyclovir, on the other hand, is the anti-viral agent used for most pathological viral infections associated with HIV/AIDS (King, 1988:176; Beers, 2006:1061).

The main problem experienced with dermal formulation, is the penetration of the SC. In mucosal skin, the principle barrier function is removed by the absence of keratinised SC as found in normal skin and is thus more permeable (Farage & Scheffler, 2011:117). Compounds are mainly delivered via passive diffusion across mucosal membranes (Patel *et al*., 2011:107).

During evaluation of the physicochemical properties of both acyclovir and ketoconazole, a number of possible obstacles could have been predicted. Firstly, acyclovir will permeate the skin more readily than ketoconazole (Sweetman, 2011). The solubility of acyclovir in water is classified as sparingly soluble and ketoconazole is seen as practically insoluble in water *(Drug bank, 2012)*. This causes difficulty in the formulation of an emulsion, as it consists of a water phase and an oil phase. Crystal formation present in dermatological products containing acyclovir, causes difficulty in the permeation of this compound through the different skin layers, as only particles smaller than 3 µm are able to penetrate the skin's follicles and SC. Particles larger than 10 µm remain on the skin's surface (Barry, 2007:573). Ketoconazole is light sensitive and degradation occurs after 24 hours of light exposure (Langer & Maibach, 2012), indicating that special precaution has to be taken when storage of the formulation is considered.

A study to determine the compatibility of acyclovir and ketoconazole was performed using microcalorimetry which is a reliable way of detecting incompatibilities mostly because all physical and chemical processes are accompanied by the exchange of heat (Phipps & Mackin, 2000:9). No incompatibilities were observed from the combination of acyclovir and ketoconazole. A cream, gel and lip balm formulation containing both acyclovir (5% w/w) and ketoconazole (2% w/w) were formulated.

Thereafter *in vitro* permeation studies were conducted using porcine vaginal mucosa in a flow-through diffusion system for a 24 hr period. The cream, gel and lip balm formulations were

compared to the commercial products Acitop® and Ketazol® containing acyclovir (5% w/w) and ketoconazole (2% w/w), respectively. All of the formulated products depicted a flux for both acyclovir and ketoconazole through the mucosa, which is in indication that both the compounds were released from each formulation. The gel formulation depicted the best flux for acyclovir when compared to Acitop®. In the case of the ketoconazole formulations, the lip balm formulation depicted a higher flux compared to the flux of of Ketazol®.

The cream, gel and lip balm formulations manufactured in this study were tested for stability over a three month period. Formulated products were stored at three different storage conditions and stability tests were conducted at months 0, 1, 2 and 3. The stability program was done in accordance with the ICH Tripartite Guidelines (2003) and the Medicines Control Council (MCC) of South Africa (2011).

Stability testing conducted over a period of three months indicated no significant change in visual appearance, pH and mass variation. Significant changes were observed in acyclovir concentration from the assay possibly due to crystal formation of acyclovir. However, significant changes in ketoconazole concentration were observed. The viscosity measurements of the cream depicted an increase, whereas the gel presented with a decrease in viscosity which could be explained due to the influence of pH, hydration and temperature of the excipients used in the formulation. Considering the zeta potential measurements, the zeta potential of the cream remained low during the stability testing. A low zeta potential may lead to coalescence in formulations. Coalescence was observed in the particle size determination of the cream, which depicted an increase in size of particles.

Due to the results obtained during this study, the following future prospects for further investigation and aspects that need to be considered were identified:

- The use of co-solvents may enhance the solubility of acyclovir in different formulations.
- A cold method should be investigated to formulate products containing acyclovir in order to avoid crystal formation observed in topical products.
- *In vitro* permeation studies should be conducted on human vaginal skin by means of flow-through diffusion, as the porcine vaginal can only be regarded as a comparative study.
- *In vivo* testing of the formulated products should be conducted in order to determine clinical efficacy of the products on the different pathogens.
- Preservative testing should be done to prevent or inhibit microbiological growth in the formulations.

- Enhancement of skin penetration should be investigated by incorporating a penetration enhancer, provided that it is safe to use on mucosal membranes.
- A study in toxicology should also be conducted.

ACKNOWLEDGEMENTS

This work has been carried out with the financial support of the National Research Foundation (NRF) of South Africa, the Unit for Drug Research and Developments of the North-West University, Potchefstroom campus and the Pharmaceutical Society of South Africa (PSSA). The Authors would like to thank Dr Armorél van Eyk for the assistance of the permeation studies conducted at the University of Witwatersrand, Gauteng, South Africa.

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FIGURE LEGENDS

EQUATION LEGENDS

$$ft = Q = A\sqrt{D(2C - C_s)C_s t}$$ **[Eq 5.1]**

FIGURES

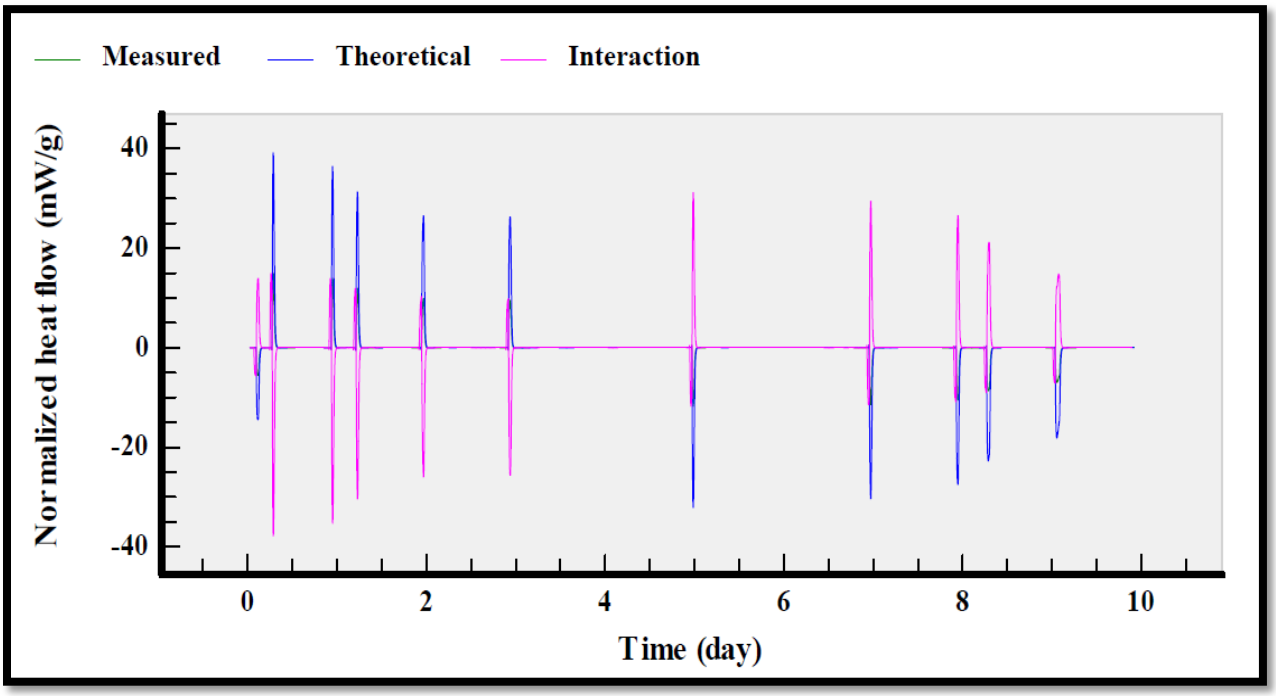

Figure 5.1: *Heat flow data of acyclovir and ketoconazole sample*.

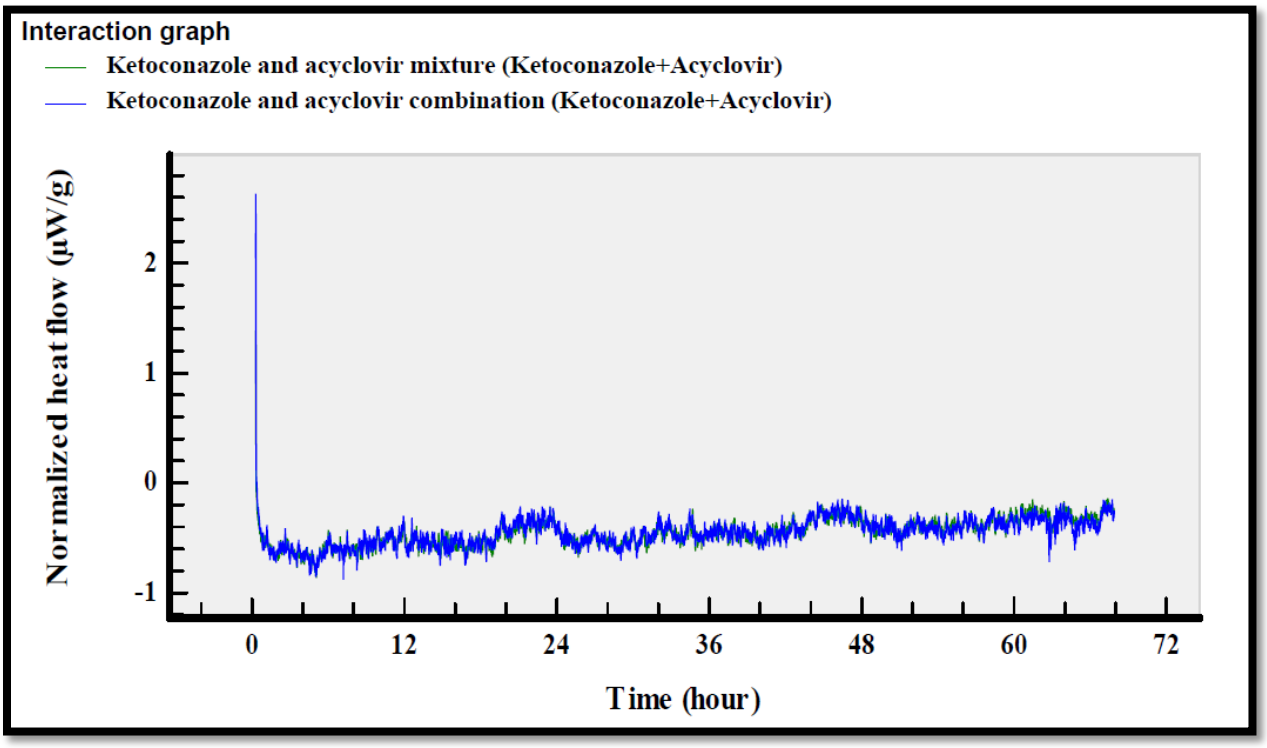

Figure 5.2: *Interaction graph for acyclovir and ketoconazole raw material.*

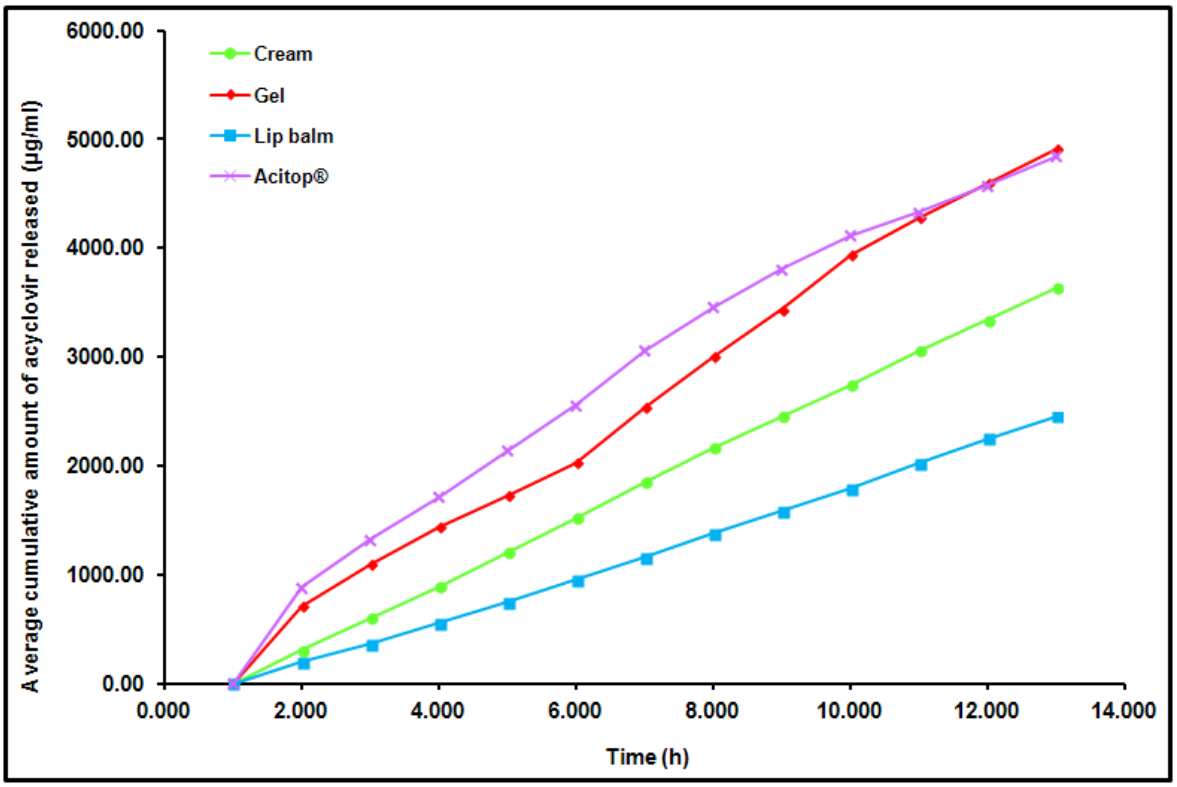

Figure 5.3: *Average cumulative amount of acyclovir permeation through the mucosa over 24 hrs.*

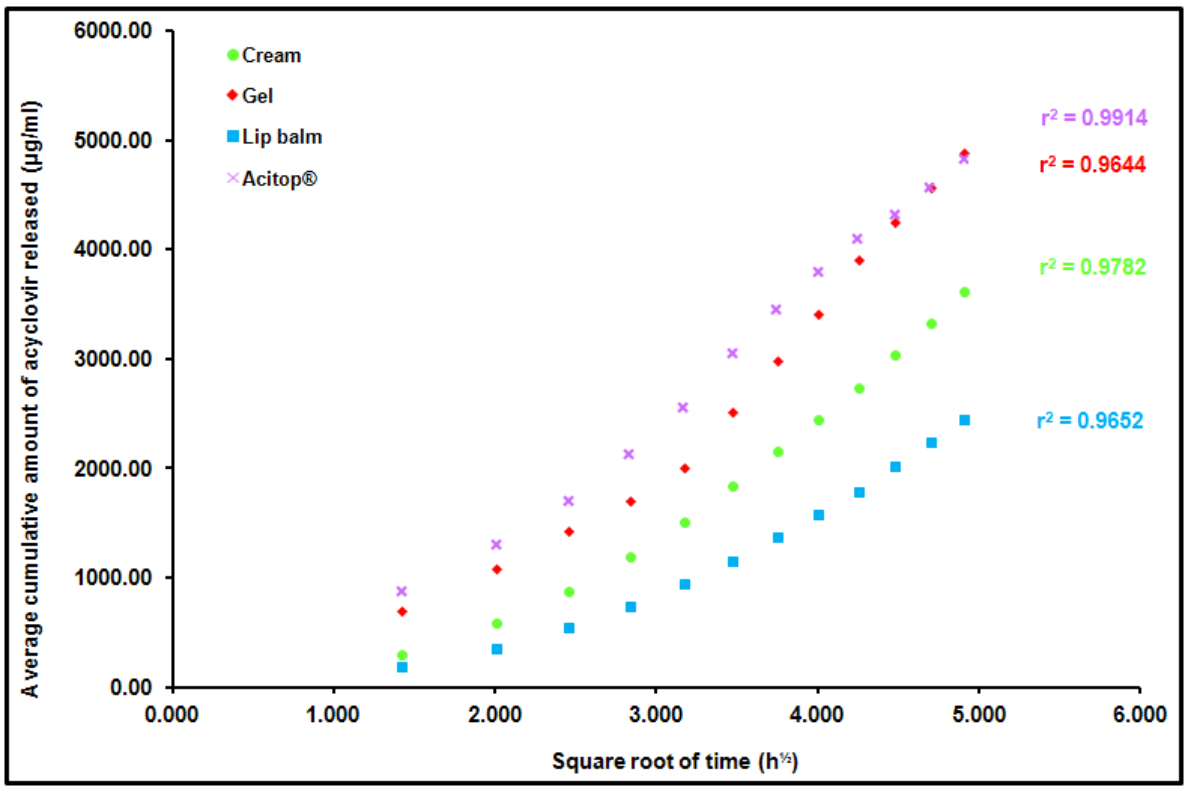

Figure 5.4: *Higuchi plot for acyclovir in different formulations by plotting the average cumulative amount released over the square root of time.*

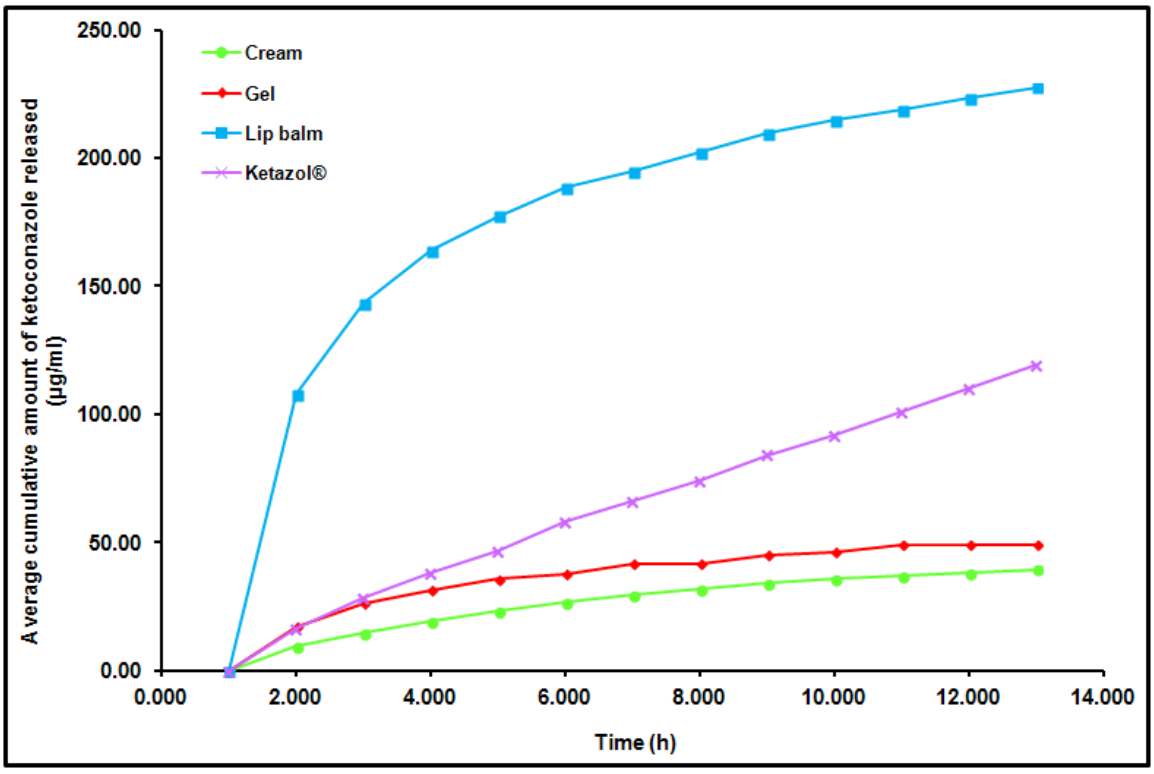

Figure 5.5: *Average cumulative amount of ketoconazole permeation through the mucosa over 24 hrs.*

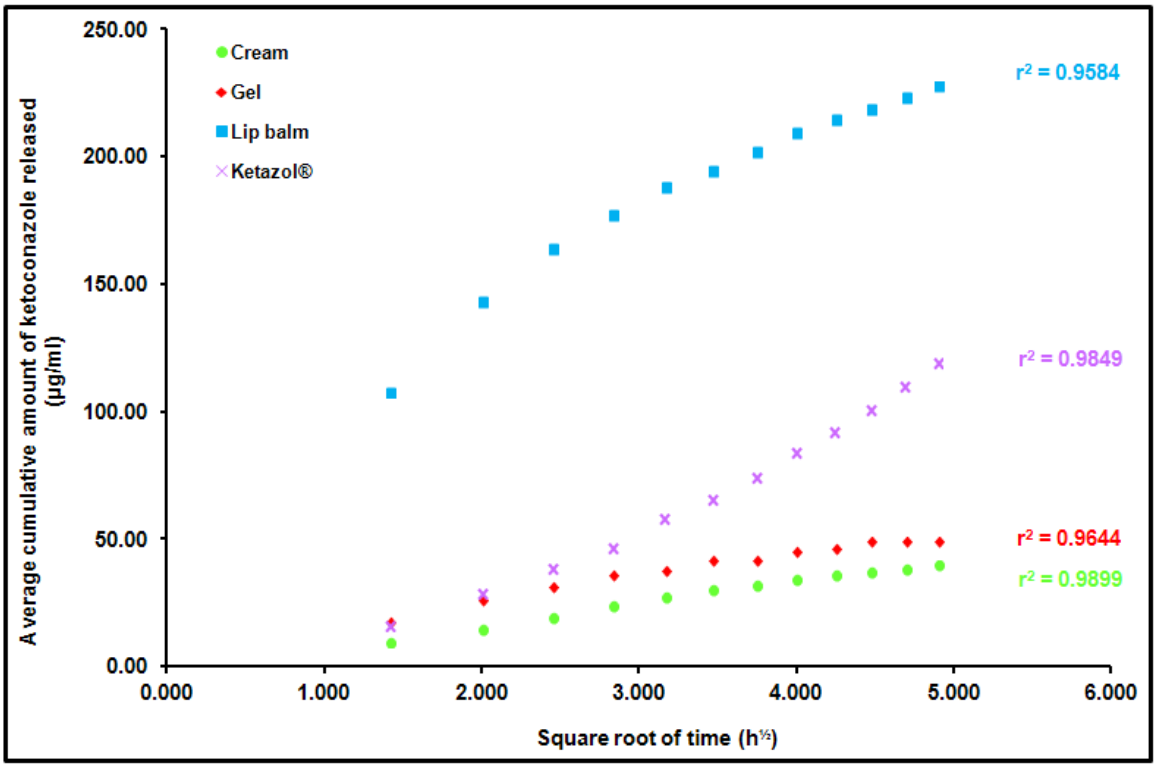

Figure 5.6: *Higuchi plot for ketoconazole in different formulations by plotting the average cumulative amount released over the square root of time.*

CHAPTER 6: SUMMARY AND FUTURE PROSPECTS

The aim of this study was to formulate a product containing both acyclovir (5% w/w) and ketoconazole (2% w/w) which could be applied to the mucosa. The rationale behind this combination lies in the synergy provided by ketoconazole in anti-viral activity when used together with acyclovir (Bickers, 1994:89). Ketoconazole has a broad anti-fungal activity and also show activity against some bacterial infections which often occur in HIV/AIDS patients (Pottage *et al.,* 1986:217; Bennet, 2006:1225). Acyclovir, on the other hand, is the anti-viral agent used for most pathological viral infections associated with HIV/AIDS (King, 1988:176; Beers, 2006:1061).

Dermatological manifestations occur in most HIV/AIDS patients as opportunistic infections and causes emotional discomfort for the patient as the skin is the most visible organ. Many of these manifestations present on mucosal tissue including the labial or genital area. Patients infected with HIV/AIDS are already using large amounts of medication by means of the oral route. It is therefore beneficial to make use of topical treatment, as it may reduce the amount of interactions between compounds and adverse reactions (Cedeno-Laurent *et al.,* 2011:5).

The main problem experienced with dermal formulation, is the penetration of the SC. In mucosal skin, the principle barrier function is removed by the absence of keratinised SC as found in normal skin and is thus more permeable (Farage & Scheffler, 2011:117). Compounds are mainly delivered via passive diffusion across mucosal membranes (Patel *et al*., 2011:107).

During evaluation of the physicochemical properties of both acyclovir and ketoconazole, a number of possible obstacles could have been predicted. Firstly, acyclovir will permeate the skin more readily than ketoconazole (Sweetman, 2011). The solubility of acyclovir in water is classified as sparingly soluble and ketoconazole is seen as practically insoluble in water (Drug bank, 2012a). This causes difficulty in the formulation of an emulsion, as it consists of a water phase and an oil phase. Crystal formation present in dermatological products containing

acyclovir, causes difficulty in the permeation of this compound through the different skin layers, as only particles smaller than 3 µm are able to penetrate the skin's follicles and SC. Particles larger than 10 µm remain on the skin's surface (Barry, 2007:573). Ketoconazole is light sensitive and degradation occurs after 24 hours of light exposure (Langer & Maibach, 2012), indicating that special precaution has to be taken when storage of the formulation is considered.

A study to determine the compatibility of acyclovir and ketoconazole was performed using microcalorimetry which is a reliable way of detecting incompatibilities mostly because all physical and chemical processes are accompanied by the exchange of heat (Phipps & Mackin, 2000:9). No incompatibilities were observed from the combination of acyclovir and ketoconazole. A cream, gel and lip balm formulation containing both acyclovir (5% w/w) and ketoconazole (2% w/w) were formulated.

Thereafter *in vitro* permeation studies were conducted using porcine vaginal mucosa in a flow-through diffusion system for a 24 hr period. The cream, gel and lip balm formulations were compared to the commercial products Acitop® and Ketazol® containing acyclovir (5% w/w) and ketoconazole (2% w/w), respectively. All of the formulated products depicted a flux for both acyclovir and ketoconazole through the mucosa, which is in indication that both the compounds were released from each formulation. The gel formulation depicted the best flux for acyclovir when compared to Acitop®. In the case of the ketoconazole formulations, the lip balm formulation depicted a higher flux compared to the flux of of Ketazol®.

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ANNEXURE A: COMPATIBILITY OF ACYCLOVIR AND KETOCONAZOLE

Table A.1: *Ketoconazole raw material and Acyclovir raw material (sample 1) - temperature program parameters and heat flow results.*

Ketoconazole raw material mass (mg)	198.63 mg
Acyclovir raw material mass (mg)	505.56 mg
Reaction start	Apr 02, 2012 10:55:37
Interaction integral	22.841 J/g
Interaction average heatflow	26.801 µW/g
Interaction error	4.2135 mW/g

Table A.2: *Ketoconazole raw material and Acyclovir raw material (sample 2) - temperature program parameters and heat flow results.*

Ketoconazole raw material mass (mg)	194.09 mg
Acyclovir raw material mass (mg)	496.84 mg
Reaction start	Apr 02, 2012 10:19:00
Interaction integral	18.871 J/g
Interaction average heatflow	22.084 µW/g
Interaction error:	2.2909 mW/g

ANNEXURE B: VALIDATION OF ACYCLOVIR AND KETOCONAZOLE

B.1 LINEARITY

Table B.1: *The mean peak area values of acyclovir.*

µg/ml	Mean
0.10	21.5
0.20	40.7
0.30	60.7
0.40	80.9
0.50	101.3
1.00	169.8
2.00	337.9
4.00	665.1
6.00	990.9
10.00	1669.4
20.00	3295.5
40.00	6530.6
60.00	9545.4
80.00	12154.7
100.00	14453.5

Table B.2: *The mean peak area values of ketoconazole.*

µg/ml	Mean
0.40	5.9
0.80	13.3
1.20	17.5
1.60	26.4
2.00	33.9
4.00	81.1
8.00	187.0
16.00	364.6
24.00	547.0
40.00	970.7
80.00	1934.5
160.00	3742.2
240.00	5403.5
320.00	7032.3
400.00	8572.7

ANNEXURE C: PERMEATION STUDIES

Table C.1: *Average cumulative amount of acyclovir released from the different formulations in 24 hrs.*

Time	$H^{1/2}$	Cream	Gel	Lip balm	Acitop®
0	0.000	0.000	0.000	0.000	0.000
2	1.414	307.146	710.620	203.031	884.084
4	2.000	601.433	1098.800	362.153	1321.483
6	2.449	885.370	1443.188	556.113	1717.548
8	2.828	1202.260	1727.238	749.029	2140.718
10	3.162	1523.729	2023.056	953.238	2564.450
12	3.464	1847.475	2530.188	1156.820	3060.218
14	3.742	2165.343	3000.680	1378.091	3459.410
16	4.000	2457.439	3435.015	1583.783	3809.757
18	4.243	2746.362	3930.356	1796.992	4117.945
20	4.472	3054.044	4273.747	2026.031	4326.972
22	4.690	3338.958	4592.698	2254.101	4576.293
24	4.899	3627.724	4903.849	2455.746	4846.206

Table C.2: *Average cumulative amount of ketoconazole released from the different formulations in 24 hrs.*

Time	$H^{1/2}$	Cream	Gel	Lip balm	Ketazol®
0	0.000	0.000	0.000	0.000	0.000
2	1.414	9.452	17.270	107.718	16.360
4	2.000	14.467	26.043	143.385	28.686
6	2.449	19.292	31.494	163.865	38.358
8	2.828	23.471	35.699	177.286	46.794
10	3.162	26.936	37.690	188.266	58.274
12	3.464	29.842	41.497	194.719	65.921
14	3.742	32.028	41.497	202.044	74.431
16	4.000	33.980	45.160	209.523	84.203
18	4.243	35.767	46.278	214.636	91.963
20	4.472	37.150	48.909	218.680	100.970
22	4.690	38.411	48.909	223.341	110.267
24	4.899	39.606	48.909	227.595	119.227

Table C.3: *Summary of the results obtained during acyclovir permeation from the cream formulation.*

Cell	Apparent release constant	Release rate (µg/cm2/h)	Lag Time	% Diffused
1	830.274	128.192	1.225	6.460
2	736.920	114.265	1.631	5.269
3	929.609	144.539	1.878	6.202
4	935.361	144.998	1.505	6.833
5	1274.406	196.958	0.000	9.328
6	767.005	116.587	1.216	5.673
7	1417.720	218.571	1.205	11.023
Average	984.471	152.016	1.436	7.255

Table C.4: *Summary of the results obtained during acyclovir permeation from the gel formulation.*

Cell	Apparent release constant	Release rate (µg/cm2/h)	Lag Time	% Diffused
1	597.179	92.685	0.896	5.088
2	814.106	126.102	0.651	7.325
3	1162.446	180.068	1.414	8.646
4	1758.989	274.734	1.304	13.590
5	532.703	83.010	0.000	4.030
6	1103.146	170.472	1.027	8.722
7	2925.871	459.322	1.415	21.252
Average	1270.634	198.056	1.239	9.808

Table C.5: *Summary of the results obtained during acyclovir permeation from the lip balm formulation.*

Cell	Apparent release constant	Release rate (µg/cm2/h)	Lag Time	% Diffused
1	565.819	87.905	1.400	4.293
2	518.689	80.076	1.224	4.049
3	1296.258	199.954	1.550	9.230
4	980.635	153.941	1.804	6.890
5	462.947	72.295	0.000	3.459
6	261.814	40.648	1.041	2.158
7	583.765	92.097	1.560	4.303
Average	667.132	103.845	1.514	4.911

Table C.6: *Summary of the results obtained during acyclovir permeation from Acitop®.*

Cell	Apparent release constant	Release rate (µg/cm2/h)	Lag Time	% Diffused
1	673.236	103.052	1.518	4.803
2	1589.038	242.201	0.347	14.629
3	960.403	146.719	0.928	7.872
4	927.111	140.203	0.086	9.018
5	1076.706	162.450	0.000	8.416
6	983.318	150.784	1.603	6.684
7	2211.211	334.940	1.129	16.423
Average	1203.004	182.907	0.896	9.692

Table C.7: *Summary of the results obtained during ketoconazole permeation from the cream formulation.*

Cell	Apparent release constant	Release rate (µg/cm2/h)	Lag Time	% Diffused
1	0.000	0.000	0.000	0.000
2	0.806	0.119	-15.642	0.079
3	0.000	0.000	0.000	0.040
4	5.617	0.790	-3.529	0.215
5	0.000	0.000	0.000	0.000
6	0.000	0.000	0.000	0.000
7	55.645	8.374	1.003	1.052
Average	8.867	1.326	0.248	0.198

Table C.8: *Summary of the results obtained during ketoconazole permeation from the gel formulation.*

Cell	Apparent release constant	Release rate (µg/cm2/h)	Lag Time	% Diffused
1	3.405	0.523	-7.425	0.195
2	14.778	2.260	-0.062	0.363
3	1.118	0.166	-19.136	0.129
4	12.932	1.842	-1.300	0.357
5	0.000	0.000	0.000	0.038
6	6.164	0.875	-2.480	0.203
7	24.041	3.688	1.197	0.426
Average	8.920	1.336	-0.938	0.245

Table C.9: *Summary of the results obtained during ketoconazole permeation from the lip balm formulation.*

Cell	Apparent release constant	Release rate (µg/cm2/h)	Lag Time	% Diffused
1	21.425	3.231	-1.899	0.708
2	36.284	5.406	-2.258	1.236
3	18.115	2.807	-0.594	0.507
4	45.266	6.712	-0.826	1.236
5	39.816	5.957	0.000	1.503
6	34.837	5.172	-2.748	1.269
7	27.894	4.272	-5.983	1.507
Average	31.948	4.794	-2.477	1.138

Table C.10: *Summary of the results obtained during ketoconazole permeation from Ketazol®.*

Cell	Apparent release constant	Release rate (µg/cm2/h)	Lag Time	% Diffused
1	27.446	4.308	1.320	0.553
2	31.396	4.768	1.291	0.577
3	23.150	3.575	1.087	0.457
4	29.459	4.571	1.043	0.614
5	35.106	5.417	0.000	0.698
6	30.110	4.670	0.939	0.634
7	30.961	4.785	0.893	0.640
Average	29.661	4.585	1.100	0.596

Table C.11: *Statistical data (ANOVA) obtained for acyclovir released from the different formulations.*

		Cream	Gel	Lip balm	Acitop®
	n*	7	7	7	7
% Diffused	Mean	7.2554	9.8080	4.9115	9.6920
	SD**	2.1159	5.9108	2.3716	4.2432
	p-value	0.7810			
Apparent Release constant	Mean	984.4707	1270.6340	667.1323	1203.0040
	SD**	261.2594	839.1027	351.0242	523.6640
	p-value	0.1117			
Release Rate	Mean	152.0157	198.0560	103.8451	182.9070
	SD**	40.4196	132.1237	54.3031	79.1518
	p-value	0.1266			
Lag Time	Mean	1.2374	0.9580	1.2255	0.8020
	SD**	0.6014	0.5094	0.5941	0.6652
	p-value	0.3823			

*n refers to the amount of cells

**SD refers to the standard deviation

Table C.12: *Statistical data (ANOVA) obtained for ketoconazole released from the different formulations.*

		Cream	Gel	Lip balm	Ketazol®
	n*	7	7	7	7
% Diffused	Mean	0.1980	0.2445	1.1379	0.5961
	SD**	0.3844	0.1411	0.3850	0.0771
	p-value	0.0003			
Apparent Release constant	Mean	8.86688	8.91966	31.94806	29.66139
	SD	20.7294	8.7321	9.8737	3.6945
	p-value	0.0020			
Release Rate	Mean	1.3262	1.3361	4.7940	4.5849
	SD**	3.1212	1.3336	1.4267	0.5582
	p-value	0.0029			
Lag Time	Mean	-2.5953	-4.1723	-2.0440	0.9389
	SD**	5.9297	7.1734	1.9932	0.4444
	p-value	0.006			

*n refers to the amount of cells

**SD refers to the standard deviation

Table C.13: *Statistical differences obtained for ketoconazole released from the different formulations by means of the Bonferonni corrections on Dunn's p-values.*

	Cream	Gel	Lip Balm	Ketazol®
% Diffused				
Cream	-	-	0.000432	0.044461
Gel	-	-	0.002456	-
Lip Balm	0.000432	0.002456	-	-
Ketazol®	0.044461		-	-
Apparent release constant				
Cream	-	-	0.008852	0.010009
Gel	-	-	0.032275	-
Lip Balm	0.008852	0.032275	-	-
Ketazol®	0.010009		-	-
Release Rate				
Cream	-	-	0.012107	0.018132
Gel	-	-	0.07082	0.05082
Lip Balm	0.012107	0.07082	-	-
Ketazol®	0.018132	0.05082	-	-
Lag time				
Cream	-	-	-	
Gel	-	-	-	0.014113
Lip Balm	-	-	-	0.004873
Ketazol®	-	0.014113	0.004873	-

ANNEXURE D: STABILITY TESTING

D.1 VISUAL APPEARANCE

Figure D.1: *Colour charts obtained from local paint store used to compare colour changes in formulations.*

D.2 MASS VARIATION

Table D.1: *Mass (g) of cream at different conditions after each time interval.*

Storage temperature	Month				Summary		
	0	1	2	3	Mean	SD	%RSD
25°C/60 % RH	112.31	112.26	112.23	112.20	112.25	0.04	0.04
30°C/60 % RH	112.28	112.28	112.26	112.24	112.26	0.02	0.02
40°C/75 % RH	112.40	112.31	112.17	112.07	112.23	0.13	0.11

Table D.2: *Mass (g) of gel at different conditions after each time interval.*

Storage temperature	Month				Summary		
	0	1	2	3	Mean	SD	%RSD
25°C/60 % RH	113.40	113.18	112.89	112.67	113.03	0.28	0.24
30°C/60 % RH	115.87	115.42	114.77	114.57	115.16	0.52	0.45
40°C/75 % RH	114.89	114.47	114.40	114.35	114.53	0.22	0.19

Table D.3: *Mass (g) of lip balm at different conditions after each time interval.*

Storage temperature	Month				Summary		
	0	1	2	3	Mean	SD	%RSD
25°C/60 % RH	113.68	113.91	114.09	114.17	113.96	0.19	0.17
30°C/60 % RH	108.58	108.76	109.01	109.06	108.85	0.20	0.18
40°C/75 % RH	112.47	113.26	113.29	113.31	113.08	0.35	0.31

D.3 ASSAY

Table D.4: *The values of compounds in cream at different conditions after each time interval.*

Ingredient	Temperature	Month				Summary		
Values are given are %		0	1	2	3	Mean	SD	%RSD
Acyclovir	25 ˚C	104.807	107.151	113.433	120.389	111.445	5.412	4.856
	30 ˚C	104.807	109.379	115.927	122.307	113.105	5.923	5.236
	40 ˚C	104.807	105.900	116.639	125.276	113.155	7.502	6.63
Ketoconazole	25 ˚C	102.945	100.977	86.194	100.895	97.753	6.014	6.152
	30 ˚C	102.945	101.617	86.429	100.341	97.833	5.946	6.078
	40 ˚C	102.945	101.65	87.063	100.853	98.128	5.753	5.863

Table D.5: *The values of compounds in gel at different conditions after each time interval.*

Ingredient	Temperature	Month				Summary		
Values are given are %		0	1	2	3	Mean	SD	%RSD
Acyclovir	25 ˚C	121.977	112.775	120.592	121.532	119.219	3.358	2.816
	30 ˚C	121.977	105.138	123.815	128.852	119.945	7.971	6.646
	40 ˚C	121.977	113.058	125.211	124.569	121.204	4.344	3.584
Ketoconazole	25 ˚C	108.778	104.513	93.231	106.268	103.198	5.322	5.157
	30 ˚C	108.778	106.564	97.794	111.518	106.163	4.598	4.331
	40 ˚C	108.778	109.219	96.164	110.272	106.108	5.158	4.861

Table D.6: *The values of compounds in lip balm at different conditions after each time interval.*

Ingredient	Temperature	Month				Summary		
Values are given are %		0	1	2	3	Mean	SD	%RSD
Acyclovir	25 ˚C	83.011	92.396	98.162	103.138	94.177	6.694	7.108
	30 ˚C	83.011	92.431	92.468	101.214	92.281	5.758	6.24
	40 ˚C	83.011	81.891	95.643	103.832	91.094	8.161	8.958
Ketoconazole	25 ˚C	86.984	91.127	80.264	91.68	87.514	4.081	4.663
	30 ˚C	86.984	86.833	74.121	91.872	84.952	5.88	6.921
	40 ˚C	86.984	81.571	73.618	89.021	82.798	5.33	6.437

D.4 PH

Table D.7: *pH of cream in the different conditions after each time interval.*

Storage temperature	Months				Summary		
	0	1	2	3	Mean	SD	%RSD
25°C/60 % RH	8.897	8.930	8.882	8.890	8.900	0.018	0.204
30°C/60 % RH	8.897	8.725	8.788	8.688	8.774	0.080	0.906
40°C/75 % RH	8.897	8.651	8.481	8.561	8.648	0.156	1.807

Table D.8: *pH of gel in the different conditions after each time interval.*

Storage temperature	Months				Summary		
	0	1	2	3	Mean	SD	%RSD
25°C/60 % RH	6.846	6.843	6.833	6.837	6.840	0.005	0.077
30°C/60 % RH	6.846	6.779	6.809	6.757	6.798	0.033	0.492
40°C/75 % RH	6.846	6.744	6.710	6.710	6.753	0.056	0.825

D.5 VISCOSITY

Table D.9: *Viscosity (cP) of cream in the different conditions after each time interval.*

Storage temperature	Month				Summary		
	0	1	2	3	Mean	SD	RSD
25°C/60 % RH	1010526.6	696921.6	564332.7	895400.1	791795.3	172711.9	21.8
30°C/60 % RH	1010526.6	869188.0	1037669.2	825481.9	935716.4	90233.9	9.6
40°C/75 % RH	1010526.6	813011.4	935581.6	1313879.9	1018249.9	184671.1	18.1

Table D.10: *Viscosity (cP) of gel in the different conditions after each time interval.*

Storage temperature	Month				Summary		
	0	1	2	3	Mean	SD	RSD
25°C/60 % RH	617641.7	981893.3	1228839.4	875106.2	925870.1	219380.2	23.7
30°C/60 % RH	617641.7	892485.3	1165704.4	910645.5	896619.2	193938.9	21.6
40°C/75 % RH	617641.7	649373.2	877058.9	639706.2	695945.0	105196.7	15.1

D.6 ZETA POTENTIAL

Table D.11: *Zeta potential (mV) of cream at different conditions after each time interval.*

Storage temperature	Month				Summary		
	0	1	2	3	Mean	SD	RSD
25°C/60 % RH	20.933	19.522	21.200	20.833	20.622	0.649	3.147
30°C/60 % RH	20.933	20.678	18.711	20.389	20.178	0.868	4.304
40°C/75 % RH	20.933	21.556	19.689	21.589	20.942	0.769	3.672

D.7 PARTICLE SIZE

Table D.12: *Average particle size (µm) of cream at different conditions after each time interval.*

Storage temperature	Month				Summary		
	0	1	2	3	Mean	SD	RSD
25°C/60 % RH	32.458	41.135	30.250	31.819	33.916	4.245	12.516
30°C/60 % RH	32.458	54.287	26.341	38.577	37.916	10.395	27.416
40°C/75 % RH	32.458	43.549	29.318	26.457	32.946	6.479	19.667

ANNEXURE E: AUTHORS GUIDE TO THE INTERNATIONAL JOURNAL OF PHARMACEUTICS

E.1 DESCRIPTION

The *International Journal of Pharmaceutics* is the journal for pharmaceutical scientists concerned with the physical, chemical and biological properties of devices and delivery systems for drugs, vaccines and biologicals, including their design, manufacture and evaluation. This includes evaluation of the properties of drugs, excipients such as surfactants and polymers and novel materials. The journal has special sections on pharmaceutical nanotechnology and personalised medicines, and publishes research papers, reviews, commentaries and letters to the editor as well as special issues.

E.1.1 Editorial policy

The over-riding criteria for publication are originality, high scientific quality and interest to a multidisciplinary audience. Papers not sufficiently substantiated by experimental detail will not be published. Any technical queries will be referred back to the author, although the Editors reserve the right to make alterations in the text without altering the technical content. Manuscripts submitted under multiple authorship are reviewed on the assumption that all listed authors concur with the submission and that a copy of the final manuscript has been approved by all authors and tacitly or explicitly by the responsible authorities in the laboratories where the work was carried out. If accepted, the manuscript shall not be published elsewhere in the same form, in either the same or another language, without the consent of the Editors and Publisher.

Authors must state in a covering letter when submitting papers for publication the novelty embodied in their work or in the approach taken in their research. Routine bioequivalence

studies are unlikely to find favour. No paper will be published which does not disclose fully the nature of the formulation used or details of materials which are key to the performance of a product, drug or excipient. Work which is predictable in outcome, for example the inclusion of another drug in a cyclodextrin to yield enhanced dissolution, will not be published unless it provides new insight into fundamental principles.

E.2 GUIDE FOR AUTHORS

E.2.1 Introduction

The *International Journal of Pharmaceutics* publishes innovative papers, reviews, mini-reviews, rapid communications and notes dealing with physical, chemical, biological, microbiological and engineering studies related to the conception, design, production, characterisation and evaluation of drug delivery systems *in vitro* and *in vivo*. "Drug" is defined as any therapeutic or diagnostic entity, including oligonucleotides, gene constructs and radiopharmaceuticals.

Areas of particular interest include: pharmaceutical nanotechnology; physical pharmacy; polymer chemistry and physical chemistry as applied to pharmaceutics; excipient function and characterisation; biopharmaceutics; absorption mechanisms; membrane function and transport; novel routes and modes of delivery; responsive delivery systems, feedback and control mechanisms including biosensors; applications of cell and molecular biology to drug delivery; prodrug design; bioadhesion (carrier-ligand interactions); and biotechnology (protein and peptide formulation and delivery).

Note: For details on pharmaceutical nanotechnology, see Editorials in 279/1-2 281/1, and 288/1.

E.2.1.1 Types of paper

- Full Length Manuscripts
- Rapid Communications

  (a) These articles should not exceed 1500 words or equivalent space.

  (b) Figures should not be included otherwise delay in publication will be incurred.

  (c) Do not subdivide the text into sections. An Abstract should be included as well as a full reference list.

- Notes

  Should be prepared as described for full length manuscripts, except for the following:

  (a) The maximum length should be 1500 words, including figures and tables.

  (b) Do not subdivide the text into sections. An Abstract and reference list should be included.

- Reviews and Mini-Reviews

  Suggestions for review articles will be considered by the Review-Editor. "Mini-reviews" of a topic are especially welcome.

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E.2.2 Before you begin

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E.2.2.2 Policy and ethics

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All authors should have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

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E.2.2.14 Referees

Please submit, with the manuscript, the names, addresses and e-mail addresses of three potential referees. Note that the editor retains the sole right to decide whether or not the suggested reviewers are used.

E.2.3 Preparation

E.2.3.1 Use of wordprocessing software

It is important that the file be saved in the native format of the wordprocessor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the wordprocessor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use

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To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your wordprocessor.

E.2.3.2 Article structure

E.2.3.2.1 Subdivision - numbered sections

Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2 ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

E.2.3.2.2 Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

E.2.3.2.3 Material and methods

Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

E.2.3.2.4 Results

Results should be clear and concise.

E.2.3.2.5 Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

E.2.3.2.6 Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

E.2.3.2.7 Appendices

If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

E.2.3.3 Essential title page information

- Title.

  Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

- Author names and affiliations.

  Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

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  Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. Ensure that telephone and fax numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address. Contact details must be kept up to date by the corresponding author.

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E.2.3.4 Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately

from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

The abstract must not exceed 200 words.

E.2.3.5 Graphical abstract

A Graphical abstract is mandatory for this journal. It should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership online. Authors must provide images that clearly represent the work described in the article. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more, but should be readable on screen at a size of 200 × 500 pixels (at 96 dpi this corresponds to 5 × 13 cm). Bear in mind readability after reduction, especially if using one of the figures from the article itself. Preferred file types: TIFF, EPS, PDF or MS Office files. See http://www.elsevier.com/graphicalabstracts for examples.

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Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

E.2.3.7 Abbreviations

Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

E.2.3.8 Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

E.2.3.9 UNITS

Follow internationally accepted rules and conventions: use the international system of units (SI). If other units are mentioned, please give their equivalent in SI.

E.2.3.10 Database linking

Elsevier encourages authors to connect articles with external databases, giving their readers oneclick access to relevant databases that help to build a better understanding of the described research. Please refer to relevant database identifiers using the following format in your article: Database: xxxx (e.g., TAIR: AT1G01020; CCDC: 734053; PDB: 1XFN). See http://www.elsevier.com/databaselinking for more information and a full list of supported databases.

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Present simple formulae in the line of normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

E.2.3.12 Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article, using superscript Arabic numbers. Many wordprocessors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

E.2.3.12.1 Table footnotes

Indicate each footnote in a table with a superscript lowercase letter.

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Whilst it is accepted that authors sometimes need to manipulate images for clarity, manipulation for purposes of deception or fraud will be seen as scientific ethical abuse and will be dealt with accordingly. For graphical images, this journal is applying the following policy: no specific feature within an image may be enhanced, obscured, moved, removed, or introduced.

Adjustments of brightness, contrast, or colour balance are acceptable if and as long as they do not obscure or eliminate any information present in the original. Nonlinear adjustments (e.g. changes to gamma settings) must be disclosed in the figure legend.

E.2.3.12.3 Electronic artwork

E.2.3.12.3.1 General points

- Make sure you use uniform lettering and sizing of your original artwork.
- Save text in illustrations as 'graphics' or enclose the font.
- Only use the following fonts in your illustrations: Arial, Courier, Times, Symbol.
- Number the illustrations according to their sequence in the text.
- Use a logical naming convention for your artwork files.
- Provide captions to illustrations separately.
- Produce images near to the desired size of the printed version.
- Submit each figure as a separate file.

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You are urged to visit this site; some excerpts from the detailed information are given here.

E.2.3.12.3.2 Formats

Regardless of the application used, when your electronic artwork is finalised, please 'save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):

EPS: Vector drawings. Embed the font or save the text as 'graphics'.

TIFF: Colour or greyscale photographs (halftones): always use a minimum of 300 dpi.

TIFF: Bitmapped line drawings: use a minimum of 1000 dpi.

TIFF: Combinations bitmapped line/half-tone (colour or greyscale): a minimum of 500 dpi is required.

If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is'.

Please do not:

- Supply files that are optimised for screen use (e.g., GIF, BMP, PICT, WPG); the resolution is too low;
- Supply files that are too low in resolution;
- Submit graphics that are disproportionately large for the content.

E.2.3.12.3.3 Colour artwork

Please make sure that artwork files are in an acceptable format (TIFF, EPS or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable colour figures then Elsevier will ensure, at no additional charge, that these figures will appear in colour on the Web (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in colour in the printed version. **For colour reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article.** Please indicate your preference for colour: in print or on the Web only. For further information on the preparation of electronic artwork, please see http://www.elsevier.com/artworkinstructions.

Please note: Because of technical complications which can arise by converting colour figures to 'gray scale' (for the printed version should you not opt for colour in print) please submit in addition usable black and white versions of all the colour illustrations.

E.2.3.12.3.4 Figure captions

Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

E.2.3.13 Tables

Number tables consecutively in accordance with their appearance in the text. Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

E.2.3.14 References

E.2.3.14.1 Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication and a copy of the title page of the relevant article must be submitted.

E.2.3.14.2 Web references

As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

E.2.3.14.3 References in a special issue

Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

E.2.3.14.4 Reference management software

This journal has standard templates available in key reference management packages EndNote (http://www.endnote.com/support/enstyles.asp) and Reference Manager (http://refman.com/support/rmstyles.asp). Using plug-ins to wordprocessing packages, authors only need to select the appropriate journal template when preparing their article and the list of references and citations to these will be formatted according to the journal style which is described below.

E.2.3.14.5 Reference style

Text: All citations in the text should refer to:

1. *Single author:* the author's name (without initials, unless there is ambiguity) and the year of publication;

2. *Two authors:* both authors' names and the year of publication;

3. *Three or more authors:* first author's name followed by 'et al.' and the year of publication.

Citations may be made directly (or parenthetically). Groups of references should be listed first alphabetically, then chronologically.

Examples: 'as demonstrated (Allan, 2000a, 2000b, 1999; Allan and Jones, 1999). Kramer et al. (2010) have recently shown ....'

List: References should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.

Examples:

Reference to a journal publication: Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2010. The art of writing a scientific article. J. Sci. Commun. 163, 51–59.

Reference to a book: Strunk Jr., W., White, E.B., 2000. The Elements of Style, fourth ed. Longman, New York.

Reference to a chapter in an edited book: Mettam, G.R., Adams, L.B., 2009. How to prepare an electronic version of your article, in: Jones, B.S., Smith , R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281–304.

E.2.3.14.6 Journal abbreviations source

Journal names should be abbreviated according to Index Medicus journal abbreviations: http://www.nlm.nih.gov/tsd/serials/lji.html;

List of title word abbreviations: http://www.issn.org/2-22661-LTWA-online.php;

CAS (Chemical Abstracts Service): http://www.cas.org/sent.html.

E.2.3.15 Video data

Elsevier accepts video material and animation sequences to support and enhance your scientific research. Authors who have video or animation files that they wish to submit with their article are strongly encouraged to include these within the body of the article. This can be done in the same way as a figure or table by referring to the video or animation content and noting in the body text where it should be placed. All submitted files should be properly labelled so that they directly relate to the video file's content. In order to ensure that your video or animation

material is directly usable, please provide the files in one of our recommended file formats with a preferred maximum size of 50 MB. Video and animation files supplied will be published online in the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. Please supply 'stills' with your files: you can choose any frame from the video or animation or make a separate image. These will be used instead of standard icons and will personalize the link to your video data. For more detailed instructions please visit our video instruction pages at http://www.elsevier.com/artworkinstructions. Note: since video and animation cannot be embedded in the print version of the journal, please provide text for both the electronic and the print version for the portions of the article that refer to this content.

E.2.3.16 Supplementary data

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E.2.3.16.1 Submission checklist

It is hoped that this list will be useful during the final checking of an article prior to sending it to the journal's Editor for review. Please consult this Guide for Authors for further details of any item.

Ensure that the following items are present:

One Author designated as corresponding Author:

- *E-mail address*
- *Full postal address*
- *Telephone and fax numbers*

All necessary files have been uploaded.

- *Keywords*
- *All figure captions*
- *All tables (including title, description, footnotes)*

Further considerations:

- *Use continuous line numbering (every 5 lines) to facilitate reviewing of the manuscript.*
- *Manuscript has been "spellchecked" and "grammar-checked"*
- *References are in the correct format for this journal*
- *All references mentioned in the Reference list are cited in the text, and vice versa*
- *Permission has been obtained for use of copyrighted material from other sources (including the Web)*
- *Colour figures are clearly marked as being intended for colour reproduction on the Web (free of charge) and in print or to be reproduced in colour on the Web (free of charge) and in black-and-white in print*
- *If only colour on the Web is required, black and white versions of the figures are also supplied for printing purposes*

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E.2.4 After acceptance

E.2.4.1 Use of the digital object identifier

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When you use a DOI to create links to documents on the web, the DOIs are guaranteed never to change.

E.2.4.2 Proofs

One set of page proofs (as PDF files) will be sent by e-mail to the corresponding author (if we do not have an e-mail address then paper proofs will be sent by post) or, a link will be provided in the e-mail so that authors can download the files themselves. Elsevier now provides authors with PDF proofs which can be annotated; for this you will need to download Adobe Reader version 7 (or higher) available free from http://get.adobe.com/reader. Instructions on how to annotate PDF files will accompany the proofs (also given online). The exact system requirements are given at the Adobe site: http://www.adobe.com/products/reader/tech-specs.html.

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E.2.5 Author inquiries

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ANNEXURE F: LANGAUGE EDITING

To Whom It May Concern

I hereby certify that I have language edited the following texts:

Aims and Objectives, Chapter 1, Abstract and Uittreksel of a Masters dissertation titled "Evaluation of the Topical Delivery of Different Dosage Forms Containing Acyclovir and Ketoconazol"

by

D. Botes

Date: 2012 12 04

Prof SW Vorster, Pr Eng
BA (Hons), MMet, MSc (Eng), PhD

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