TRANSDERMAL DELIVERY OF ACYCLOVIR AND KETOCONAZOLE BY PHEROID™ TECHNOLOGY

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This dissertation is presented in the so-called article format, which includes an introductory chapter with sub-chapters, a full length article for publication in a pharmaceutical journal and appendices containing relevant experimental data. The article contained in this dissertation is to be published in the European Journal of Pharmaceutical Sciences of which the complete guide for authors is included in the appendices.

ABSTRACT

The aim of this study was to investigate *in vitro* transdermal delivery of the antiviral drug, acyclovir and the antifungal drug, ketoconazole, with the aid of the novel Pheroid™ drug delivery system.

Since its appearance in the early 1980's, human immunodeficiency virus (HIV) infection has had a major impact on the field of dermatology. The skin is amongst the organs where HIV disease and immunosuppression usually manifest, and diseases that were once rare have become more common. HIV is associated with a variety of infectious diseases, some of which are of viral and fungal origin. Acyclovir, an antiviral drug is active against numerous viruses of the herpes viridae family including herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), varicella zoster virus, and to a lesser extent against Epstein-Barr virus and cytomegalovirus. Ketoconazole, an antifungal drug, is effective against the majority of pathogenic fungi, including dermatophytes and yeasts and has *in vitro* activity against a number of gram-positive bacteria. When combined with acyclovir, ketoconazole also displays antiviral activity against HSV-1 and HSV-2 and synergistic antiviral activity. It would be appropriate to design a single topical dosage form containing both an antiviral and antifungal drug, which could be used in the treatment of the cutaneous manifestations commonly seen in HIV and acquired immunodeficiency syndrome (AIDS).

The application of transdermal delivery to a wide variety of drugs is however limited due to the significant barrier to penetration across the skin that is associated mainly with the outermost stratum corneum (SC) layer of the epidermis. The systemic absorption of acyclovir and ketoconazole after topical administration is minimal. Acyclovir therapy has insufficient effectiveness due to the failure of the drug to traverse the SC, lack of its reach at the target site; the basal epidermis and diverse distribution of the drug in the skin layers. In contrast to acyclovir, the target site for ketoconazole is the SC. In order to inhibit the growth of fungal pathogens, sufficient concentrations of the drug should be delivered to this layer.

A large variety of additives have been tested to enhance transdermal penetration. Usually, penetration enhancers promote drug diffusion by disturbing the structure of the SC and/or deeper layers of the skin. Improved antiviral results have been achieved for acyclovir by using dimethyl sulfoxide (DMSO), modified aqueous cream (MAC) and the addition of oleic acid and oleyl alcohol in 5% concentrations to propylene glycol bases. Transdermal penetration of ketoconazole has also been enhanced by 10% lauramide-diethanolamine.

Pheroid™ technology can enhance the absorption and/or efficacy of a selection of active ingredients and other compounds. Pheroids™ contain ethyl esters of the essential fatty acids, linoleic acid and linolenic acid, as well as oleic acid. Penetration enhancement of acyclovir has been achieved by addition of oleic acid to drug formulations, therefore it was hypothesized that it could be possible to achieve at least the same results by using Pheroids™.

Vertical Franz cell diffusion studies were conducted over 12 hours, using female abdominal skin. As donor phase, 5% acyclovir and 2% ketoconazole in phosphate buffered solution was compared with 5% acyclovir and 2% ketoconazole in Pheroids™. *In vitro* penetration of acyclovir and ketoconazole was directly assayed by high pressure liquid chromatography (HPLC).

The Pheroids™ proved to be advantageous for transdermal diffusion of acyclovir but not for ketoconazole when used as delivery system.

Keywords: acyclovir, ketoconazole, transdermal diffusion, Pheroids™, delivery system

UITTREKSEL

Die doel van hierdie studie was om die *in vitro* transdermale aflewering van die antivirale geneesmiddel asiklovir en die antifungale geneesmiddel ketokonasool met behulp van die Pheroid™ geneesmiddel aflewering sisteem te ondersoek.

Menslike immuniteit gebrek virus (MIV) het 'n belangrike impak op die veld van dermatologie sedert sy verskyning in die vroeë 1980's. Die vel is een van die organe waar MIV en immuun onderdrukking gewoonlik manifesteer en siektes wat voorheen skaars was, kom nou alledaags voor. MIV word geassosieer met 'n verskeidenheid infektiewe siektes, waarvan sommiges van virale en fungale oorsprong is. Asiklovir, 'n antivirale geneesmiddel, is aktief teen 'n aantal virusse van die herpes familie, insluitend herpes simplex tipe 1 (HSV-1), herpes simplex tipe 2 (HSV-2), varicella zoster virus, en tot 'n mindere mate teen Ebstein-Barr virus en cytomegalovirus. Ketokonasool, 'n antifungale geneesmiddel, is effektief teen die meeste patogeniese fungusse, insluitend dermatofiete en gisse en het *in vitro* antivirale aktiwiteit teen 'n aantal gram positiewe bakterieë. Ketokonasool toon ook antivirale aktiwiteit teen HSV-1 en HSV-2 en sinergistiese antivirale aktiwiteit indien dit met asiklovir gekombineer word. Die ontwerp van 'n enkele topikale doseervorm wat beide 'n antivirale en antifungale geneesmiddel bevat, sal bruikbaar wees in die behandeling van die kutaneuse manifestasies wat algemeen gesien word in MIV en verworwe immuniteit gebrek sindroom (VIGS).

Toepassing van transdermale aflewering vir 'n verskeidenheid van geneesmiddels is beperk weens die effektiewe weerstand teen vel penetrasie wat geassosieer word met die buitenste stratum corneum (SC) laag van die epidermis. Die sistemiese absorpsie van asiklovir en ketokonasool na topikale aanwending is minimaal. Asiklovir terapie toon onvoldoende effektiwiteit weens die onvermoë van die geneesmiddel om deur die SC te dring, die teiken area, naamlik die basale epidermis te bereik en ook as gevolg van 'n gebrek aan homogene verspreiding van die geneesmiddel in die verskillende vel lae. In teenstelling met asiklovir, is die teiken area vir ketokonasool die SC self. Voldoende konsentrasies van die geneesmiddel moet hierdie laag van die vel bereik om die groei van fungale patogene te inhibeer.

'n Groot verskeidenheid byvoegings is al ondersoek ten einde transdermale penetrasie te verbeter. Gewoonlik bevorder penetrasie bevorderaars geneesmiddel diffusie deurdat dit die struktuur van die SC en die dieper lae van die vel versteur. Verbeterde antivirale resultate is

reeds vir asiklovir verkry deur die byvoeging van dimetiel sulfoksied (DMSO), gemodifiseerde "aqueous" room (MAC) en oliënsuur en oleïel alkohol in konsentrasies van 5% in 'n propileen glikol basis. Transdermale penetrasie van ketokonasool kan ook deur die byvoeging van 10% lauramied dietanol amien verbeter word.

Pheroid™ tegnologie kan die absorpsie en/of effektiwiteit van 'n verskeidenheid aktiewe bestanddele en ander verbindings verhoog. Pheroids™ bevat etiel esters van die essensiële vetsure, linoliensuur, linoleensuur, sowel as oliënsuur. Penetrasie bevordering van asiklovir is deur die byvoeging van oliënsuur by geneesmiddel formules verkry; vandaar die hipotese dat dit moontlik kan wees om ten minste dieselfde resultate te verkry met die Pheroids™.

Vertikale Franz sel diffusie studies oor 12 uur deur vroulike abdominale vel is onderneem. As donor fase is 5% asiklovir en 2% ketokonasool in fosfaat buffer oplossing en 5% asiklovir en 2% ketokonasool in Pheroids™ met mekaar vergelyk. *In vitro* penetrasie van asiklovir en ketokonasool is direk met hoëdrukvloeistofchromatografie (HDVC) bepaal.

Die Pheroids™ as aflewering sisteem het wel tot die transdermale diffusie van asiklovir bygedra maar nie tot die van ketokonasool nie.

Sleutelwoorde: asiklovir, ketokonasool, transdermale diffusie, Pheroids™, aflewering sisteem

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CHAPTER 1: INTRODUCTION AND STATEMENT OF THE PROBLEM

The acquired immunodeficiency syndrome (AIDS) epidemic and human immunodeficiency virus (HIV) have had an intense impact on the spectrum and diagnosis of cutaneous disease. Up to 92% of HIV-positive patients may present with skin disorders. The majority of HIV-induced cutaneous diseases is not life-threatening, but is cosmetically disfiguring and jeopardizes the quality of life of HIV infected patients (Ramdial, 2000).

Herpes simplex has always been associated with immunodepression, a fact that became more evident with the onset of the AIDS epidemic (Trope & Lenzi, 2005). It was one of the earliest infections seen in AIDS patients (Panasiti *et al.*, 2007). Cutaneous herpes simplex virus (HSV) infections occur in about 20% of patients with HIV/AIDS; with reactivation of HSV often occurring in these patients at all stages of immunocompetency, resulting in chronic mucocutaneous disease with severe and widespread skin ulcers (Ramdial, 2000). Ulcerated perianal lesions are frequent in patients with HIV/AIDS, where the HSV has been shown to be the major etiologic agent, causing 22-76% of all cases (Panasiti *et al.*, 2007).

Acyclovir, a synthetic purine nucleoside analogue antiviral agent derived from guanine (McEvoy, 2002), is effective against herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), varicella zoster virus (VZV), and to a lesser extent against Epstein-Barr virus and cytomegalovirus (CMV) (Díez-Sales *et al.*, 2005).

Opportunistic fungal infections are also frequent in HIV/AIDS patients and fungal infections in these patients are one of the main causes of morbidity and mortality (Durden & Elewski, 1997). Candida species are mainly the causes of fungal infections in patients with AIDS, with *Candida albicans* the most common species. Other species include *C. tropicalis*, *C. krusei*, and *C. glabrata*. Mucous membrane infection rather than disseminated candidiasis occur in HIV-infected persons because of the intact humoral immune response and mucocutaneous candidiasis frequently indicates rapid progression to AIDS (Durden & Elewski, 1997). Dermatophytes, which infect the keratinized epidermis, nails, and hair, are also common opportunistic pathogens in HIV/AIDS disease (Johnson, 2000). Seborrhoeic dermatitis has an occurrence of 46 to 83.3% in patients with HIV/AIDS (Ramdial, 2000). It occurs with greater frequency in the HIV/AIDS patients, and is usually more rigorous than in the non-HIV/AIDS patients. Extensive cutaneous association is common, with a tendency in the trunk, extremities and groin (Durden & Elewski, 1997).

According to McEvoy (2002) ketoconazole, a synthetic imidazole derivative and antifungal agent, is active against most pathogenic fungi, including dermatophytes and yeasts. It also has *in vitro* activity against several gram-positive bacteria, including *Staphylococcus areus*, *S. epidermidis*, enterococci, Nocardia, and *Actinomadura* (McEvoy, 2002). In addition, ketoconazole displays antiviral activity against HSV-1 and HSV-2 and synergistic antiviral activity when combined with acyclovir (Pottage *et al.*, 1986).

Panasiti *et al.* (2007) reported a case where a 37-year-old patient suffered from multiple painful ulcerative lesions of the perianal regions. Their laboratory examinations showed positively for HIV infection. They suggested that in HIV-positive patients, perianal HSV-2 can have atypical manifestations, especially if co-infection by *Candida albicans* occurs (Panasiti *et al.*, 2007).

In this study, the transdermal delivery of a combination of acyclovir and ketoconazole was investigated, in order to formulate a product containing both actives which can be effective in treating patients who suffers from co-infections of HSV and *Candida albicans*.

Transdermal drug delivery has a number of advantages over conventional oral dosage forms: (1) it avoids peaks and valleys in serum levels frequently seen with distinct oral dosages, (2) it avoids first-pass metabolism because of terrifically low skin metabolism, (3) in many occasions zero-order delivery is sustained and can be maintained for a longer period of time, leading to a less frequent dosing regimen and (4) there is relatively less intersubject variability as compared to oral drug administration because of inevitable food effects and adverse physiological conditions that might hinder the oral absorption process (Roy, 1997).

As with the other routes of drug delivery, transport across the skin is also associated with several disadvantages, the main disadvantage being that not all compounds are suitable candidates. A number of physicochemical parameters have been identified that influence the diffusion process, and differences in permeation rates can occur between individuals, different races, and between the elderly and young (Roberts *et al.*, 2002). According to Bouwstra (1997) the natural function of the skin is to protect the body against the loss of endogenous substances such as water and against undesired influences from the environment caused by exogenous substances. The major problem in dermal and transdermal delivery of drugs, is overcoming the natural barrier of the skin (Bouwstra, 1997).

According to Sweetman (2002) systemic absorption of both acyclovir and ketoconazole after topical administration is minimal. This could be due to the physicochemical properties of the drugs and the complexity of the skin.

Previous studies done by Jiang *et al.* (1998) concluded that the limited efficiency of topical acyclovir therapy is due, at least in part, to the inability of acyclovir to penetrate through the stratum corneum (SC) barrier layer of the skin, and an inability to reach the target site; the basal epidermis (Jiang *et al.*, 1998). According to Freeman *et al.* (1986), topical acyclovir in polyethylene glycol ointment has been disappointing in the treatment of recurrent HSV infections in immunocompetent patients. The effect of polyethylene glycol on skin penetration has been postulated to be due to a drug-vehicle interaction that results in a lower thermodynamic activity of the drug. Other researchers have suggested that the impeding effect of polyethylene glycol is due to its inability to hydrate the SC or to a relative osmotic effect which tends to dehydrate the SC (Freeman *et al.*, 1986).

Díez-Sales *et al.* (2005) reported that propylene glycol (PG) could have an enhancing effect in the permeation of acyclovir across human epidermis. This enhancing effect depended on the concentration of PG used. It is suggested that PG diffuses into the SC, interacts with the polar group regions of the lipids by replacing bound water, resulting in a slight shortening of the mean acyl chain length in the bilayers (Díez-Sales *et al.*, 2005).

In this study, we conducted vertical Franz cell diffusion studies with female abdominal skin, and applied a combination of acyclovir and ketoconazole in micro-sponges of a novel therapeutic drug delivery system, Pheroid™, as well as in phosphate buffered solution (PBS) as control. Oleic acid, a fatty acid which has previously been shown to enhance the transdermal delivery of acyclovir, is one of the components of the Pheroid™ delivery system. It is thus expected that the Pheroids™ will enhance the penetration of acyclovir across the skin.

The aim of this study was to determine whether the Pheroid™ delivery system can be employed to deliver acyclovir and ketoconazole transdermally.

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CHAPTER 2: TRANSDERMAL DELIVERY OF ACYCLOVIR AND KETOCONAZOLE

1 INTRODUCTION

Diseases of the skin and mucous membranes were amongst the first recognized clinical manifestations of acquired immunodeficiency syndrome (AIDS) in the early 1980s. Hundreds of disorders occurring on the skin and mucosa have been reported since then in human immunodeficiency virus (HIV) disease (Fiallo & Talhari, 2007). The changes in the skin are mainly attributable to the alteration in immune function (Criton *et al.*, 1995) and it is renowned that more than 90% of HIV-infected patients will develop at least one type of dermatologic disorder during the course of their HIV infection (Zancanaro *et al.*, 2006).

2 CUTANEOUS DISEASES COMMON IN HIV AND AIDS

The cutaneous manifestations of HIV infection have been the subject of intense scrutiny, since the skin is the most commonly affected organ in HIV-infected individuals (Ramdial, 2000). Infectious diseases are the main category of cutaneous disorders associated with HIV infection. The majority of these infections are either fungal or viral (Kar *et al.*, 1996). Goodman *et al.* (1987) studied skin disease in patients with acquired immunodeficiency syndrome (AIDS) and with AIDS-related complex. The most common cutaneous findings were candidiasis (47%), seborrhoeic dermatitis (32%), dermatophytosis (30%), acquired ichthyosis or xerosis (30%), herpes simplex infections (22%) and molluscum contagiosum (9%) (Goodman *et al.*, 1987) In the context of HIV/AIDS infections, the clinical presentation of dermatoses assumes either a classic or an uncommon form. This will depend, in most cases, on the patients immunological status, represented mainly by the CD4 lymphocyte count and associated viral load (Trope & Lenzi, 2005).

HIV produces cellular immune deficiency characterized by the depletion of helper T lymphocytes ($CD4^+$ cells). Most infections and neoplastic processes in the skin of a patient infected with HIV are altered or facilitated by the loss of $CD4^+$ cells of the immune system (Erdal *et al.*, 2007).

2.1 VIRAL INFECTIONS

Numerous viruses of the herpes viridae family may lead to cutaneous disease, including herpes simplex virus (HSV), herpes zoster virus (HZV) and disseminated cytomegalovirus (CMV) infection (Erdal *et al.*, 2007). All of these organisms are double-stranded DNA

viruses, which permanently infect their target cells (Nadelman & Newcomer, 2000) and cause frequent morbidity in HIV-infected persons (Harris & Saag, 1997).

2.1.1 HERPES SIMPLEX VIRUS INFECTION

Mucocutaneous lesions of HSV are by far the most common infestation in AIDS (Criton *et al.*, 1995). There are two types of HSV, HSV-1 and HSV-2 (Beers, 2007). HSV-1 is transmitted primarily by contact with infected saliva (Lutwick & Seenivasan, 2006), it is acquired in childhood (Baeten & Celum, 2006) and causes cold sores on the lips (herpes labialis) and sores on the cornea of the eye (herpes simplex keratitis) (Beers, 2007). Conversely, HSV-2 is transmitted sexually and causes anogenital ulcers (Baeten & Celum, 2006). Nevertheless, HSV-1 has been found in genital lesions, and HSV-2 has been found in oral lesions (Nadelman, & Newcomer, 2000). The increased observation of crossover infections is probably secondary to orogenital intercourse (Lin *et al.*, 2003).

Primary HSV infection results in the establishment of a lifelong latent infection in sensory ganglion neurons innervating the site of inoculation (Weber & Cinatl, 1996). HSV invades and replicates in neurons as well as in epidermal and dermal cells. Virions travel from the preliminary site of infection on the skin or mucosa to the sensory dorsal root ganglion, where latency is established (Torres, 2007). Once reactivated, replicated virus travels down the sensory neuron, and is clinically manifest as epidermal vesicles or mucosal ulcers (Lin *et al.*, 2003). Periodic reactivation of the latent virus may result in recurrent mucocutaneous facial, ophthalmic, or genital herpetic infections (Weber & Cinatl, 1996). A variety of stimuli, such as trauma, ultraviolet radiation, and extremes in temperature, stress, immunosuppression, or hormonal fluctuations can induce recurrent clinical outbreaks (Torres, 2007).

2.1.1.1 SYMPTOMS AND SIGNS OF HSV INFECTIONS

The clinical symptoms differ significantly according to the patient's immune status. Typical localized infections with itching, erythema and grouped vesicles will appear and heal spontaneously within a few days as long as the cell-mediated immune functions are normal. In patients with advanced HIV infection and severe immunodeficiency, deep and large ulcerations of the anogenital region, but also the face and other parts of the body will appear (Schoefer *et al.*, 2006). The first oral infection with HSV generally causes sores inside the mouth (herpetic gingivostomatitis), which last 10 to 14 days and are often very severe, making eating and drinking exceptionally uncomfortable (Beers, 2007). Constitutional symptoms such as fever, malaise, and anorexia may accompany the primary infection (Van Hees & Naafs, 2001).

Recurrent herpes labialis occurs in 20-40% of the population (Woo & Challacombe, 2007) and is often heralded by a sensation of burning or hyperesthesia in the affected area before the appearance of lesions (Harris & Saag, 1997). New lesions appear over 1 to 2 days, the vesicles rapidly become pustules which usually become crusty within 48 hours. Viral shedding occurs over 3 to 5 days. The lesions last 2 to 10 days and heal without scarring (Nadelman & Newcomer, 2000). Infectivity is highest within the first 24 hours of the appearance of lesions with 80% of the vesicles and 34% of the ulcer/crust lesions yielding positive HSV cultures (Woo & Challacombe, 2007). Fig. 2.1 (a) and Fig. 2.1 (b) illustrate the clinical representation of herpes gingivostomatitis (National Skin Centre, 2007) and herpes simplex labialis (DermNet NZ, 2006).

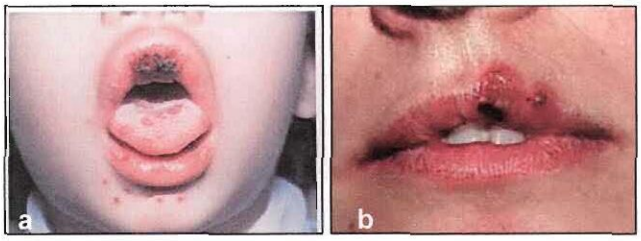

Fig. 2.1 (a): Herpes gingivostomatitis, Fig. 2.1 (b): Herpes simplex labialis.

The first episode of HSV-2 infection can cause more extensive and severe symptoms than those of HSV-1 and may include systemic problems. Incubation lasts 2 to 10 days, with viral shedding occurring for about 15 days (Nadelman & Newcomer, 2000). Painful, erythematous, vesicular lesions that ulcerate most commonly occur on the penis, but they can also occur on the anus and the perineum in men. In women, primary herpes genitalis presents as vesicular/ulcerated lesions on the cervix and as painful vesicles on the external genitalia bilaterally. In addition they can occur on the vagina, the perineum, the buttocks, and sometimes the legs in a sacral nerve distribution (Torres, 2007). Fever and malaise are common and some people experience a burning sensation during urination. Occasionally, an infected person may have no symptoms (Beers, 2007).

After primary infection, the virus may be latent for months to years until a recurrence is triggered. Recurrent clinical outbreaks are milder and often preceded by a prodrome of tingling, itching, burning, pain, or paresthesia (Torres, 2007), which precedes the blisters by several hours to 2 to 3 days. A typical episode of recurring genital herpes lasts a week (Beers, 2007).

Seroprevalence in people co-infected with HIV has recently been reported in international studies to be close to 90% for HSV-1 and up to 77% for HSV-2 (Torres, 2007). Impaired immunity in immunocompromised individuals, as in the case of persons with HIV-1 infection, leads to more frequent and severe symptomatic and asymptomatic HSV reactivation (Baeten & Celum, 2006). Ulcerated perianal lesions are commonly observed in patients with AIDS and in advanced stages of disease, prevalence rates as high as 10-14% has been reported (Nascimento *et al.*, 2002). As the immunodeficiency progresses, HSV infection becomes persistent and progressive. Erosions enlarge and deepen into painful, non-healing ulcers (Fiallo & Talhari, 2007). The recurrence of asymptomatic HSV shedding occurs 3-5 times more frequent in patients with HIV than in immunocompetent persons (Rigopoulos *et al.*, 2004). Figure 2.2 (a), (b), and (c) illustrates the clinical representation of primary herpes simplex in males and females (Dermnet, 2007) and herpes simplex in a HIV infected female (Fiallo & Talhari, 2007).

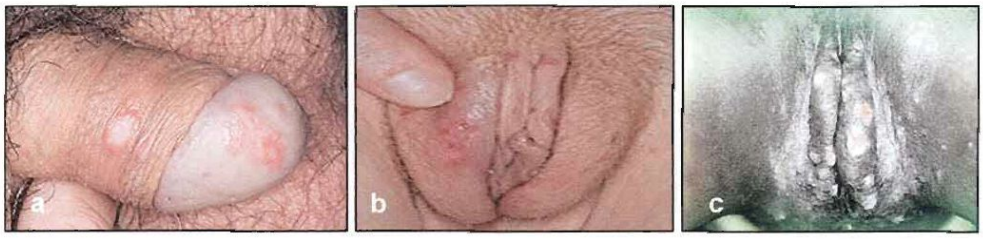

Fig. 2.2 (a): Primary herpes genitalis in males, Fig. 2.2 (b): Primary herpes genitalis in females, Fig. 2.2 (c): Herpes simplex in a HIV infected female.

2.1.1.2 COMPLICATIONS OF HSV INFECTIONS

Encephalitis is one of the most life-threatening complications of HSV infection. Without therapy, the mortality rate exceeds 70% and only about 9% of surviving patients return to normal health. Another important consequence of HSV-1 infection is an allergic response called erythema multiforme (Nadelman & Newcomer, 2000). HSV-2 infection in pregnancy can have devastating effects on the foetus. Neonatal HSV generally manifests within the first 2 weeks of life and clinically ranges from localized skin, mucosal, or eye infections to pneumonitis, disseminated infection, encephalitis and death (Torres, 2007).

2.1.1.3 OTHER MANIFESTATIONS OF HSV

Herpetic whitlow, herpes keratoconjunctivitis, herpes keratitis, eczema herpeticum, and herpes gladiatorum are other manifestations of herpes (Lin *et al.*, 2003). Herpetic whitlow is a cutaneous infection, generally on the hands, caused by HSV-1 or HSV-2. A localized,

painful, erythematous swelling with vesicle formation occurs often with lymphadenopathy (Harries & Lear, 2004).

HSV-2 may rarely infect the eye by means of direct contact with infectious genital secretions and occasionally is transmitted to neonates as they pass through the birth canal of a mother with genital HSV-2 infection. Although more common as a manifestation of recurrent HSV infection, HSV keratitis may also be seen during a primary infection (Wang & Ritterband, 2007). Herpes keratoconjunctivitis and herpes keratitis may present with superficial or deep ulcers on the conjunctiva or cornea (Lin *et al.*, 2003).

Eczema herpeticum is a devastating herpes virus infection on skin already affected by atopic dermatitis; it is a dermatologic emergency and untreated infections may lead to complications, including herpes keratitis and disseminated HSV infections with visceral involvement. The rash starts as dome-shaped vesicles, which later disappear and become punched-out excoriations, crusts, and erythematous plaques. The most commonly affected areas include the head, neck and trunk. Systemic symptoms such as fever and malaise normally accompany the rash (Buccolo, 2004).

Herpes gladiatorum occurs when abraded skin of one person is inoculated with the active herpetic lesions of another (Lin *et al.*, 2003). According to Perriello (2007) the lesions are usually located on exposed areas of the body where the most skin-to-skin contact occurs. Vesicular eruptions present on the head, trunk and extremities (Lin *et al.*, 2003). Figure 2.3 (a), (b), (c), (d) and (e) illustrates the clinical representation of herpetic whitlow (DermNet NZ, 2007), herpes simplex keratitis (Wang & Ritterband, 2007), eczema herpeticum on the face and back (Buccolo, 2004) and herpes gladiatorum (Emedicine, 2007) respectively.

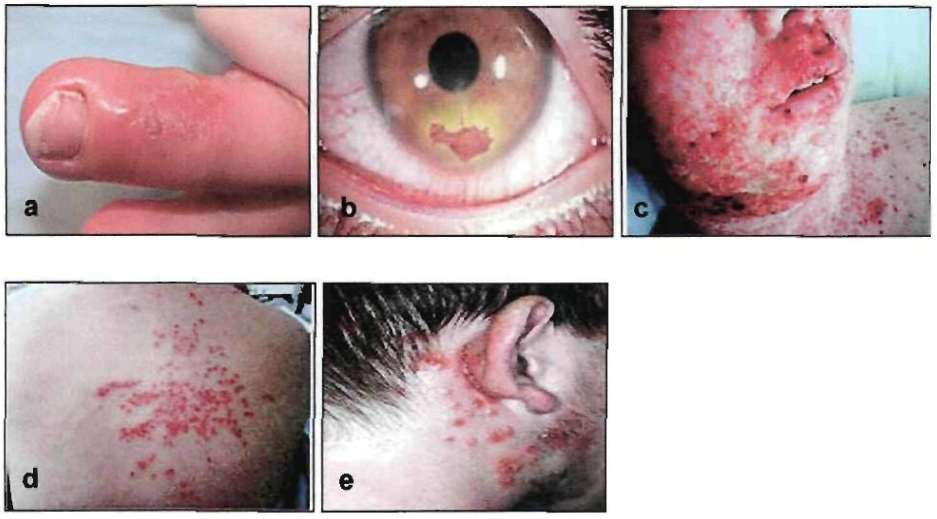

Fig. 2.3 (a): Herpetic whitlow, Fig. 2.3 (b): Herpes simplex keratitis, Fig. 2.3 (c): Eczema herpeticum on the face, Fig. 2.3 (d): Eczema herpeticum on the back, Fig. 2.3 (e): Herpes gladiatorum.

2.1.2 VARICELLA ZOSTER VIRUS AND HERPES ZOSTER VIRUS INFECTION

Chickenpox and shingles are caused by the varicella zoster virus (VZV) (Beers, 2007). Primary infection presents as varicella (or chickenpox), a contagious and usually benign illness that occurs in epidemics among susceptible children (Gnann & Whitley, 2002). After primary infection of chickenpox, VZV establishes a latent infection in the trigeminal and dorsal root ganglia (Kennedy *et al.*, 1998). Subsequent reactivation of latent VZV in dorsal root ganglia results in a localized cutaneous eruption termed "herpes zoster" (HZ) or "shingles" (Gnann & Whitley, 2002).

VZV infection usually recurs with advancing age in immunocompetent hosts, but may occur earlier in life as a result of decreased specific VZV humoral or cellular-mediated immunity (Erlich & Safrin, 2007). The increased incidence of HZ in the elderly is related to the selective decline in cell-mediated immunity against VZV due to advancing age (Mandal, 2006). Shingles is common in HIV patients and may be the earliest sign of immunosuppression, but it can occur at any stage of HIV disease (Fiallo & Talhari, 2007). VZV reactivation occurs maximally after the period of most severe immunosuppression, where reactivation of HSV also typically occurs during this period (Mandal, 2006).

2.1.2.1 SYMPTOMS AND SIGNS OF VZV AND HZV INFECTION

Some people experience pain, a tingling sensation, or itching in an area of skin in the 3 or 4 days before shingles develop (Beers, 2007). The typical lesion of primary varicella (chickenpox) has been described as a "dewdrop on a rose petal" and appears initially as

erythematous macules over the face and trunk, sparing the extremities. The lesions progress to pruritic papules, vesicles and pustules with crusting. Systemic symptoms of fever, headache, myalgia, anorexia, and vomiting often occur with these dermatologic symptoms (Lin *et al.*, 2003).

The outbreak of shingles is almost always limited to a strip of the skin on one side of the body that contains a group of infected nerve fibres; the dermatome (Beers, 2007). The manifestation of HZ is reported as more frequent, more severe, and of longer duration in HIV patients compared to immunocompetent persons (Rigopoulos *et al.*, 2004). The vesicles frequently become chronic ulcerative, necrotic, or verrucous. Progressive neuronal inflammation and necrosis lead to severe pain (post-herpetic neuralgia), which increases as the infection travels down the nerve (Trent & Kirsner, 2004). Atypical and complicated HZ forms are more common in HIV patients and recurrent HZ appears in 20-30% of these patients (Rigopoulos *et al.*, 2004). In immunocompromised hosts, both varicella and shingles may occur as severe illness, occasionally with dissemination to the lungs, liver or central nervous system (Harris & Saag, 1997). Fig. 2.4 (a), (b) and (c) illustrates the clinical representation of chickenpox or varicella (Van Hees & Naafs, 2001), disseminated zoster in a HIV patient (Fiallo & Talhari, 2007) and shingles (National Skin Centre, 2007) respectively.

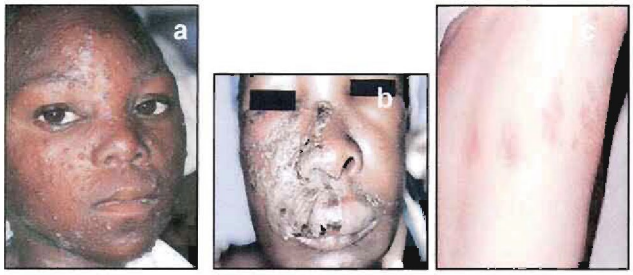

Fig. 2.4 (a): Chickenpox or varicella, Fig.2.4 (b): Disseminated zoster in a HIV patient, Fig. 2.4 (c): Shingles

2.1.2.2 COMPLICATIONS OF VZV AND HZV INFECTION

Post-herpetic neuralgia, delayed healing or locally progressive disease are the most upsetting problems of HZ in the immunocompromised. The frequency of postherpetic neuralgia is reported to be 23% in HIV, whereas 9% is noted in the general population. Furthermore, intensely immunosuppressed patients are at risk from visceral disease that is potentially fatal, especially if the presentation is atypical (Mandal, 2006).

2.1.3 CYTOMEGALOVIRUS

Cytomegalovirus (CMV) is perhaps the most common virus to co-infect HIV-infected persons (Harris & Saag, 1997). CMV does not generally manifest in or on the skin; only a few reports in the literature discuss CMV-induced skin lesions, such as perianal and oral ulcerations that may occur as an extension of pre-existing CMV-induced gastrointestinal disease (Trent & Kirsner, 2004). Non-specific maculopapular eruptions similar to those affecting patients with Epstein-Barr virus or papulovesicular, nodular, purpuric, and ulcerative lesions of CMV infection are observed in patients who are immunocompromised (Erdal *et al.*, 2007).

2.2 FUNGAL INFECTIONS

Candida spp, dermatophytes and Malassezia furfur infections are the most common pathogens responsible for superficial mycoses in HIV infected patients. Their clinical course is often atypical, and can be masked by other infections (Fiallo & Talhari, 2007). Recurrent and persistent mucocutaneous candidiasis is frequent in patients with HIV infection. In adults, generalized dermatophytosis, or tinea capitis, which is usually caused by *Trichophyton rubrum,* may suggest HIV infection (Erdal *et al.*, 2007).

2.2.1 CANDIDIASIS

Mucocutaneous candidiasis is one of the most common manifestations of HIV disease, affecting most patients at some time during their illness (Harris & Saag, 1997). Candida is a resident yeast of the mucous membranes. It becomes pathogenic under favourable host conditions, for instance when host immunity is decreased such as in HIV-infected patients (Van Hees & Naafs, 2001). Candida fungal infection develops in 30% to 50% of HIV patients (Trent & Kirsner, 2004), and causes significant morbidity and mortality in these patients (Johnson, 2000). *Candida albicans* is the most common species, but other species include *C. tropicalis, C. krusei* and *C. glabrata* (Durden & Elewski, 1997). The most common forms of mucocutaneous candidiasis include oropharyngeal and vulvovaginal disease (Fichtenbaum & Aberg, 2006).

2.2.1.1 SYMPTOMS AND SIGNS OF CANDIDIASIS

Oropharyngeal candidiasis, or thrush, develops as one of four patterns: (1) pseudomembranous, with removable whitish patches; (2) hyperplastic, with thickened plaques; (3) atrophic, which manifests only as erythematous patches; and (4) angular cheilitis, characterized by erythema, fissuring, and scaling at the corners of the mouth (Harris & Saag, 1997).

Candidiasis or thrush presents on the skin as red macules often with small pustules on their periphery which break down as the lesion spreads outwards. Redness, superficial erosions and white adherent plaques, which may be itchy and painful, can be seen on the oral and vulvo-vaginal mucosa (Van Hees & Naafs, 2001). Cutaneous candidiasis is found most commonly in the diaper area and skin folds (Kekitiinwa & Schwarzwald, 2007). Skin involvement includes intertrigo, folliculitis, paronychia, and/or onychomycosis (Durden & Elewski, 1997).

Candidiasis in HIV patients is strongly related to deficiency of anti-candida defence mechanisms, both topical and systemic, due to the induced immunodeficiency related to HIV infection (Rigopoulos *et al.*, 2004). Mucocutaneous candidiasis occurs in 3 forms in persons with HIV infection: oropharyngeal, oesophageal, and vulvovaginal disease (Fichtenbaum & Aberg, 2006). Mucocutaneous candidiasis in untreated HIV-infected individuals heralds rapid progression to AIDS (Fiallo & Talhari, 2007). Up to 90% of persons with advanced untreated HIV infection develop oropharyngeal candidiasis, with 60% having at least 1 episode per year with frequent recurrences (50-60%). Oesophageal candidiasis occurs less frequently (10-20%) but is the leading cause of oesophageal disease. Vaginal candidiasis has been noted in 27-60% of women, similar to the rates of oropharyngeal disease (Fichtenbaum & Aberg, 2006). Fig. 2.5 (a), Fig. 2.5 (b) and Fig. 2.5 (c) illustrate the clinical presentation of oral candidiasis (Beers, 2007), oral candidiasis in a HIV patient (Van Hees & Naafs, 2001) and candidiasis in the diaper area (Dermatlas, 2007).

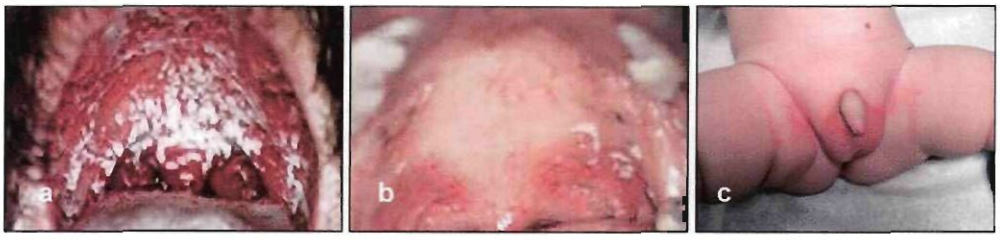

Fig. 2.5 (a): Oral candidiasis, Fig. 2.5 (b): Oral candidiasis in a HIV patient, Fig. 2.5 (c): Candidiasis in diaper area

2.2.2 SEBORRHOEIC DERMATITIS

The incidence of seborrhoeic dermatitis in the general population is approximately 3-5% of all young men (Schoefer *et al.*, 2006). In the HIV infected population on the other hand, seborrhoeic dermatitis is very common, affecting up to 85% of patients at some time. The extent and severity of disease may be exaggerated (Rodwell & Berger, 2000). Areas rich in

sebaceous glands, such as the forehead, scalp, eyebrows, nasolabial folds, external ear canal, between the shoulder blades, over the sternum, and retroauricular area, develop yellowish, oily scales and crusts on slightly erythematous to very red plaques. The lesions may be pruritic (Schoefer *et al.*, 2006).

2.2.3 DERMATOPHYTOSIS

Dermatophytosis is an infection of the skin, hair or nails caused by dermatophytes, a group of related filamentous fungi also known as ringworm fungi. These organisms attack the keratinized tissue of the host (Rinaldi, 2000). Dermatophyte infections are extremely common in AIDS patients and are reported to affect 30-50% of this population (Criton *et al.*, 1995). Dermatophytosis generally occurs as tinea corporis or tinea capitis. The lesions are extensive and noncompliant to treatment in HIV-infected persons (Kekitiinwa & Schwarzwald, 2007).

2.2.3.1 TINEA CORPORIS

Tinea corporis most commonly occur on the exposed surfaces of the body, namely the face, arms and shoulders. Lesions are usually round, showing scaling at the periphery or in concentric rings. In immunosuppressed persons multiple, large or widespread lesions may be seen (Van Hees & Naafs, 2001).

2.2.3.2 TINEA CAPITIS

Tinea capitis are normally seen in children. Severe pustular forms exist with follicular pustules and nodules and often immense purulent secretion. The patient may have a fever and headache, the lymphnodes in the neck swell and there may be bacterial superinfection (Van Hees & Naafs, 2001). Fig. 2.6 (a), Fig. 2.6 (b) and Fig. 2.6 (c) illustrates the clinical representation of seborrhoeic dermatitis (Beers, 2007), tinea capitis and tinea corporis (Van Hees & Naafs, 2001) respectively.

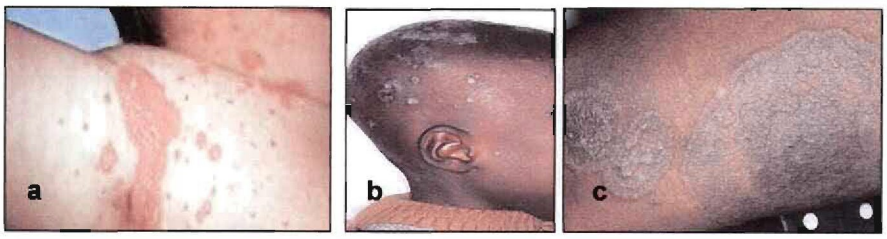

Fig. 2.6 (a): Seborrhoeic dermatitis, Fig. 2.6 (b): Tinea capitis, Fig. 2.6 (c): Tinea corporis

2.3 CONCLUSION

Skin diseases tend to be more chronic, more severe, more resistant to conventional treatments, and often display unusual clinical presentations during the course of HIV infection, compared to those seen in the non-HIV infected population (Fiallo & Talhari, 2007).

3 TREATMENT OF CUTANEOUS DISEASE

Effective therapy of cutaneous disease requires that the active agent is delivered to the site of infection in adequate concentrations to produce a pharmacologic effect (Pershing *et al.*, 1994). Adequate amounts of drug delivered to skin basal epidermis is necessary for the treatment of HSV skin infections because major virus-induced epidermal pathology occurs in the basal epidermis (Jiang *et al.*, 1998). In the case of superficial dermatophyte infections, where the pathogen resides on or within the outermost layer of the skin, the antifungal therapeutic agent must be delivered to the SC in adequate concentrations to inhibit the growth of the fungal pathogen (Pershing *et al.*, 1994).

Common HIV-associated dermatoses, such as candidiasis, dermatophyte infections, and all HSV infections have decreased since the introduction of highly active antiretroviral therapy (HAART) (Zancanaro *et al.*, 2006). This treatment consists of at least 3 different antiretroviral drugs, often with a combination of 2 nucleoside analogues with a protease inhibitor or a nucleoside reverse transcriptase inhibitor (Lin *et al.*, 2003). Although striking improvement in survival and quality of life has occurred with HAART, less than 10% of HIV-infected people in the world have access to advanced therapies (Johnson, 2000).

3.1 TREATMENT OF VIRAL INFECTIONS

Table 1 indicates the drugs generally used in the treatment of viral infections which are common in HIV/AIDS patients.

Table 1: Drugs used in the treatment of viral infections common in HIV/AIDS (compiled from Nadelman & Newcomer, 2000)

Disease	Drugs
Herpes simplex	Foscarnet, ganciclovir, acyclovir, valaciclovir, famciclovir.
Varicella zoster	Acyclovir, valaciclovir, famciclovir (oral).
Herpes zoster	Acyclovir (parenteral and oral).

Acyclovir, however, is more potent against HSV-2 than famciclovir, and the parenteral and oral forms decrease both healing time and viral shedding if taken within 24 hours of the first signs of recurrent episodes (Nadelman & Newcomer, 2000).

Acyclovir is one of the most effective and selective agents against viruses of the herpes group (Diez-Sales *et al.*, 2005). It inhibits replication of human herpes viruses in cell culture, with herpes simplex type 1 being the most susceptible, followed in descending order of susceptibility by herpes simplex type 2, varicella zoster virus, Epstein-Barr virus and cytomegalovirus (O'Brien & Campoli-Richards, 1989). Antiviral activity of acyclovir depends on the intracellular activation of the drug to acyclovir triphosphate. The thymidine kinases encoded by HSV, VZV and to a smaller degree, Epstein-Barr virus, convert acyclovir to the monophosphate. Normal cellular enzymes then phosphorylate the monophosphate to the diphosphate and triphosphate (Dollery, 1999a). The specificity of acyclovir is achieved because the phosphorylation required for its activation occurs only in cells infected with herpes viruses (Griffiths, 2005). Acyclovir triphosphate functions as a substrate for and preferential inhibitor of herpes simplex DNA polymerase. It therefore inhibits viral DNA replication. In addition it inhibits viral DNA polymerase and consequently cellular DNA replication to a smaller extent (Dollery, 1999a). Since acyclovir is selectively converted to its active form in herpes virus-infected cells, it is not toxic to normal, uninfected cells (Jiang *et al.*, 1998).

Topical acyclovir is indicated for the treatment of limited non-life threatening initial and recurrent mucocutaneous herpes simplex virus (HSV-1 and HSV-2) infections in immunocompromised patients. It is also used as adjunctive therapy to improve cutaneous healing of localized HZ in immunosuppressant persons being treated systemically with other treatment regimens for HZ (USP DI, 1998a). Table 2 summarizes the adverse reactions associated with parenteral, oral and topical administration of acyclovir.

Table 2: Summary of adverse reactions associated with parenteral, oral and topical administration of acyclovir (compiled from Gennaro *et al.*, 2000)

Route of administration	Adverse reaction
Parenteral	Irritation at the site of injection (9%) is the most common adverse effect. The drug may crystallize in the urine and impair renal function if fluid intake is inadequate, glomerular filtration rate is low, the dosage interval is too short or the drug is given as a bolus. Metabolic encephalopathy (1%) with hallucinations, confusion, tremors and seizures, bone-marrow depression and alterations in hepatic function can also occur.
Oral	In the short term nausea, vomiting (2.7%), headache (0.6%), diarrhoea, dizziness, fatigue, skin rash, sore throat (all 0.3%), anorexia, oedema, lymphadenopathy and leg pain can occur. In the long term there may be headache (1.9%), diarrhoea (2.4%), nausea and vomiting (2.7%), arthralgia, vertigo (both 3.6%), insomnia, fatigue, irritability, depression, rash, acne, alopecia, fever, palpitations, sore throat, muscle cramps and lymphadenopathy.
Topical	Local stinging, burning or pain (28%), itching (4%), vulvitis (0.3%) and rash (0.3%)

Due to the number of adverse reactions associated with parenteral and oral administration of acyclovir, topical delivery of the drug could be feasible. However, the topical application of acyclovir has proven clinically disappointing in the therapy of HSV skin infections compared with oral or intravenous administration. The limited efficiency of acyclovir therapy is due, at least in part, to the inability of acyclovir to penetrate the SC barrier layer of the skin, and lack of its reach at the target site, the basal epidermis (Jiang *et al.*, 1998). As will be discussed in § 4.6.1 to § 4.6.8, the physicochemical properties of a drug also play an important role in the transdermal delivery of drugs. These physicochemical properties can either be beneficial or it can be a limiting factor in the transdermal delivery of drugs.

In several studies using a cytopathic effect inhibition assay (CPE-inhibition assay), the concentration of acyclovir required to produce 50% inhibition of viral cytopathic effect or plaque formation (ID_{50}) ranged from 0.02 – 0.7 µg/ml and 0.018 – 0.043 µg/ml respectively for susceptible strains of HSV-1 and HSV-2 respectively. In several studies using a plaque inhibition assay, the ID_{50} of acyclovir reported for susceptible strains of HSV-1 and HSV-2 ranged from 0.01 – 3.2 µg/ml and 0.027 – 0.36 µg/ml respectively (McEvoy, 2002).

3.2 TREATMENT OF FUNGAL INFECTIONS

Table 3 indicates the drugs that are commonly used in fungal infections associated with HIV/AIDS.

Table 3: Drugs used in the treatment of fungal infections common in HIV/AIDS (compiled from Durden & Elewski, 1997)

Disease	Drugs
Oral candidiasis	Clotrimazole (topical), fluconazole (systemic)
Cutaneous candidiasis	Topical azoles such as clotrimazole, oxiconazole, ketoconazole. Topical allylamines such as terbinafine and naftitine, or topical nystatin.
Seborrhoeic dermatitis	Topical ketoconazole

Ketoconazole was the drug of choice since it is readily available in South Africa. It also demonstrates synergistic antiviral activity when combined with acyclovir (McEvoy, 2002). If formulated successfully in combination with acyclovir, it could be of benefit for HIV-patients suffering from co-infections.

Ketoconazole is a synthetic imidazole compound; it has a broad spectrum of activity against both dermatophytes and yeasts (Daniel, 1996). Ketoconazole inhibits the biosynthesis of ergosterol, a key component of the cell membrane of yeast and fungal cells. It replaces the precursor lanosterol as a substrate for the fungal cytochrome P450 enzyme lanosterol-14α-demethylase, which catalyzes the conversion of lanosterol to ergosterol. This action modifies the permeability of yeast and fungal cell membranes. Inhibition of ergosterol biosynthesis, the most important sterol of these cell membranes, is accompanied by accumulation of 14α-methylsterol (Dollery, 1999b).

Ketoconazole is used as the principal agent in topical treatment of tinea corporis (ringworm of the body) and tinea cruris (ringworm of the groin) caused by *Trichophyton rubrum*; *Trichophyton mentagrophytes*; and *Epidermophyton floccosum*, tinea pedis (athletes foot), tinea versicolor (pityriosis versicolor or "sun fungus") caused by Malassezia furfur, cutaneous candidiasis caused by Candida species and Paronychia. In addition, it is used for the treatment and prophylaxis of seborrhoeic dermatitis; it reduces the scaling due to dandruff and it is used as the secondary agent in topical treatment of tinea barbae and tinea capitis (USP DI, 1998b). Table 4 summarizes the adverse reactions associated with oral and topical administration of ketoconazole.

Table 4: Summary of adverse reactions associated with oral and topical administration of ketoconazole

Route of administration	Adverse reaction
Oral	Nausea and vomiting are the most frequent adverse reactions (3-10%), followed by pruritis (1.5%) and muscle cramps (1.2%). Other adverse effects may be sleepiness, headache, diarrhoea, photophobia, fever, thrombocytopenia, gynecomastia, impotence and oligospermia (Gennaro *et al.*, 2000).
Topical	Local reactions have been reported in 3-5% of patients, consisting of severe irritation, pruritis and stinging (McEvoy, 2002).

Due to the number of adverse reactions and drug interactions which are associated with oral administration of ketoconazole, topical delivery of the drug could be feasible. Formulation of the drug in a topical vehicle, can overcome the problems which are associated with drug interactions and adverse reactions which occurs in the gastrointestinal tract.

Ketoconazole interferes with hepatic microsomal enzymes and demonstrates interaction with a wide variety of drugs (Dollery, 1999b). Table 5 summarizes the drug interactions that occur with concomitant oral administration of ketoconazole.

Table 5: Summary of the drug interactions which occur with concomitant oral administration of ketoconazole (compiled from McEvoy, 2002)

Drug	Drug interaction
Antimuscarinics, antacids, cimetidine, ranitidine	The absorption of ketoconazole is decreased since gastric acidity is necessary for the dissolution and absorption of ketoconazole.
Antituberculosis agents, rifampicin	Serum concentrations of ketoconazole are decreased.
Antiviral agents, acyclovir	There is a dose dependant synergistic antiviral activity against HSV-1 and HSV-2.
Cisapride	Ketoconazole inhibits the metabolism of cisapride, resulting in serious cardiovascular effects, including ventricular tachycardia and ventricular fibrillation.
Coumarin anticoagulants	Ketoconazole may enhance the anticoagulant effect of coumarin drugs.
Cyclosporine	Ketoconazole may interfere with the metabolism of cyclosporine *via* hepatic microsomal enzyme inhibition, causing an increase in the plasma levels of cyclosporine and serum creatinine.
Tacrolimus	There is an increase in plasma concentrations of tacrolimus, the immunosuppressive agent.
Phenytoin	The metabolism of both drugs is altered.
Alcohol	A disulfiram reaction, including flushing, rash, peripheral oedema, nausea, headache can occur when alcohol is consumed during ketoconazole therapy.
Corticosteroids, methylprednisolone, prednisolone	The plasma concentration of the corticosteroid may be increased, possibly due to the decreased clearance of the corticosteroid. Ketoconazole may enhance the adrenal suppressive effects of corticosteroids.
Theophylline	The serum concentration of theophylline is decreased.
Benzodiazepines, midazolam, triazolam	There is an increase in the peak plasma concentrations and prolongation of the plasma half-life of these benzodiazepines, leading to prolonged hypnotic and sedative effects.

A wide range of minimum inhibitory concentration (MIC) values of ketoconazole has been reported for *Candida*. In some *in vitro* studies on *C. albicans*, *C. parapsilosis*, and *C. tropicalis*, the minimum inhibitory concentration of ketoconazole at which the growth of 90% of strains tested was inhibited (MIC_{90}) was 1 – 16 µg/ml. However, in other studies, these organisms required ketoconazole concentrations of 25 µg/ml or greater for *in vitro* inhibition. The (MIC_{90}) of ketoconazole for dermatophytes is generally reported to be between 0.25 -2 µg/ml (McEvoy, 2002).

3.3 CONCLUSION

Although transdermal drug delivery offers a suitable approach of administration for a variety of clinical indications (Benson, 2005) and offers an alternative for the conventional drug delivery methods of oral and parenteral administration (Singh, 2005), not all compounds are suitable candidates for transdermal drug delivery (Roberts *et al.*, 2002). The limitations of transdermal drug delivery are governed largely by skin anatomy (Prausnitz *et al.*, 2004) and the application of transdermal drug delivery to a wide range of drugs is limited due to the significant barrier to penetration across the skin, which is associated primarily with the outermost SC layer of the epidermis (Benson, 2005).

In the following sections the structure of the skin, the mechanism of drug transport through the skin, the various pathways across the skin, the physiological and physicochemical factors influencing transdermal drug delivery, and penetration enhancement as applicable to acyclovir and ketoconazole will be discussed in short.

4 TRANSDERMAL PENETRATION

4.1 INTRODUCTION

As the largest organ of the body, human skin provides around 10% of the body mass of an average person (Williams, 2003). The natural function of the skin is to protect the body against the loss of endogenous substances such as water (Bouwstra, 1997) and to prevent the intrusion of microbes, chemicals and different forms of radiation (Zatz, 1993a). The skin also mediates the sensations of touch, pain, heat and cold (Barry, 1983), preserves itself and repairs its own wounds rapidly and effectively (Stewart *et al.*, 1974).

4.2 STRUCTURE OF SKIN

Human skin consists mainly of four main layers:

- The most superficial layer of the epidermis, the SC or horny layer (Lund, 1994). The SC is the end product of epidermal cell differentiation (Williams, 2003); it consists of 10-15 layers of corneocytes, and has a thickness varying from around 10-15 μm in the dry state, to 40 μm when hydrated (Benson, 2005). According to Mukhtar (1992) it is the least permeable layer of the skin for all but the most lipophilic compounds.

- The viable epidermis which is situated directly beneath the SC and contains keratinocytes at varying stages of differentiation (Asbill & Michniak, 2000). The actively dividing cells migrate upwards to successively form the spinous, granular and clear layers. As part of this process, the cells gradually lose their nuclei and undergo changes in composition. The role of the viable epidermis in skin barrier function is mainly related to the intercellular lipid channels and to several partitioning phenomena (Foldvari, 2000).

- The dermis, or corium; which is located between the epidermis and subcutaneous fat (Hunter *et al.*, 1995). It is usually 3-5 mm thick (Williams, 2003) and consists primarily of a matrix of connective tissue woven from fibrous protein which is embedded in an amorphous ground substance of mucopolysaccharide (Barry, 1983). The immense network of fibrous, filamentous and amorphous connective tissue determines the flexibility and tensile strength of the skin and provides the physical support for the widespread nerve and vascular networks (Schaefer & Redelmeier, 1996). Nerves, blood vessels and lymphatics pass through the dermis and skin appendages perforate it (Barry, 2002).

- The subcutaneous fat layer or hypodermis which lies between the overlaying dermis and the underlying body constituents (Williams, 2003). It is the deepest layer of the skin (Walters & Roberts, 2002) and generally contains abundant fat (Hunter *et al.*, 1995). One of the most important roles of the hypodermis is to carry the vascular and neural systems for the skin. It also attaches the skin to underlying muscle (Walters & Roberts, 2002). According to Barry (2002) the hypodermis provides a mechanical cushion, thermal barrier, and synthesizes and stores readily available high-energy chemicals.

The skin appendages include the hair follicles with their related sebaceous glands, eccrine sweat glands, apocrine sweat glands and the nails (Walters & Roberts, 2002). The ducts of

these appendages perforate the SC and may function as shunts for diffusion (Franz & Lehman, 2000).

4.3 DRUG TRANSPORT THROUGH THE SKIN

According to Mukhtar (1992) molecules cross membranes either by passive diffusion or by active transport. The successful delivery of a drug from a topical formulation into and/or through the skin necessitate the following sequential steps: (1) dissolution if required and then diffusion of drug molecules in the vehicle to the vehicle/skin interface; (2) partitioning of the drug from the vehicle into the SC; (3) diffusion of the drug through the SC; (4) partitioning of the drug from the lipophilic SC into the underlying viable epidermis; (5) diffusion through the viable epidermis and upper dermis and (6) uptake of the drug by the cutaneous microcirculation (Guy & Hadgraft, 1992).

According to Williams (2003) there are other potential fates for molecules entering human skin: permeants may bind with a variety of elements of the skin, the potential exists for drugs to be degraded or activated (as with prodrugs) at metabolic sites, they may also bind to receptors within the skin, and depending on the nature of the drug, the permeant may partition into the subcutaneous fat layer, and not enter the systemic circulation.

4.4 PENETRATION PATHWAYS ACROSS THE SC

There are three pathways postulated for the diffusion of solutes through the SC: transcellular, intercellular (paracellular), and transappendageal (Roberts *et al.*, 2002). The route of major significance will differ depending on drug polarity and whether steady state conditions have been achieved (Zatz, 1985).

4.4.1 THE INTERCELLULAR ROUTE

The intercellular lipid route provides the most important pathway by which most small, uncharged molecules cross the SC, except for some specialized cases (Williams, 2003). With the intercellular route, solutes diffuse around the corneal cells in a tortuous manner, remaining continuously in the intercellular matrix (Abraham *et al.*, 1995). In this instance the path length taken by the molecule is significantly greater than that of the SC thickness (Williams, 2003).

4.4.2 THE TRANSCELLULAR ROUTE

The transcellular route may predominate for highly hydrophilic molecules at pseudo-steady state (Williams, 2003). According to Abraham *et al.* (1995) solutes move directly through the corneal cells and intermediary intercellular lipid matrix. Thus, with transcellular permeation,

the pathway is directly across the SC and therefore the path length for permeation is generally regarded as the thickness of the SC (Williams, 2003).

4.4.3 THE TRANSAPPENDAGEAL PATHWAY (SHUNT ROUTE)

Generally, the transappendageal pathway contributes negligibly to the steady-state flux of a drug (Barry, 2001a); however, transport through the appendageal route has been shown to be significant during the non-steady-state period of percutaneous penetration (Mukhtar, 1992). It offers a parallel pathway by which solutes can be absorbed by sweat ducts and hair follicles without interference by the SC (Abraham *et al.*, 1995). Although the skin appendages contribute a small fractional area, they may offer the most important portal of entry into the subepidermal layers of the skin, for ions and large polar molecules (Moghimi *et al.*, 1999). For large molecules with low diffusional constants or poor solubility, transfollicular penetration may be the only absorption mechanism (Lund, 1994). In addition, colloidal particles and polymers can target the follicle (Barry, 2001a).

4.4.4 CONCLUSION

Generally, for polar drugs at least, it is probable that the transfollicular route and the transcellular route provide the principal pathway during percutaneous absorption. When penetrants become more non-polar, the intercellular route becomes more considerable, although it possibly does not dominate (Ghosh & Pfister, 1997).

Since acyclovir is a polar drug, it is expected that the route of major importance would be the transfollicular and transcellular route. Ketoconazole on the other hand is non-polar, and the intercellular route would be considered during its delivery through the skin.

4.5 PHYSIOLOGICAL FACTORS INFLUENCING TRANSDERMAL DRUG DELIVERY

4.5.1 DAMAGE AND DISEASE OF THE SKIN

Normal skin is extremely impermeable to most substances (Cevc *et al.*, 1996) and percutaneous penetration through diseased or damaged skin has been shown to differ from that through intact tissue (Mukhtar, 1992). According to Williams (2003) various disorders result in an eruption of the skin surface, and in such situations the barrier properties of the SC is compromised, allowing easier movement of drugs into and through the skin. According to the above, it is thus reasonable to assume that acyclovir and ketoconazole delivery would be higher through diseased skin than normal skin.

4.5.2 SKIN AGE

It is generally presumed that the skin of the foetus, the young, and the elderly are more permeable than adult tissue (Barry, 1983). The skin structures, including the barrier, are not yet fully developed in neonates and infants (Schalla & Schaefer, 1982), the skin is comparatively a much larger organ in infants than in adults and the epidermal enzymes able to metabolize applied medicines may not be completely developed (Lund, 1994). In the elderly, age-related changes in the skin occur in the dermis (MacLauglin & Holick, 1985) and according to Schaefer & Redelmeier (1996) there is an increase of the replacement time of the SC which is equal to reduced repair rate after wounding, an increase in corneocyte area and related decrease in the SC thickness, although there are no changes in the number of corneocyte layers.

4.5.3 TEMPERATURE

The temperature of the SC generally falls between 30°C and 37°C (Mukhtar, 1992). In *in vitro* experiments, alterations in the permeability coefficient appear to be small for temperatures up to 70 °C. Above this temperature irreversible denaturation takes place and large increases in permeability coefficient can be seen (Wiechers, 1989).

4.5.4 HYDRATION

Hydration of the skin is an important factor influencing the rate and degree of percutaneous absorption. It may influence the partitioning and concentration gradient of the penetrating molecule in the SC, and the overall thickness of the effective barrier (Riviere, 1993). If the SC is in a hydrated state rather than in its natural dry state, it is generally accepted that the majority of substances are absorbed through the tissue faster (Zatz, 1993b). In fact, there is a positive relationship between the extent of hydration of the SC and the percutaneous absorption rate of both hydrophilic and lipophilic compounds (Bodde *et al.*, 1990). According to Barry (1983) it is possible that some drugs which rapidly penetrate the skin to produce tissue concentrations that are high enough to exercise an osmotic effect may enhance skin hydration.

4.5.5 ANATOMICAL SITE

According to Lund (1994) Fick's law of diffusion proclaims that the diffusion of a solute will be inversely proportional to the thickness of the SC. Differences in penetration rates have been shown for full thickness cadaver skin, isolated from different sites, with rates increasing in the following anatomical order: plantar, anterior forearm, instep, scalp and ventral thigh, scrotum, and posterior auricular (Idson, 1975).

4.5.6 SEX AND RACE

According to Williams (2003) keratinocytes tend to be larger in females (37-46 μm) than in males (34-44 μm); however there are no reports of considerable dissimilarities in drug delivery between equivalent sites in the two sexes. Dissimilarity in the thickness of the SC vary among the Caucasian and Negroid races to such an extent that the thicker SC in the Negroid may lead to a slightly decreased percutaneous rate for certain substances (Jackson, 1993).

4.5.7 SKIN METABOLISM

Given that skin possesses significant enzymatic activities, a number of drugs applied topically are metabolized through the skin (Bando *et al.*, 1997). According to Barry (2002) it has been estimated that the skin can metabolize some 5% of candidate topical drugs. Studies by Bando *et al.* (1997) established that skin metabolism directly affects the total amount of drug that penetrates in the case of highly lipophilic drugs. It was found that the higher the metabolic rate to hydrophilic drugs is, the greater the amount that penetrated the skin would be (Bando *et al.*, 1997).

Since some of the above mentioned factors could have played a role in the permeation of both acyclovir and ketoconazole during our *in vitro* transdermal studies, human abdomen skin of adult Caucasian females, obtained after cosmetic surgery were used for the transdermal diffusion studies. During the preparation of the skin, care was taken to ensure intactness of skin samples. All experiments were also conducted in a water bath with a temperature of 37 °C to provide an epidermal surface temperature of 32 °C, which imitates the *in vivo* condition.

4.6 PHYSICOCHEMICAL PROPERTIES INFLUENCING TRANSDERMAL DRUG DELIVERY

4.6.1 PARTITION COEFFICIENT (P)

According to Williams (2003) the partition coefficient is a measure of the distribution of molecules between two phases. For transdermal delivery studies a partition coefficient between octanol and water is frequently used as a guide as to how well a molecule will spread between SC lipids and water (Williams, 2003). The absorption rate increases with P below the optimum log P ~ 2.5, due to the higher partition coefficient which provides a larger concentration gradient across the SC (Hadgraft & Wolff, 1993). Molecules demonstrating intermediate partition coefficients (log P between 1 and 3) have sufficient solubility within the lipid domains of the SC to allow diffusion through this domain, whilst still having adequate hydrophilic nature to permit partitioning into the viable tissues of the epidermis (Benson,

2005). Compounds with a low log P display low permeability since there is little partitioning into the skin lipids. On the other hand, compounds with high log P also give low permeability owing to their incapability to partition out of the SC (Thomas & Finnin, 2004).

The partition coefficient of acyclovir between n-octanol and 0.2 M phosphate buffer is 0.018 (Dollery, 1999a) while the partition coefficient of ketoconazole between octanol and water is 3.73 (Mannisto *et al.*, 1982). Since acyclovir has a very low partition coefficient and ketoconazole has a high partition coefficient, it is likely that these two drugs may exhibit poor skin penetration. It is thus necessary to use some kind of penetration enhancer to promote delivery of these two drugs through the skin.

4.6.2 DIFFUSION COEFFICIENT (D)

According to Williams (2003) the diffusion coefficient of a penetrant is sometimes a term used interchangeably with diffusivity. The diffusivity in liquid mediums usually has a tendency to decrease with an increase in the molecular volume and diffusivity in liquids is expected to differ only slightly with increased molecular size. However, diffusivities are more sensitive to molecular size in the more structured medium, semi-crystalline lipid regime in the SC (Roy, 1997). In the case of constant temperature, the diffusion coefficient of a drug in a topical vehicle or in skin depends on the properties of the drug and the diffusion medium; and the interaction between them (Barry, 2002).

4.6.3 PERMEABILITY COEFFICIENT (Kp)

The permeability coefficient describes the rate of penetrant transport per unit concentration and depends on the SC/vehicle partition coefficient of a drug (Williams, 2003). In addition it changes systemically with melting point; the compounds having the lowest melting points displaying the highest permeability coefficient (Roy, 1997) (see also § 4.6.6).

4.6.4 MOLECULAR SIZE AND SHAPE

According to Barry (2002) it appears that absorption is inversely associated with molecular weight: small molecules penetrate more rapidly than larger ones. As molecular weight increases, solute diffusivity reduces exponentially, imposing a size limitation on favourable transport across the skin (Naik *et al.*, 2000). The majority conventional therapeutic agents that are chosen as candidates for transdermal delivery have a tendency to lie within a rather narrow range of molecular weights (100-500 Dalton) (Williams, 2003). Acyclovir has a molecular weight of 225.2 (Lund, 1994) and falls in the range mentioned above. Ketoconazole has a molecular weight of 531.4 (British Pharmacopoeia, 2007) and just falls

out of the range mentioned above. The large molecular weight of ketoconazole can be a limiting factor in the transdermal delivery of the drug.

4.6.5 STATE OF IONISATION

The majority of drugs are weak acids or bases and, according to the pH-partition theory, as proposed by Shore *et al.* (1957), may exist in an ionized or non-ionized form, depending upon the pH of the vehicle. A change in solution pH alters the state of ionization of a drug and therefore its hydrophilicity/lipophilicity as it crosses the membrane (Malan *et al.*, 2002). The solubility of ionized species will be significantly higher than for unionized species (Hadgraft & Valenta, 2000) and membranes are more permeable to the nonionized forms, since they have greater lipid solubility (Malan *et al.*, 2002). It is believed that the nonionized molecule travels by the intracellular route through the SC, while the ionized molecule will more probably pass through the intercellular spaces (Wiechers, 1989). The pKa-values of acyclovir are 2.27 and 9.25 and the drug exists as the unionized form at physiological pH (Dollery, 1999a). The pKa-values of ketoconazole are 6.51 and 2.94 (Dollery, 1999b). It is thus likely that acyclovir would penetrate the skin *via* the intracellular route.

4.6.6 MELTING POINT

According to Roy (1997) compounds with lower melting points would generally be more permeable through human skin due to their intrinsic higher solubility in the SC. Acyclovir has a melting point of 257 °C (Sciencelab, 2005) whereas ketoconazole has a melting point of 148 °C to 152 °C (British Pharmacopoeia, 2007). The relative high melting point of acyclovir can be a limiting factor in its transdermal delivery. On the contrary, and other factors excluded, it is likely that ketoconazole would be able to penetrate the skin more rapidly than acyclovir, since the melting point of ketoconazole is much lower than the melting point of acyclovir.

4.6.7 SOLUBILITY

Solubility is a dominant factor in skin penetration; its significance was recognized when it was established that compounds soluble in both lipid and water penetrate better than substances displaying either high water or high lipid solubility (Malan *et al.*, 2002). According to Naik *et al.* (2000) a drug must preferably have both lipoidal and aqueous solubilities. If it is too hydrophilic, the molecule will not be capable to transfer into the SC. If it is too lipophilic, the drug will tend to remain in the SC layers. Aqueous solubility of a drug determines the concentration available to the absorption site, whereas the lipid solubility specified by the partition coefficient determines the rate of transport of the drug across the absorption site (Singh, 2005). The aqueous solubility of a drug molecule is partially dependant on other

physicochemical properties, for instance, the partition coefficient and molecular surface features that are relatable to drug absorption (Malan *et al.*, 2002). According to Hadgraft & Wolff (1993) the solubility parameter of the skin has been estimated as ~ 10 and consequently, drugs which have similar values would likely dissolve readily in the SC. Drugs with high lipid solubility, in general, permeate the skin at better rates (Cleary, 1993).

Acyclovir is stated to be slightly soluble in water, insoluble in ethanol, practically insoluble in most organic solvents, soluble in dilute aqueous solutions of alkali hydroxides and mineral acids (Lund, 1994). It has a solubility of 1.3 mg/ml in water at 25°C and 0.2 mg/ml in alcohol (McEvoy, 2002). Ketoconazole is practically insoluble in water, freely soluble in methylene chloride, soluble in methanol and sparingly soluble in alcohol (British Pharmacopoeia, 2007). It has a solubility of 40 µg/ml in water at 23°C and is relatively insoluble in alcohol at 23°C (McEvoy, 2002). Since ketoconazole is lipophilic (Dollery, 1999b) it is suspected that it would remain within the SC. This will be ideal since the target site of ketoconazole is the SC.

4.6.8 CONCENTRATION

The partitioning of the drug into the skin can be affected by its thermodynamic activity in the application vehicle. This can be improved by increasing the concentration of the drug in the vehicle or by manipulating the vehicle to decrease drug solubility (Thomas & Finnin, 2004). According to Lund (1994) an increase in the concentration of an active substance in a vehicle generally produces an increase in the thermodynamic activity of the system and subsequent increase in the amount of material absorbed. When a drug is at its highest thermodynamic activity, the maximum skin penetration rate is acquired, for instance in a supersaturated solution (Benson, 2005).

4.7 SKIN MATHEMATICS

According to Barry (2002) the fundamental hypothesis underlying the mathematical theory for isotropic materials (which have identical structural and diffusional properties in all directions) is that the rate of transfer of diffusing material per unit area of a section is proportional to the concentration gradient measured normal to the section (Barry, 2002). Fick's first and second laws describe diffusion of uncharged compounds across a membrane or any homogenous barrier (Schaefer & Redelmeier, 1996). Fick's laws are relevant whenever the chemical or physical nature of the membrane controls the rate of diffusion and only if the distribution coefficient favours the SC. Fick's laws include a diffusion coefficient which is assumed to produce a linear concentration gradient of the permeant in the SC (Rieger, 1993).

4.7.1 FICK'S FIRST LAW

The amount (M) of a material flowing through a unit cross-section (S) of a barrier in unit time (t) is known as the flux (J).

$$J = \frac{dM}{S.dt} \qquad \text{(Eqn.1)}$$

The flux in turn is proportional to the concentration gradient dC/dx:

$$J = -D\frac{dC}{dx} \qquad \text{(Eqn.2)}$$

in which D is the diffusion coefficient of a penetrant in cm^2/sec, C is its concentration in g/cm^3, and x is the distance in cm of the movement vertical to the surface of the barrier (Martin, 1993).

4.7.2 FICK'S SECOND LAW

According to Tojo (1997) drug movement in the skin can be described by Fick's second law of diffusion if the skin contains no drug molecules prior to the application of the delivery device:

$$\frac{\partial C}{\partial x} = D\frac{\partial^2 C}{\partial x^2} \qquad \text{(Eqn. 3)}$$

where D is the diffusion coefficient in the SC, C is the concentration, t is the time and x is the distance from the surface of the skin (Tojo, 1997).

According to Schaefer & Redelmeier (1996) the concentration gradient, the path length and the diffusion coefficient are the most important factors determining the flux of a compound between two points in an isotropic medium.

4.8 PENETRATION ENHANCERS

4.8.1 CHEMICAL ENHANCERS

Because the skin provides such a formidable barrier to delivery of most drugs, a broad range of additives have been tested to enhance transdermal penetration (Prausnitz *et al.*, 2004). Penetration enhancers, in general, promote drug diffusion by disturbing the structure of the SC and/or deeper layers. The specific mechanism can fall into one of three categories: (1) disruption of the highly ordered structure of intercellular lipid channels, (2) interaction with

corneocyte intracellular protein components, and (3) enhanced partitioning of the drug in the presence or absence of the enhancer compound (Foldvari, 2000). These three possible mechanisms of skin penetration enhancer activity represent the essential aspects of the lipid-protein-partitioning (LPP) theory (Goodman & Barry, 1989).

Classes of chemical penetration enhancers include sulfoxides, alcohols, polyols, alkanes, fatty acids, esters, amines and amides, terpenes, surface active agents and cyclodextrins (Walker & Smith, 1996). Since acyclovir demonstrates poor skin penetration, the following penetration enhancers have been previously studied with regard to the possible enhancement of the penetration of acyclovir through skin:

- *Polyols*: Díez-Sales *et al.* (2005) studied the mechanism underlying propylene glycol (PG) effects on acyclovir penetration through human epidermis. The results presented in their work show quantitatively that the enhancer effect of PG in the permeation of acyclovir across human epidermis from solvent systems and gels depends on the concentration of PG. PG probably diffuses into the skin and enhances the partition of the drug into it, thus providing higher permeability coefficients (Díez-Sales *et al.*, 2005).

- *Fatty acids:* Cooper *et al.* (1985) studied the effect of fatty acids and alcohols on the penetration of acyclovir across human skin *in vitro*. Both oleic acid and oleyl alcohol at the 5% level in propylene glycol markedly increased the penetration of acyclovir (Cooper *et al.*, 1985). Oleic acid pools within the lipid domains (Barry, 2001a) and induces lipid fluidization as well as phase separation within the membrane (Naik *et al.*, 2000).

- *Terpenes:* Terpene compounds are derived from plant essential oils and combine good penetration enhancing abilities with low skin irritancy and low systemic toxicity. It appears that for hydrophilic drugs, the primary effect of terpene enhancer treatment is to increase drug diffusivity in the horny layer, i.e. to reduce the barrier properties of the skin. For more lipophilic drugs, in most instances, terpenes not only increase drug diffusivity but also enhance drug partitioning into the SC (Cornwell *et al.*, 1996). Myburg (2003) investigated the influence of terpenes on the transdermal permeation of acyclovir. Penetration of acyclovir was significantly enhanced by two terpenes, *viz.* 1,8-cineole and menthol. The extent of enhancement was, however, not large enough to be of clinical use (Myburg, 2003).

- *Surfactants:* Surfactants are potent transport enhancers by virtue of their ability to solubilize/extract lipids and denature proteins (Prausnitz *et al.*, 2004). Polysorbates (Tween) and polyoxyethylene alkylphenols (Brij) penetrate into the skin and cause micellar solubilization of SC lipids. Dodecyltrimethyl ammonium bromide (DTA-B) penetrates into the skin and extracts lipids from the SC. Sodium lauryl sulphate binds to intracellular keratin in corneocytes, removes some of the intercellular lipid, increases transepidermal water loss (TEWL) and alters the processing of epidermal lipids (Foldvari, 2000). Roestorf (2006) studied the effect of Brij 97 on the transdermal delivery of acyclovir. The flux of acyclovir increased as the concentration of the surfactant that was incorporated in the emulsion increased. This could be due to the surfactant that increases the solubility of the drug in the skin (Roestorf, 2006).

- *Polyoxypropylene-polyoxyethelene polymers:* Piret *et al.* (2000) evaluated the topical efficacy of acyclovir incorporated into a polyoxypropylene-polyoxyethelene polymer and compared it to that of 5% acyclovir ointment. They found that the acyclovir formulation reduced the viral titers below detectable levels. Reducing the number of treatments to a single application given 24 hour post-infection resulted in a significantly higher efficacy of the formulation of acyclovir than the acyclovir ointment. Acyclovir incorporated within the polymer was also significantly more effective than the acyclovir ointment when treatment was initiated on day 5 post-infection. The higher efficacy of the acyclovir formulation than of the acyclovir ointment is attributed to the semi-viscous character of the polymer, which allows better penetration of the drug into the skin (Piret *et al.*, 2000).

However, oleic acid and Brij 97 can cause slight skin irritation, whereas oleyl alcohol and polyethylene glycol can cause irritation and act as permeators (Sciencelab, 2005).

4.8.2 PHYSICAL ENHANCERS

The enhancement of permeation of all drug molecules may not be accomplished effectively with chemicals. Specific physical techniques of enhancement may provide an effective alternative method for improved permeation in such cases (Walker & Smith, 1996). Physical enhancement of absorption of drug molecules through skin includes iontophoresis, electroporation, sonoporation, thermal poration and microneedles (Chan, 2005).

4.8.2.1 Iontophoresis

Iontophoresis uses a small direct current (approximately 0.5 mA/cm^2) to drive charged molecules into the skin (Benson, 2005). Three important mechanisms enhance drug transport: (1) the driving electrode repels oppositely charged species, (2) the electric current

increases skin permeability, and (3) electro-osmosis moves uncharged molecules and large peptides (Barry, 2001b). Efficiency of transport depends mainly on polarity, valency and mobility of the charged species, the composition of the delivery formulation and the current profile (Naik *et al.*, 2000). Volpato *et al.* (1998) studied the *in vitro* distribution of acyclovir in human skin layers after iontophoresis. The data they obtained indicated that transdermal iontophoresis dramatically improves the amount of acyclovir present in the skin (Volpato *et al.* 1998). They concluded that acyclovir can be accumulated at the target site more quickly and maintained at higher level through application of an iontophoretic pulse and keeping the drug reservoir on the skin (Volpato *et al.*, 1998).

However, there are some disadvantages to iontophoresis. They include the following: there is a lack of adequate delivery control and the cost is relatively high. A long lasting power source is also required (Rhodes, 2007) and outpatient use is problematic (Barry, 2001b).

4.8.2.2 Electroporation

Electroporation uses high-voltage short pulses to create localized regions of membrane permeabilization by producing aqueous pathways in lipid membrane bilayers (Naik *et al.*, 2000). During the pulse, drug moves *via* iontophoresis and/or electro-osmosis. Significant penetration also occurs between pulses by simple diffusion because relatively persistent changes in the SC lower its resistance. However, outpatient use of electroporation is problematic (Barry, 2001b).

4.8.2.3 Ultrasound (sonophoresis)

Ultrasound at various frequencies in the range of 20 kHz to 16 MHz has been used to enhance skin permeability by a method called sonophoresis (Prausnitz *et al.*, 2004). This technique applies a preparation topically and massages the site with an ultrasonic source (Barry, 2001b). Mechanistically, sonophoresis is considered to enhance drug delivery through a combination of thermal, chemical and mechanical alterations within the skin tissue (Naik *et al.*, 2000). It disturbs lipid-packing in the SC and the increased free volume enhances drug penetration. Ultrasound however, requires validation for patient efficiency and safety, and it is not readily available for home use (Barry, 2001b).

4.8.2.4 Microneedles

The SC can be bypassed by injection (Barry, 2001a). Microneedles, a device containing 400 solid or hollow silicon needles, approximately 150 μm in length, has been used to penetrate through the SC into the upper epidermis (Benson, 2005). These needles of micron dimensions create holes large enough for molecules to enter, but small enough to avoid pain

or significant damage. *In vitro* experiments have shown inserting microneedles into skin can increase permeability by orders of magnitude for small drugs, large macromolecules and nanoparticles (Prausnitz *et al.*, 2004).

4.8.2.5 Conclusion

Although physical enhancement can be used to promote penetration of certain drugs through the skin, it is not readily accessible to the majority of people. Since these methods are very expensive and outpatient use is problematic, people having a background of poverty will not benefit from these methods. It would thus be more appropriate to design a drug formulation which is more affordable and more available to the majority of the population.

4.8.3 METABOLIC AND BIOCHEMICAL ENHANCERS

Chemicals that provoke biochemical and metabolic events within the skin can potentially be used to alter skin permeability. For example, these types of enhancers can reduce the barrier properties of the skin by either inhibiting enzymes responsible for the synthesis of specific SC lipids during SC repair or by promoting the metabolism of existing skin lipids that are responsible for skin barrier function. Although promising, both of these approaches need to undergo further *in vivo* investigation of their enhancement effects and their potential to produce skin irritation (Finnin & Morgan, 1999).

4.9 PHEROIDS™ AS DRUG DELIVERY VEHICLE FOR ACYCLOVIR AND KETOCONAZOLE

4.9.1 BACKGROUND

Pheroid™ technology, derived from what was formerly known as Emzaloid™ technology, is capable of enhancing the absorption and/or efficacy of a variety of categories of active ingredients and other compounds (Grobler *et al.*, 2007). Currently, research on the possible employment of Pheroid™ technology in the treatment strategies of diseases such as malaria, HIV/AIDS and tuberculosis are under investigation at the North-West University of South-Africa.

4.9.2 INTRODUCTION

The Pheroid™ delivery system is a colloidal system that has distinctive and stable lipid-based submicron and micron sized structures, called Pheroids™, which are homogeneously dispersed in a dispersion medium. These dispersed structures can be manipulated in terms of morphology, structure, size and function (Grobler *et al.*, 2007). The components of Pheroids™ are manipulated in a very specific manner to ensure its high entrapment

capabilities, very fast rate of transport, delivery and stability. The absorption capabilities and drug release characteristics of the Pheroid™ can thus be controlled (Grobler, 2004).

4.9.3 COMPOSITION OF PHEROIDS™

In general, Pheroids™ have a lipid bilayer, but contains no phospholipids or cholesterol. They are formed by a self-assembly process comparable to that of low-energy emulsions and micro-emulsions and no lyophilization or hydration of the lipid components is required (Grobler *et al.*, 2007). Pheroids™ contain ethyl esters of the essential fatty acids, linoleic acid and linolenic acid, as well as oleic acid, which are emulsified in water saturated with nitrous oxide (Saunders *et al.*, 1999). Since the fatty acids are in the *cis*-formation, they are compatible with the orientation of the fatty acids in man (Grobler *et al.*, 2007). The association of nitrous oxide with the dispersed phase has been shown to contribute to the following: the miscibility of the fatty acids in the dispersal medium, the self-assembly process of the Pheroids™, and the stability of the formed Pheroids™ (Grobler *et al.*, 2007).

4.9.4 CHARACTERISTICS AND ADVANTAGES OF PHEROIDS™

Various types of Pheroids™, each with its own morphology and size, can be manufactured. Some of these types include bilayer membrane vesicles, highly elastic or fluid bilayered vesicles, pro-Pheroids™, reservoirs, micro-sponges, and depots (Grobler, 2004). The basic Pheroid™ has a vesicular structure with size ranging from 200-440nm. The membranes of the Pheroids™ contain pores and a model of the fatty acid packing of the membrane has been proposed (Grobler *et al.*, 2007). Table 6 depicts some of the characteristics of Pheroids™ and the advantages which are associated with each characteristic.

Table 6: Characteristics of Pheroids™ and advantages associated with each characteristic (adapted from Grobler, 2004)

Characteristic	Advantage
The Pheroid™ can be manipulated in terms of size, charge, lipid composition and membrane packaging.	The Pheroid™ delivery system can be optimized for the active compound and indication of the drug.
The Pheroid™ is composed principally of fatty acids.	An affinity exists between the Pheroid™ and cell membranes.
Various combinations of fatty acids and/or other added molecules are used.	To some degree, the Pheroid™ is targeted to subcellular level.
The Pheroid™ is part of the natural biochemical pathways.	The Pheroid™ causes no cytotoxicity and assists in the maintenance of cell membrane.
The Pheroid™ is polyphilic.	Drugs that have dissimilar solubilities as well as insoluble drugs can be entrapped within the Pheroid™.
Entrapment of active compounds in Pheroids™ decreases the volume of distribution and as a result the concentration at the target site is increased.	An improved but narrow therapeutic index can be reached, with a reduction in specific toxicity.
The Pheroid™ protects drugs from metabolism, opsonization and inactivation in the plasma and other body fluids.	Only slight leakage of the drug from the Pheroid™ before the target site has been reached has been observed.
The Pheroid™ is designed to show a high degree of elasticity and fluidity, with a relatively high phase transition temperature.	Fast release of the active compounds from macromolecular carriers has been associated with the extent of fluidity of the membranes, making the Pheroid™ vesicles ideal for quick release, whilst preserving the possibility of sustained release from pro-Pheroid™ depots.
Entrapment in Pheroids™ changes the pharmacokinetics of active compounds.	There is a reduction in the time needed to achieve maximum concentration levels.
Micro-sponges are ideal for combination therapies.	One drug can be entrapped in the interior volume and the other drug can be entrapped in the sponge spaces.

In this study, micro-sponges containing the water soluble acyclovir in the interior aqueous volume and ketoconazole in the sponge membrane spaces were used. This type of Pheroid™ was used since the acyclovir is a hydrophilic molecule and the ketoconazole is a hydrophobic molecule. Both the drugs could thus be incorporated into a single dosage form, enabling the possibility of combination therapy.

4.9.5 UPTAKE AND METABOLISM OF PHEROIDS™ BY CELLS AND RELEASE OF ENTRAPPED COMPOUNDS

The Pheroid™ is stearically stabilized by electro-chemical interaction, which makes the Pheroid™ an extremely elastic vesicular structure (Grobler *et al.*, 2007). Even though the mechanism of uptake of the Pheroids™ by cells is still speculative, preliminary proof

proposes that the uptake is actively facilitated by the fatty acid membrane binding proteins usually present within lipid rafts in the cell membrane (Grobler *et al.*, 2007). According to Grobler *et al.* (2007) the cellular uptake of the Pheroid™ is based on a variety of local interactions between fatty acids and cells, amongst these the binding between fatty acids and the fatty acid binding proteins in the cell membrane and the interaction between the Pheroids™ and the lipid rafts present in the cell membrane. The Pheroids™ are metabolized in either the mitochondria or the peroxisomes of the cell, depending on the composition; with subsequent release of the active compound (Grobler *et al.*, 2007).

4.9.6 RECENT STUDIES ON THE POSSIBLE APPLICATION OF PHEROID™ TECHNOLOGY ON ANTIVIRAL AND ANTIFUNGAL ACTIVES

MeyerZall Laboratories (2002) compared the effect of miconazole nitrate, an antifungal agent, on cultured human cells and fungal growth, to the effect of the same antifungal at the same concentrations, but entrapped in Pheroids™. The highly toxic effect of the antifungal compound on human cells was neutralized, and the Pheroid™ formulation acted as an extremely effective fungicide (Grobler, 2004).

Reynecke (2004) compared lamellar gel phase systems and Pheroids™ as transdermal drug delivery vehicles for acyclovir. Significant enhancement of transdermal flux of acyclovir ($p < 0.1$) was observed with Pheroid™ as drug delivery vehicle. This was due to the fact that Pheroid™ entraps and deliver drug with higher efficiency than the ordinary drug delivery vehicles (Reynecke, 2004).

4.10 SUMMARY

As described earlier, mucocutaneous disorders are exceptionally common throughout the course of HIV disease, and is seen in more than 90% of HIV/AIDS patients. Although the majority of these skin disorders are not life-threatening, they may be cosmetically disfiguring and as such, practically destroy an individual's quality of life. Contagious diseases are the most important group of cutaneous disorders related to HIV/AIDS infection, with the mainstream of these infections being either fungal or viral.

Some of the viral infections which can be seen in HIV/AIDS include HSV infection, HZ infection or shingles, and CMV infection. HSV infections occur in about 20% of patients with HIV/AIDS. Reactivation of HSV often occurs in these patients at all stages of immunocompetency and results in chronic mucocutaneous disease with severe and extensive skin ulcers. HZ or shingles, also common in HIV/AIDS patients, may be the earliest sign of immunosuppression, but it can occur at any stage of HIV/AIDS disease. The symptoms of HZ are more frequent and severe; and have a longer duration in HIV/AIDS

patients with the vesicles frequently becoming chronic ulcerative and necrotic. Atypical and complicated HZ forms are also more common among these patients.

Some of the fungal infections commonly seen in HIV/AIDS include candidiasis, seborrhoeic dermatitis and dermatophytosis. Candidiasis, an opportunistic infection in HIV/AIDS disease, is well renowned for its significant morbidity and mortality. Mucocutaneous candidiasis occurs in 3 forms in persons with HIV infection: oropharyngeal, oesophageal, and vulvovaginal disease. Seborrhoeic dermatitis is also very common in patients with HIV/AIDS, affecting up to 85% of patients at some stage of their disease. With seborrhoeic dermatitis, areas rich in sebaceous glands develop yellowish, oily scales and crusts on slightly erythematous to very red plaques and the lesions may be pruritic. Dermatophytosis on the other hand affects 30-50% of HIV/AIDS patients and usually occurs as tinea corporis or tinea capitis. In HIV/AIDS patients the lesions are very widespread and noncompliant to treatment.

Acyclovir, an antiviral agent, is effective against HSV-1 and HSV-2, VZV and to a lesser extent against Epstein-Barr virus and CMV. Topically it is indicated for the treatment of limited non-life threatening initial and recurrent mucocutaneous HSV-1 and HSV-2 infections in immunocompromised patients and it is used as adjunctive therapy in immunosuppressant persons with HZ. Ketoconazole, an antifungal agent, is on the contrary active against dermatophytes and yeasts, but also displays antiviral activity against HSV-1 and HSV-2 and synergistic antiviral activity when combined with acyclovir. It is used as the primary agent in the topical treatment of tinea corporis, tinea cruris, tinea pedis, tinea versicolor, cutaneous candidiasis and paronychia, and as secondary agent in the topical treatment of tinea barbae and tinea capitis. Additionally, ketoconazole is used for the treatment and prophylaxis of seborrhoeic dermatitis.

Transdermal drug delivery offers a variety of advantages over the conventional oral and parenteral dosage forms. There is, however, some disadvantages associated with transdermal drug delivery; the major disadvantage being the fact that not all compounds are suitable candidates. The restrictions of transdermal drug delivery are governed mainly by the skin's anatomy and the application of transdermal delivery to a wide range of drugs is restricted due to the significant barrier to penetration across the skin, which is associated mainly with the outermost layer of the epidermis, the SC. Topical application of acyclovir has unfortunately proven clinically disappointing in the therapy of HSV skin infections when compared with oral or intravenous administration. This is due to the inability of acyclovir to penetrate the SC barrier layer of the skin, and lack of its reach at the target site; the basal epidermis.

Fortunately, there are various techniques which can be employed to promote transdermal drug delivery. Some of these techniques include the use of chemical enhancers, physical enhancement strategies and formulation of the drug in a delivery vehicle. In this study, the use of Pheroids™ as delivery vehicle was investigated.

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CHAPTER 3: ARTICLE FOR PUBLICATION IN THE EUROPEAN JOURNAL OF PHARMACEUTICAL SCIENCES

TRANSDERMAL DELIVERY OF ACYCLOVIR AND KETOCONAZOLE BY PHEROID™ TECHNOLOGY

TRANSDERMAL DELIVERY OF ACYCLOVIR AND KETOCONAZOLE BY PHEROID™ TECHNOLOGY

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Abstract

The aim of this study was to investigate *in vitro* transdermal diffusion of the antiviral drug, acyclovir and the antifungal drug, ketoconazole, with the aid of the novel Pheroid™ drug delivery system. Since viral and fungal cutaneous manifestations are commonly seen in HIV/AIDS, transdermal diffusion of a combination of acyclovir and ketoconazole, which can be useful in treatment of these cutaneous diseases, was investigated.

Transdermal application of acyclovir has proven clinically disappointing in the treatment of herpes simplex virus infections, due to the inability of acyclovir to reach the target site; the basal epidermis. Previous studies done at the North-West University have shown that Pheroids™ enhance the skin penetration of acyclovir. Therefore we decided to investigate the possibility to deliver a combination of acyclovir and ketoconazole *via* this technology. Vertical Franz cell diffusion studies were conducted over 12 hours, using female abdominal skin and 20:80 v/v ethanol and phosphate buffered solution (PBS) as receptor phase. Concentrations of 5% acyclovir and 2% ketoconazole were applied to the donor phase suspended in either PBS or Pheroids™. *In vitro* penetration of acyclovir and ketoconazole was directly assayed by HPLC.

The Pheroids™ proved advantageous for transdermal diffusion of acyclovir but not for ketoconazole.

Keywords : acyclovir, ketoconazole, transdermal diffusion, Pheroids™, delivery system

1. Introduction

The human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS) epidemic has had a profound effect on the extent and diagnosis of cutaneous disorders. The majority of HIV-positive patients may present with skin disorders, which in most cases are not life-threatening, but are cosmetically mutilating and endanger the quality of life of these patients (Ramdial, 2000). Herpes zoster is common in HIV/AIDS patients and may be the earliest indication of immunosuppression (Fiallo and Talhari, 2007). According to Van Hees and Naafs (2001) severe and chronic seborrheic eczema may also be an early manifestation. Molluscum contagiosum, papular pruritic eruption, severe herpes simplex or human papilloma infection, severe bacterial, mycobacterial and fungal infections and Kaposi's sarcoma are other cutaneous manifestations of HIV/AIDS (Van Hees and Naafs, 2001). Panasiti et al. (2007) reported a case where a 37-year-old patient experienced multiple painful ulcerative lesions of the perianal regions. Their laboratory assessments confirmed HIV infection of the patient. They suggested that in HIV-positive patients, perianal HSV-2 can have unusual manifestations, particularly if co-infection by *Candida albicans* occurs (Panasiti et al., 2007).

Acyclovir, a synthetic purine nucleoside analogue antiviral agent derived from guanine (McEvoy, 2002), is active against herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), varicella zoster virus, and to a lesser extent against Epstein-Barr virus and cytomegalovirus (Díez-Sales et al., 2005). Ketoconazole, a synthetic imidazole derivative, antifungal agent, is effective against the majority of pathogenic fungi, including dermatophytes and yeasts. Furthermore it has *in vitro* activity against a number of gram-positive bacteria, including *Staphylococcus areus*, *S. epidermidis*, enterococci, Nocardia, and *Actinomadura* (McEvoy, 2002) and demonstrates antiviral activity against HSV-1 and HSV-2 and synergistic antiviral activity when combined with acyclovir (Pottage et al., 1986).

It is well known that transdermal delivery of drugs offers a variety of advantages over and above the usual oral and parenteral dosage forms. It would thus be reasonable to combine an antiviral agent and antifungal agent in a transdermal delivery system to treat both viral- and fungal infections common in HIV/AIDS infected patients. Unfortunately the systemic absorption of acyclovir and ketoconazole after topical administration is reported to be minimal (Sweetman, 2002). Acyclovir therapy has inadequate effectivity due to the inability of the drug to traverse the outermost layer of the skin; the stratum corneum (SC), lack of its reach at the target site; the basal epidermis (Jiang et al., 1998) and heterogeneous distribution of the drug in the skin layers (Volpato et al., 1998). Fortunately, the target site for ketoconazole is the SC; since the pathogen resides on or within this layer and adequate concentrations of the drug should be delivered to this layer of the skin to inhibit the growth of fungal pathogens (Pershing et al., 1994).

It would be preferable to avoid systemic absorption of ketoconazole after topical application in HIV/AIDS patients since ketoconazole may interact with antiretroviral drugs. Such interactions have been shown between ketoconazole and nevirapine (nevirapine may decrease concentrations of ketoconazole) and protease inhibitors such as amprenavir, indinavir, nelfinavir, ritonavir and saquinavir (may increase serum concentrations of ketoconazole) (Anon, 2007).

The application of transdermal delivery to a wide variety of drugs is limited due to the significant barrier to penetration across the skin, which is associated principally with the outmost SC layer of the epidermis (Benson, 2005). The physicochemical properties of a drug also play an important role in transdermal delivery. Some of the physicochemical properties of a drug molecule that may have an effect on membrane penetration include the partition coefficient and diffusion coefficient, which in turn are dependant on variables such as molecular weight, size and shape, and degree of ionization (Wiechers, 1989). The drug solubility (Malan et al., 2002), melting point and concentration also play a role in transdermal

diffusion (Naik et al., 2000). The ideal properties of a drug penetrating the SC well include the following: an aqueous solubility larger than 1 mg/ml, a molecular weight less than 500 Da, a melting point less than 200 °C (Naik et al., 2000) and an octanol water partition coefficient between 1 and 3 (Hadgraft, 2004). In addition the drug should be in the unionized form since it then has greater lipid solubility (Malan et al., 2002). Table 1 indicates the physicochemical properties of acyclovir and ketoconazole as applicable to transdermal delivery.

When considering the aqueous solubility and molecular weight of acyclovir, transdermal diffusion of acyclovir should be feasible. However, the high melting point and low partition coefficient of acyclovir could be a limiting factor in the transdermal diffusion of the drug. Ketoconazole on the other hand, has a very low aqueous solubility, high molecular weight and high partition coefficient, which could have a negative impact on the transdermal penetration of the drug. On the other hand, when considering the melting point of ketoconazole, the penetration of the drug should be feasible. Thus, overall it is expected that transdermal delivery of ketoconazole should be minimal, due to the physicochemical properties of the drug.

Because the skin provides such a formidable barrier, a great variety of additives have been tested with the aim of enhancing transdermal penetration (Prausnitz et al., 2004). Usually, penetration enhancers encourage drug diffusion by disturbing the structure of the SC and/or deeper layers of the skin. The precise mechanism can fall into one of three categories: (1) disruption of the highly ordered structure of intercellular lipid channels, (2) interaction with corneocyte intracellular protein components, and (3) enhanced partitioning of the drug in the presence or absence of the enhancer compound (Foldvari, 2000). Classes of penetration enhancers include: sulfoxides, alcohols, polyols, alkanes, fatty acids, esters, amines and amides, terpenes, surface active agents and cyclodextrins (Walker and Smith, 1996). Improved antiviral results have been achieved for acyclovir by using dimethyl sulfoxide

(DMSO), modified aqueous cream (MAC) and addition of oleic acid and oleyl alcohol in 5% concentrations in propylene glycol bases. Transdermal penetration of ketoconazole has also been enhanced by addition of 10% lauramide-diethanolamine to Durotak 87-2516 adhesive (Venkateshwaron and Quan, 2004).

Pheroid™ technology can enhance the absorption and/or efficacy of a selection of active ingredients and other compounds. The Pheroid™ delivery system has distinctive and stable lipid-based submicron- and micron-sized structures, called Pheroids™, which are uniformly dispersed in a dispersion medium. These Pheroids™ can be manipulated in terms of morphology, structure, size and function (Grobler et al., 2007). Pheroids™ consists primarily of plant and essential fatty acids (Grobler, 2004). According to Saunders et al. (1999) Pheroids™ contain ethyl esters of the essential fatty acids, linoleic acid and linolenic acid, as well as oleic acid (Saunders et al., 1999). Penetration enhancement of acyclovir has been achieved by addition of oleic acid to drug formulations (Cooper et al., 1985), therefore it was hypothesized that it could be possible to achieve at least the same enhancement when using Pheroids™ to deliver the two proposed drugs.

In this study the *in vitro* transdermal delivery of acyclovir and ketoconazole entrapped in the Pheroid™ delivery system was investigated.

2. Materials and methods

2.1 Materials

Acyclovir; (9-(2-hydroxyethoxymethyl)guanine (MW = 225.20) and ketoconazole; piperazine, cis-1-acetyl-4-[4-[[2-(2-,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl] (MW = 531.4) were kindly donated by Kirsch Pharma (Isando, Farrarmere, South Africa). Potassium dihydrogen orthophosphate (KH_2PO_4) (Merck, South Africa), disodium hydrogen orthophosphate dihydrate ($Na_2HPO_4.2H_2O$) (Saarchem, South Africa) and sodium chloride (NaCl) (Merck, South Africa) were used during the preparation of

phosphate buffered solution (PBS) which served in conjunction with absolute ethanol AR (Labchem, South Africa) as components of the receptor phase during the *in vitro* permeation studies. Pheroids™ were prepared by the Unit for Drug Research and Development, North-West University. Ammonium phosphate monobasic ($(NH_4)H_2$) (Sigma, St Louis, USA) and 'Far UV' grade for HPLC acetonitrile (Acros organics, New Jersey, USA) were used in the mobile phase of the HPLC analysis. A Restek Ultra® C18 (150 mm x 4.6 mm, 5 µm particle size) HPLC column was used. Water used during the preparation of solutions was purified by a Milli-Q Academic purification system (Millipore, Milford, USA). Vertical Franz diffusion cells (PermeGear Inc., Bethlehem, PA, USA) and a type JB5 Grant Water bath (Grant instruments, Cambridge, England) were used during the *in vitro* permeation studies.

2.2 Methods

2.2.1 Determination of ketoconazole crystal size

In order to prepare a homogenous formulation of insoluble or slightly soluble compounds for entrapment into Pheroid™ micro sponges, it is preferable that the size of the drug raw material should be smaller than 50 µm. With the aim of establishing the particle size of the supplied ketoconazole, random light micrographs of the ketoconazole raw material were captured and the average size of the ketoconazole crystals was determined. For this purpose, a Nikon eclipse TE 300 microscope, with a Nikon DXM 1200 camera for realtime imaging, a Nikon Plan Apo 60 x 1.40 oil immersion objective and immersion oil for microscopy, 50cc Type A, nd = 1.515 (23°C) (Nikon, Japan) were used. The total amount of ketoconazole crystals were plotted against the crystal size. After calculation of the percentage of ketoconazole crystals in the various size ranges, the percentage of the ketoconazole crystals were plotted against the crystal size.

2.2.2 Entrapment of acyclovir and ketoconazole in Pheroids™

Entrapment of acyclovir and ketoconazole in the Pheroids™ was monitored with the aid of confocal laser scanning microscopy (CLSM). For this purpose a Nikon PCM 2000 confocal

laser scanning microscope, with a Nikon DXM 1200 camera, realtime imaging, a medium (10µm) pinhole and Nikon Plan Apo 60 x 1.40 oil immersion objective was used. The microscope was equipped with a argon laser (wavelengths: excitation 488 nm, emission 515 nm) and a helium/neon laser (wavelengths: excitation 505 nm, emission 564 nm).

According to protocol, Pheroids™ are generally labelled with the fluorophore Nile Red. Nile Red mainly fluoresces when accumulated in lipid rich areas, and as a result readily stains the fatty acid components of the Pheroid™ formulation (Saunders et al., 1999). Since there are no fluorophores commercially available to label the acyclovir and ketoconazole, micrographs were obtained by labelling the Pheroids™ only. Although not labelled, the active ingredients could be visualised by a combination of fluorescent and reflected light and were clearly visible as green dots in the solution and/or as crystals, which precipitated in the solution. 4 µl of Nile Red was added to 50 µl of the Pheroids™ containing 5% acyclovir and 2% ketoconazole. The sample was mixed using a vortex mixer, and left to stand for 15 minutes to ensure labelling of the Pheroids™ with Nile Red. 20 µl of the sample was placed on a microscope slide and covered with a cover slip. The samples were subjected to laser excitation at 488 and 505 nm, together with reflected light. Micrographs of emission at 515 and 568 nm were captured according to standard operating procedures developed for Pheroid™ technology analysis.

2.2.3 *In vitro* permeation studies

In order to reduce the variability in skin permeability properties between different anatomical sites, *in vitro* permeation studies were only performed on Caucasian female abdominal skin samples obtained after cosmetic surgery. Ethical approval for the proclamation and exploitation of the skin was provided by the Research Ethics Committee of the North-West University (reference number 04D08). Informed consent was obtained from the patients beforehand and their identities were masked to assure anonymity. The full-thickness skin was frozen at -20 °C within 24 hours after removal. Before separation of the epidermis from

the dermis, the skin was thawed at room temperature and residual blood was removed with tissue paper. Subcutaneous fat was dissected from the dermal side as thoroughly as possible, taking care not to damage the dermal side (Freeman et al., 1986). After the skin had been immersed in water at 60 °C for 1 minute, the epidermal layer and dermal layers were carefully separated (Bronaugh and Collier, 1993). Following successful removal, the skin sections were drifted on top of Whatman® filter paper with the stratum corneum side of the epidermal layer facing upwards and were subsequently left to air dry. The prepared skin samples were covered in aluminium foil and were kept frozen at -20 °C until used. The frozen skin was thawed at room temperature and examined for defects, and then cut into circles with a diameter of ± 10 mm before mounting them on the diffusion apparatus.

2.2.4 Franz cell diffusion method

In vitro permeation studies were carried out using vertical Franz diffusion cells with a receptor capacity of approximately 2 ml and a diffusion area of 1,075 cm^2. The skin pieces were mounted between the donor and receptor compartments with the dermal side in contact with the receptor phase. The two compartments were sealed with vacuum grease and clamped together with a metal clamp. The receptor compartments were filled with receptor solution consisting of 20:80 v/v ethanol and PBS. The donor compartments were filled with PBS only, since ethanol enhances the solubility of drugs by disruption of the SC integrity through extraction of biochemicals (Purdon et al., 2004). The Franz cells were placed in the water bath and subsequently equilibrated for 1 hour. The receptor phase of each receptor compartment was stirred at 750 rpm by means of small magnetic stirring bars and the aid of a Variomag® magnetic stirring plate throughout the entire experiment. In order to attain 32°C at membrane surface, the receptor phase was maintained at 37 ± 0.5 °C in the water bath (Cleary, 1993). Following equilibration, air bubbles were removed from the receptor phase, and the PBS solution in the donor phase was removed and replaced with 1 ml of the two separate test solutions (PBS containing 5% acyclovir and 2% ketoconazole, and the

Pheroid™ micro sponges containing 5% acyclovir and 2% ketoconazole). The donor compartments were covered with Parafilm® to avoid evaporation.

The entire content of the receptor compartments were withdrawn at predetermined intervals (0.25; 0.5; 0.75; 1; 1.25; 1.5; 1.75; 2; 4; 6; 8; 10; and 12 hours), followed by replacement with an equivalent amount of fresh receptor phase. One hundred microlitres (100 µl) of each sample was directly assayed by high-performance liquid chromatography (HPLC) to determine the concentration of the actives in the receptor fluid.

2.2.5 High-performance liquid chromatography (HPLC) analyses

The HPLC analysis of acyclovir and ketoconazole was performed by employing a method developed and validated at the North-West University, Potchefstroon Campus, South Africa. The equipment used is described under materials above. Chemstation Rev. A.06.02 data acquisition and analysis software was used for data acquisition and processing. HPLC analysis was performed at 25 °C.

As mobile phase a mixture of 0.5 volumes of acetonitrile (ACN) and 9.5 volumes of a 2.3 g/L solution of ammonium phosphate were used, which changed by linear-gradient elution after 2 minutes to a mixture of 8.0 volumes of ACN and 2.0 volumes of a 2.3 g/L solution of ammonium phosphate over 9 minutes, followed by the final elution mixture (0.5 volumes of ACN and 9.5 volumes of a 2.3 g/L solution of ammonium phosphate) for 5 minutes. A flow rate of 1 ml/min was maintained. Runtime was set to 14 minutes. The detector was set to 252 nm for the first 6 minutes, where after it was set to 243 nm. The detection time of acyclovir was approximately 3.4-3.9 minutes and that of ketoconazole approximately 6.95-7.02 minutes. Injection volume was set at a default value of 100 µl.

2.2.6 Data analysis

The cumulative amount of acyclovir and ketoconazole which permeated the skin per unit time area was plotted against time. The yield of each cell was depicted as a percentage of the applied concentration and based on these values, data of cells with yield values of 0.004% and less for acyclovir and 0.005% and less for ketoconazole was selected for inclusion in the dataset.

3. Results and discussion

3.1 Determination of ketoconazole crystal size

Figure 1 illustrates the percentage of ketoconazole crystals within the various size ranges, as calculated from random microscopic imaging of a sample of ketoconazole raw material. Most of the ketoconazole crystals (95.55%) were smaller than 5 µm and only 0.20% of the crystals were between the size ranges of 30 µm - 35 µm. None of the crystals observed exceeded 35 µm. Since the ketoconazole crystals were smaller than 50 µm, it was decided that it was not necessary to grind the ketoconazole in order to produce smaller particles. The crystals of size smaller than 5 µm can be and was entrapped within the Pheroid™ micro sponges (see 3.2).

3.2 Entrapment of acyclovir and ketoconazole in Pheroids™

Figure 2 illustrates the vesicular structures of the Pheroid™ delivery system and the entrapment of acyclovir and ketoconazole inside the lipid structures of this system. Micrograph A and Micrograph B represents the control: Pheroid™ sponges analysed without the inclusion of acyclovir and ketoconazole. The Pheroids™ are clearly visible as red spheres. Micrographs C represent a sample of the Pheroids™ with an acyclovir and ketoconazole concentration of 0.05 g/ml and 0.02 g/ml respectively. The entrapment is confirmed by the change in the appearance of the micro sponges. Figure 3 illustrates ketoconazole crystals which were observed in the Pheroids™ formulation. Since both actives were present in relatively high concentrations and the solution was oversaturated, it

is possible that crystallization of the actives could have occurred. Acyclovir has characteristic needle-shaped crystals and on closer investigation it was found that the crystals which were observed within the Pheroids™ did not exhibit this characteristic. Consequently it was presumed that the crystals were ketoconazole and it was reasonable to assume that the acyclovir was completely dissolved within the aqueous phase of the Pheroids™ since no acyclovir crystals was observed in the solution. It was also presumed that the majority of ketoconazole was entrapped within the sponge spaces of the Pheroids™ with the crystals observed being the result of the oversaturated preparation.

3.2 Permeation of acyclovir and ketoconazole with the aid of the Pheroid™ delivery system

The *in vitro* permeation of acyclovir and ketoconazole with the aid of the Pheroid™ delivery system was investigated and compared to the permeation of acyclovir and ketoconazole whilst dissolved in PBS, which served as positive control. The *in vitro* permeation profiles of acyclovir and ketoconazole in the different preparations are shown in Fig. 4 and Fig. 5. Graph 1 in Fig. 4 represents the average data obtained for acyclovir in PBS from 8 Franz cells and graph 2 for acyclovir in Pheroids™ from 9 Franz cells. Graph 1 in Fig. 5 represents the average data obtained for ketoconazole in PBS from the same 8 Franz cells used for acyclovir and graph 2 for ketoconazole in Pheroids™ from the same 9 Franz cells used for acyclovir. *In vitro* permeation profiles of acyclovir demonstrated biphasic flux patterns. Consequently, the flux (Js ± standard deviation) of acyclovir was calculated for time intervals 0 – 2 hours, and 2 – 12 hours. Ketoconazole, however, did not demonstrate biphasic flux patterns, and the flux of ketoconazole was determined from the slope of the linear portion of cumulative amount (µg/ml) of drug versus time (hours) plot. The enhancement ratios (ER_{Flux}) of acyclovir and ketoconazole were calculated from the flux observed in Pheroids™ divided by the flux observed in the control.

The average flux obtained for acyclovir in both PBS and Pheroids™ for time interval 0 – 2 hours were 0.161 ± 0.085 and 0.180 ± 0.162 respectively, with the Pheroids™ demonstrating an enhancement ratio of 1.12. The average flux obtained for acyclovir in both PBS and Pheroids™ for time interval 2 – 12 hours were 0.054 ± 0.040 and 0.088 ± 0.040 respectively, with the Pheroids™ demonstrating an enhancement ratio of 1.63.

During the analysis of the amount of ketoconazole that permeated the skin, a number of problems were encountered. The HPLC method was validated to detect larger concentrations of ketoconazole. As a result, the method was not sensitive enough to detect the small concentrations of ketoconazole which did permeate the skin. There was no interference between the blank injection of the HPLC system (baseline noises, etc.) and the solvent peaks with the peaks obtained for ketoconazole during the validation. However, the validation was performed with larger concentrations of the drug. During the analysis of the samples obtained from the diffusion studies, there was great difficulty in identifying the ketoconazole peaks, since the concentration of drug which permeated the skin was very small and resolution between the solvent, blank, and ketoconazole peaks was poor. The skin components and components of the Pheroids™ could also have been the cause of poor resolution between peaks, since either the Pheroids™ or skin components caused a shift in the baseline of the HPLC chromatograms. In order to assure that the correct peaks were integrated, the retention times of the standard for each day were used as control. Peaks with retention times close to the control were chosen, and any other peak was rejected. Although the flux could be calculated, there is not a hundred percent certainty whether the data obtained for ketoconazole is acceptable, and the results are only included to place emphasis on the problems that were encountered during the HPLC analysis of ketoconazole.

The average flux obtained for ketoconazole in PBS and Pheroids™ was 0.023 ± 0.010 and 0.007 ± 0.004 respectively. The decrease in flux of ketoconazole obtained with the Pheroids™ may perhaps be ascribed to the crystallization of the drug which occurred within

the Pheroid™ delivery system, causing retardation of ketoconazole diffusion through the skin. As expected, the low aqueous solubility, high molecular weight and high partition coefficient of ketoconazole could also have been limiting factors in the transdermal diffusion of the drug.

Table 2 indicates the average flux values for acyclovir in PBS and Pheroids™ for time interval 0 – 2 hours and 2 – 12 hours, the % yield and the enhancement ratios for each of the time intervals. Table 3 indicates the average flux values for ketoconazole in PBS and Pheroids™, the % yield and the enhancement ratio.

Table 4 is a summary of some studies that were previously done in an effort to enhance the delivery of acyclovir across the skin. Freeman et al. (1986) studied the penetration of acyclovir through excised human skin and guinea pig skin from three vehicles; polyethylene glycol (PEG) ointment, modified aqueous cream (MAC) and dimethyl sulfoxide (DMSO). Delivery of acyclovir from PEG was very slow for both human and guinea pig skin (flux 0.055 and 0.047 μg/cm²/h). Formulation of acyclovir in modified aqueous cream and in DMSO resulted in an 8- and 60-fold increase respectively in the flux through human skin (flux 0.42 and 3.31 μg/cm²/h) (Freeman et al., 1986). The studies of Freeman et al. (1986) were conducted over 120 hours. The structure and integrity of the skin may have been compromised during such a long diffusion period. However, the flux obtained with DMSO is much larger than the flux of acyclovir in Pheroids™ obtained in the present study. DMSO is harmful to the skin and not acceptable for use on patients with skin lesions.

Volpato et al. (1998) studied the effect of iontophoresis on transdermal delivery of acyclovir at various pH (pH = 3.0 and pH = 7.4) through excised human skin. The passive flux obtained at pH 3.0 was 0.000006 μg/cm²/h. When 1.54 mg/ml acyclovir in a solution with pH 3.0 was applied by means of iontophoresis over a 7 hour period, the flux obtained was 0.00542 μg/ml. The passive flux obtained at pH 7.4 was 0.0003 μg/cm²/h. When 1.74 mg/ml

acyclovir in a solution with pH 7.4 was applied by means of iontophoresis over a 7 hour period, the flux obtained was 0.0027. The flux values obtained in the present study was obtained after 12 hours, and by application of 50 mg/ml acyclovir. When taking the amount of drug applied into consideration, the flux obtained in the present study is approximately equal to the flux obtained with iontophoresis by Volpato et al. (1998) with the solution at pH 7.4.

Cooper et al. (1986) studied the effect of addition of various concentrations of oleic acid and oleyl alcohol to the penetration of acyclovir across dermatomed human cadaver skin over 24 hours. They concluded that percentages of 5% oleic acid and 5% oleyl alcohol significantly increased the amount of acyclovir which penetrated the skin. The nearly unbelievable flux values obtained after application of 2.5 mg/ml acyclovir with 5% oleic acid and 5% oleyl alcohol were 58.9 (+ 4.7, - 4.0) and (89.1 + 7.0, -6.4) (Cooper et al., 1986). The flux obtained for acyclovir during the present study was much lower, when considering that 50 mg of acyclovir was applied. On the other hand, their studies were conducted by using human cadaver skin of which the integrity could have been compromised. Human cadaver skin samples are typically obtained from a variety of anatomical sites and after many different diseased states, which might alter the percutaneous delivery of the drugs (El-Kattan et al., 2000). Another fact to bear in mind is that oleyl alcohol can cause skin irritation (Sciencelab, 2005) and although it enhances skin penetration of acyclovir significantly, it would not be practical to formulate the drug in such a dosage form, since patients suffering from chronic ulcerated lesions as seen in HIV would experience more discomfort.

Free fatty acids make up more or less 10% of SC lipids. The only unsaturated fatty acids identified free in the SC includes oleic acid (6%) and linoleic acid (2%) (Menon, 2002). Essential fatty acids are one of the main components of the Pheroids™. Pheroids™ contain ethyl esters of the essential fatty acids, linoleic, linolenic, and oleic acid (Saunders et al., 1999). The increased flux value for acyclovir in the Pheroid™ delivery system may be

attributed to the fact that fatty acids are one of the essential compounds in the Pheroid™ delivery system. The major mode of the enhancing activity of the fatty acids appears to be by the selective perturbation of the intercellular lipid bilayers in the SC (Walker and Smith, 1996). Oleic acid disrupts the normal intercellular lipid arrangement of the SC by introduction of "fluid-like oleic acid channels" within the SC lipids (Walker & Hadgraft, 1991), pools within the lipid domains (Barry, 2001) and induces lipid fluidization as well as phase separation within the membrane (Naik et al. 2000:322).

Table 5 indicates previous studies on the transdermal delivery of ketoconazole. Venkateshwaron and Quan (2004) studied the effect of ketoconazole penetration through human skin from Durotak 87-2516 adhesive, and from Durotak 87-2516 adhesive with the addition of the penetration enhancer 10% lauramide-diethanolamine over 24 hours by using human cadaver skin. The flux obtained after application of 30 mg/ml ketoconazole was 1.81 ± 0.62 and 3.41 ± 1.83 (Venkateshwaron and Quan, 2004). In the present study there was in effect no penetration of ketoconazole through the skin and the flux of ketoconazole is not nearly comparable to the flux values obtained by Venkateshwaron and Quan (1994). This could be due to the formulation of the ketoconazole in the Pheroids™, or the oversaturated solution causing crystallization of the drug with consequent retardation of penetration across the skin.

The results presented in this study indicate that the Pheroid™ delivery system is capable of enhancing delivery of acyclovir across the skin *in vitro*. However, due to the formulation problems and the problems with the HPLC analysis, it is uncertain whether the Pheroids™ had any significance with regards to the delivery of ketoconazole across the skin *in vitro*. As mentioned earlier, the target area for ketoconazole is the SC and thus the drug should be delivered to this layer of the skin. It would also be more appropriate to avoid systemic absorption of the drug, since ketoconazole demonstrates interactions with a wide variety of

antiretrovirals, which could have a detrimental effect on HIV/AIDS patients already receiving these drugs.

Conclusions

HPLC methods for two different drugs have been outlined. The HPLC method was validated and was proven to be simple, rapid, selective and sensitive in the instance of acyclovir. However, the HPLC method proved disappointing in the selectivity and sensitivity of ketoconazole analysis. The transdermal diffusion of acyclovir and ketoconazole in PBS and Pheroids™ were investigated. The following observations were made:

- Microscopy of the Pheroids™ with the inclusion of acyclovir and ketoconazole revealed crystallization of one of the actives within the Pheroids™; presumably ketoconazole, since acyclovir has distinctive needle-shaped crystals and the crystals observed in the Pheroids™ did not exhibit this characteristic.
- The Pheroid™ microsuspension was oversaturated, since large concentrations of both acyclovir and ketoconazole was incorporated within the Pheroid™ delivery system. The oversaturation is most possibly the cause of ketoconazole crystallization within the Pheroids™.
- An enhancement of transdermal flux of acyclovir was observed with the Pheroids™. This was due to the fact that Pheroids™ entrap and delivers drugs with higher efficiency than the PBS.
- The flux of ketoconazole showed no improvement due to the crystallization of the drug within the Pheroids™, leading to retardation of diffusion of ketoconazole across the skin. This observation, however, is not a disadvantage, since the target site of ketoconazole is the SC.
- The Pheroids™ proved to be advantageous for transdermal diffusion of acyclovir but not for ketoconazole when used as delivery system.

- Despite of the small percentage yields obtained for acyclovir, the concentrations obtained still fall within the range of 0.02 – 0.7 µg/ml and 0.018 – 0.043 µg/ml, which has been described to produce 50% inhibition of viral cytopathic effect or plaque formation (ID_{50}) for susceptible strains of HSV-1 and HSV-2 respectively (McEvoy, 2002).

The following aspects render further investigation:

- The concentrations of acyclovir and ketoconazole within the Pheroid™ microsuspension should be reduced to avoid oversaturation and crystallization of ketoconazole within the Pheroids™.
- The HPLC method should be validated over a wider range of concentrations to ensure selectivity and sensitivity during analysis of *in vitro* diffusion studies. Standards of the Pheroids™ and skin components within the appropriate receptor solution obtained after a diffusion study should be included in the HPLC validation to ensure that no interference with these components and the investigated drugs occur.
- Tape-stripping of skin may be used to determine the degree of ketoconazole distribution within the SC.

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Tables

Table 1: Physicochemical properties of acyclovir and ketoconazole

Physicochemical property	Acyclovir	Ketoconazole	References
Aqueous solubility	1.3 mg/ml in water	40 µg/ml in water	McEvoy et al. (2002)
Molecular weight	225.2	531.4	British Pharmacopoeia, 2007
Melting point	257 °C [1]	148 °C – 152 °C[2]	1. Sciencelab, 2005 2. British Pharmacopoeia, 2007
Partition coefficient (log P)	0.018 [a,1]	3.73 [b,2]	1. Dollery, 1999 2. Mannisto et al. (1982)
pKa	2.27, 9.25 [1]	2.94, 6.51[2]	1. Dollery, 1999 2. Mannisto et al. (1982)

[a] log P between n-octanol and 0.2M phosphate buffer

[b] log P between n-octanol and water

Table 2: Average flux of acyclovir in PBS and the Pheroid™ delivery system (mean ± S.D.), the % yield and the enhancement ratio of acyclovir flux for time intervals 0 – 2 hours and 2 – 12 hours. Numbers in brackets refer to graph numbers in Figure 3.

Drug in solution or delivery system	Flux (μg/cm²/h) 0 – 2 hours	Flux (μg/cm²/h) 2 – 12 hours	% Yield 0 – 2 hours	% Yield 2 – 12 hours	ER_{Flux} 0 – 2 hours	ER_{Flux} 2 – 12 hours
Acyclovir in PBS (1)	0.161 ± 0.085	0.054 ± 0.040	0.00067 ± 0.0003	0.0011 ± 0.0008	-	-
Acyclovir in Pheroids™ (2)	0.180 ± 0.162	0.088 ± 0.040	0.0007 ± 0.00063	0.00175 ± 0.0008	1.12	1.63

Table 3: Average flux of ketoconazole in PBS and the Pheroid™ delivery system (mean ± S.D.), the % yield, and the enhancement ratios of ketoconazole flux. Numbers in brackets refer to graph numbers in Figure 4.

Drug in solution or delivery system	Flux (μg/cm²/h)	% Yield	ER_{Flux}
Ketoconazole in PBS (1)	0.023 ± 0.010	0.0017 ± 0.0012	-
Ketoconazole in Pheroids™ (2)	0.007 ± 0.004	0.0006 ± 0.0005	0.3

Table 4: Data from transdermal studies with the model compound acyclovir. Data presented in the literature are compared to data of the present study in terms of enhancement methods, acyclovir concentration, the diffusion membrane, duration of the studies and fluxes obtained with various methods of penetration enhancement.

Study	Method of enhancement	Acyclovir concentration	Membrane	Time (hours)	Flux (µg/cm²/h)				
					Iontophoresis	Chemical enhancers	Acyclovir in Pheroids™	Passive	Reference
1	Chemical enhancement (MAC)	50 mg/ml	Excised human abdominal or facial skin	120	-	0.42 ± 0.05[1]	-	0.055 ± 0.044[1]	Freeman et al. (1986)
2	Chemical enhancement (MAC)	50 mg/ml	Guinea pig skin	120	-	0.36 ± 0.12[1]	-	0.047 ± 0.011[1]	Freeman et al. (1986)
3	Chemical enhancement (95% DMSO)	50 mg/ml	Excised human abdominal or facial skin	120	-	3.31 ± 0.79[1]	-	0.055 ± 0.044[1]	Freeman et al. (1986)
4	Chemical enhancement (95% DMSO)	50 mg/ml	Guinea pig skin	120	-	4.10 ± 0.26[1]	-	0.047 ± 0.011[1]	Freeman et al. (1986)
5	Iontophoresis[2] (pH = 3.0)	1.54 mg/ml	Excised human abdominal skin	7	0.00542	-	-	0.00006	Volpato et al. (1998)
6	Iontophoresis[2] (pH = 7.4)	1.74 mg/ml	Excised human abdominal skin	7	0.0027	-	-	0.0003	Volpato et al. (1998)
9	Chemical enhancement (5% oleic acid)	2.5 mg/ml	Dermatomed human cadaver skin (~0.25mm)	24	-	58.9 ($+4.7_{-4.0}^{\div 3}$)	-	None	Cooper et al. (1985)
10	Chemical enhancement (5% oleyl alcohol)	2.5 mg/ml	Dermatomed human cadaver skin (~0.25mm)	24	-	89.1 ($+7.0_{-6.4}^{\div 3}$)	-	None	Cooper et al. (1985)
11	Pheroid™ delivery system	50 mg/ml	Human epidermis	12	-	-	0.180 ± 0.162[4] 0.088 ± 0.040[5]	0.161 ± 0.085[4] 0.054 ± 0.040[5]	Present study

[1] Values are the mean ± standard error of the mean (n = 3 to 5)

[2] A constant direct current of 0.3 mA (0.5 mA/cm²) was applied for 15 minutes, 30 minutes and 7 hours

[3] Geometric mean ± standard error of the mean

[4] Flux calculated for time period 0 – 2 hours

[5] Flux calculated for time period 2 – 12 hours

Table 5: Comparison between data from transdermal studies with the model compound ketoconazole. Data presented in the literature are compared to data of the present study in terms of enhancement methods, ketoconazole concentration, the diffusion membrane, duration of the studies and fluxes obtained with the various methods of penetration enhancement.

Study	Method of enhancement	Ketoconazole concentration	Membrane	Time (hours)	Flux (µg/cm²/h)			Reference
					Formulation	Ketoconazole in Pheroids™	Passive	
1	Formulation (Durotak 87-2516 adhesive)	30mg/ml	Human cadaver skin	24	1.81 ± 0.62	-	-	(Venkateshwaron and Quan, 2004)
2	Formulation and chemical enhancer (Durotak 87-2516 adhesive and 10% lauramide-diethanolamine)	30mg/ml	Human cadaver skin	24	3.41 ± 1.83	-	-	(Venkateshwaron and Quan, 2004)
3	Pheroid™ delivery system	20mg/ml	Human abdominal epidermis	12	-	0.007 ± 0.004	0.023 ± 0.010	Present study

Figure legends

Fig. 1: Percentage ketoconazole crystals within various size ranges (μm) as determined by polarised light microscopic analysis.

Fig. 2: Confocal laser scanning micrographs of Pheroids™ and Pheroids™ with the inclusion of acyclovir and ketoconazole. The Pheroids™ were labelled with Nile Red which emits in the red spectrum when excited by laser at 505nm. A and B: Pheroid™ sponges, C: acyclovir and ketoconazole within the Pheroids™.

Fig. 3: Light microscopic images of ketoconazole crystals in the Pheroid™ preparation.

Fig. 4: *In vitro* permeation profiles of acyclovir

◆ (1): Acyclovir dissolved in PBS (control flux of acyclovir). The average cumulative concentration (μg/cm²) of acyclovir versus time (hours) is shown.

■ (2): Acyclovir dissolved in Pheroids™. The average cumulative concentration (μg/cm²) of acyclovir versus time (hours) is shown.

Fig. 5: *In vitro* permeation profiles of ketoconazole

◆ (1): Ketoconazole dissolved in PBS. The average cumulative concentration (μg/cm²) of ketoconazole versus time (hours) is shown.

■ (2): Ketoconazole dissolved in the Pheroids™. The average cumulative concentration (μg/cm²) of ketoconazole versus time (hours) is shown.

Figure 1

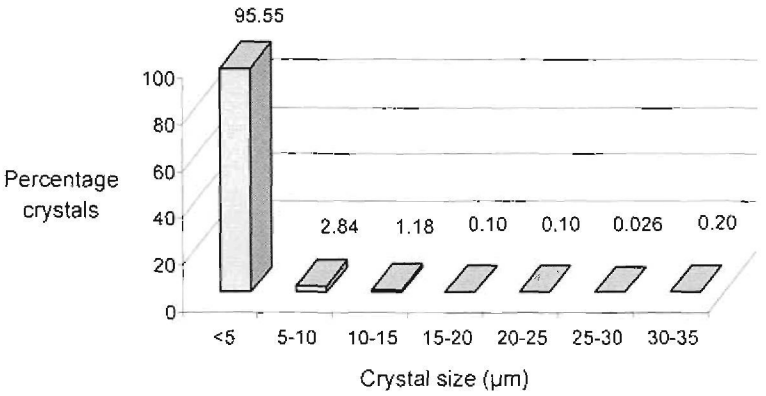

95.55
100
80
60
40
20
0
Percentage crystals
2.84
1.18
0.10
0.10
0.026
0.20
<5
5-10
10-15
15-20
20-25
25-30
30-35
Crystal size (µm)

Figure 2

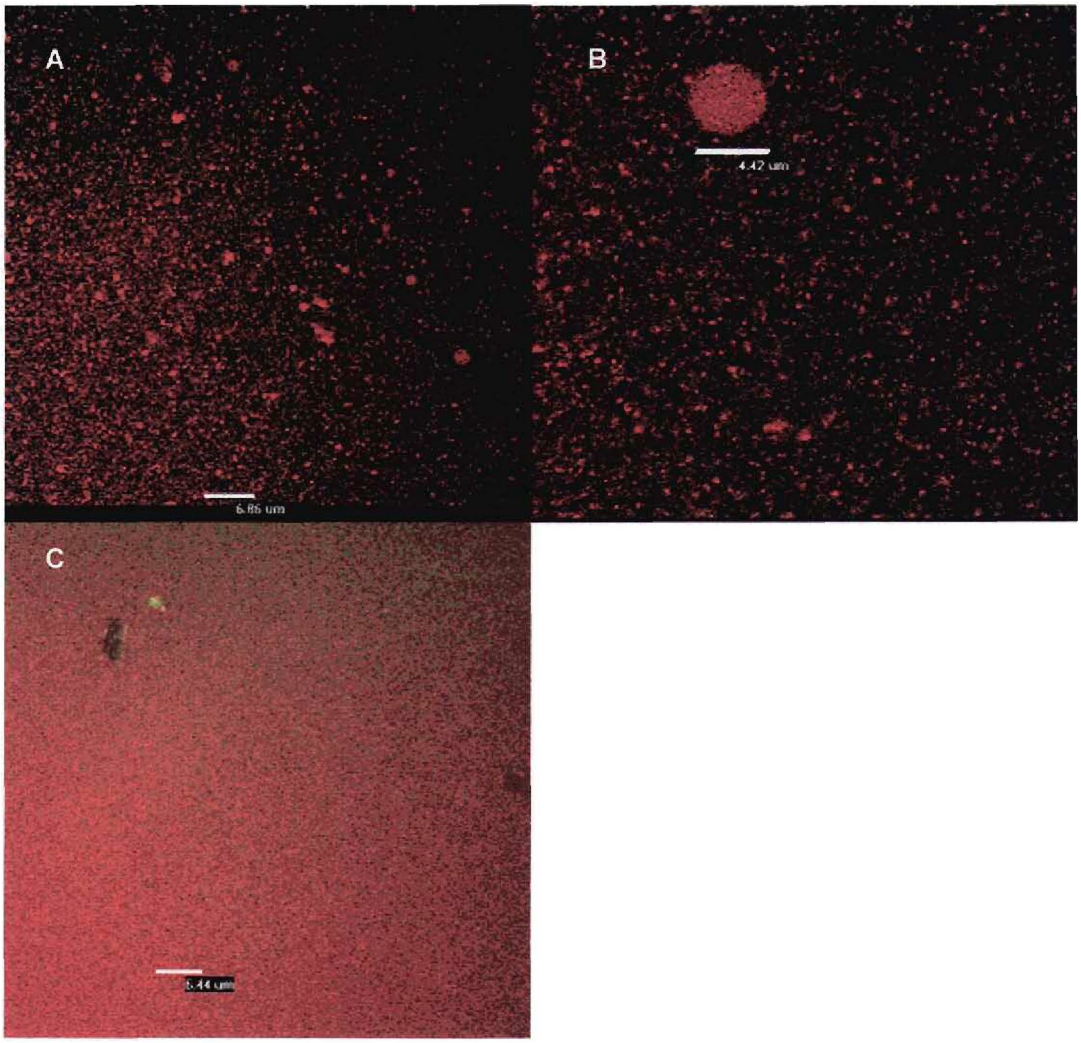


Figure 3

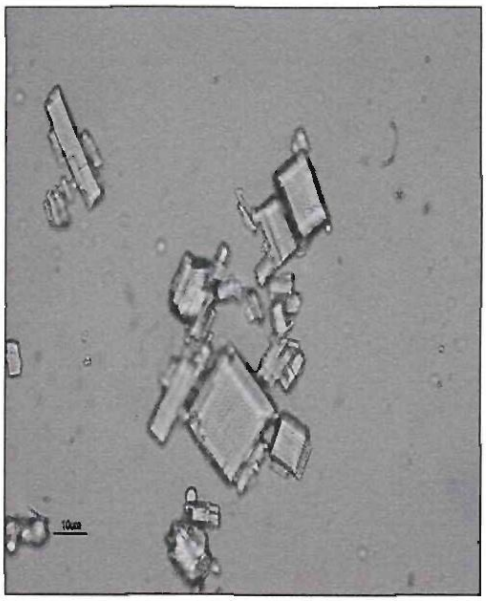


Figure 4

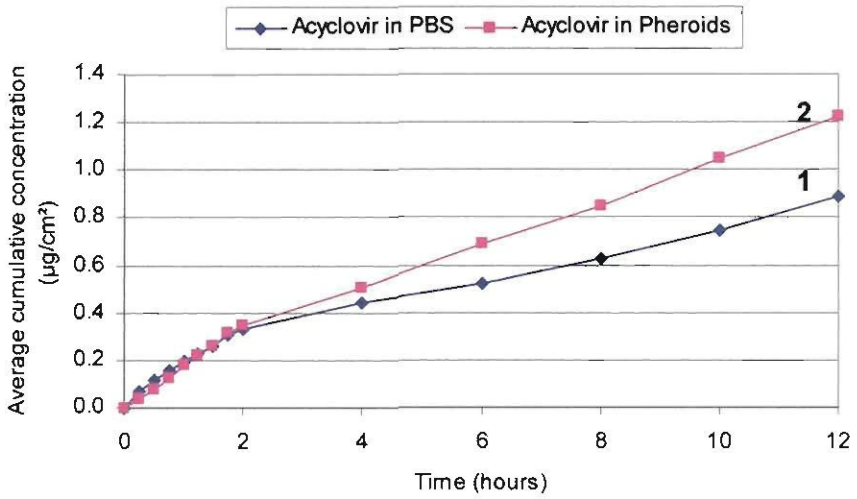
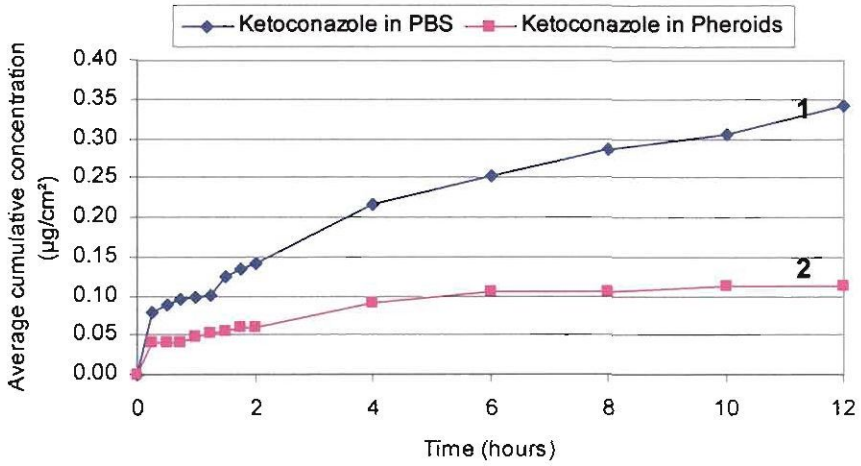

Acyclovir in PBS
Acyclovir in Pheroids
Average cumulative concentration (µg/cm²)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
Time (hours)
2
1

Figure 5


Ketoconazole in PBS
Ketoconazole in Pheroids
Average cumulative concentration (µg/cm²)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
2
4
6
8
10
12
Time (hours)
1
2

CHAPTER 4: SUMMARY AND FUTURE PROSPECTS

Since the early 1980's, when acquired immunodeficiency syndrome (AIDS) was first discovered, more than 40 million people have become infected with the immunodeficiency virus (HIV) (Johnson, 2000). HIV infection and AIDS are often associated with a broad range of skin and mucosae manifestations (Rigopoulos *et al.*, 2004). Decreased mucocutaneous immunity often accounts for the early clinical signs seen in HIV infected patients. As HIV disease advances, these manifestations may become more severe and diverse, causing physical discomfort and psychological distress in otherwise healthy individuals. In advanced immunosuppression, opportunistic pathogens can present as atypical cutaneous lesions, presenting challenges in both diagnosis and treatment (Uthayakumar *et al.*, 1997).

A spectrum of infections of viral, fungal, bacterial and parasitic origins occurs alone or in combination with each other (Ramdial, 2000). The majority of these infections, however, are either fungal or viral (Kar *et al.*, 1996). Numerous viruses of the herpes viridae can cause cutaneous disease in HIV infected patients, including herpes simplex virus (HSV), herpes zoster virus (HZV) and disseminated cytomegalovirus (CMV) infection (Erdal *et al.*, 2007). Fungal infections among people with HIV/AIDS include candidiasis and dermatophytosis. Dermatophytosis normally occurs as tinea corporis or tinea capitis (Kekitiinwa & Schwarzwald, 2007). Seborrhoeic dermatitis is also very common in the HIV infected population (Rodwell & Berger, 2000).

Acyclovir was the first specific antiviral drug to become widely used against herpes viruses, particularly herpes simplex virus type 1 and 2 and varicella zoster virus (Dollery, 1999). Ketoconazole, a synthetic imidazole antifungal compound, has a broad spectrum of activity against both dermatophytes and yeasts (Daniel, 1996). Systemic absorption after topical application of both acyclovir and ketoconazole, however, appears to be minimal (McEvoy, 2002). Acyclovir is unable to penetrate through the stratum corneum (SC) barrier layer of the skin and reach the target site; the basal epidermis (Jiang *et al.*, 1998). The target site for ketoconazole, however, is the SC and the drug should be delivered to this layer of the skin to inhibit growth of fungal pathogens (Pershing *et al.*, 1994).

One of the major functions of skin is to prevent the body from losing water into the environment and to block the entry of exogenous agents. Therefore, the skin and, in particular, the stratum corneum (SC) forms an effective barrier to drug permeation.

Currently, the most widely used approach to drug permeation-enhancement across the SC barrier is the use of chemical penetration enhancers (Asbill & Michniak, 2000).

In this study the effect of the Pheroid™ delivery system on the transdermal diffusion of acyclovir and ketoconazole was investigated. Pheroids™ contain essential fatty acids, amongst which is oleic acid that has previously been shown to enhance the penetration of acyclovir across the skin. For this reason it was hypothesized that it could be possible to achieve at least the same results by using Pheroids™.

The permeability of acyclovir and ketoconazole through human abdominal skin was determined using the *in vitro* Franz diffusion cell method. Transdermal diffusion of 5% acyclovir and 2% ketoconazole in Pheroids™ were compared to 5% acyclovir and 2% ketoconazole in phosphate buffered solution (PBS).

The following observations were made:

- Microscopy of the Pheroids™ with the inclusion of acyclovir and ketoconazole showed crystallization of ketoconazole.
- The Pheroid™ microsuspension was oversaturated, since large concentrations of both acyclovir and ketoconazole was incorporated within the Pheroid™ delivery system. The oversaturation was most possibly the cause of ketoconazole crystallization within the Pheroid™ preparation.
- An enhancement of transdermal flux of acyclovir was observed with the Pheroids™. This was due to the fact that Pheroids™ entrap and delivers drugs across the SC with higher efficiency than the PBS.
- Although validated for larger concentrations, the HPLC method for ketoconazole analysis proved disappointing in sensitivity and selectivity of the smaller concentrations of the drug.
- The flux of ketoconazole showed no improvement due to the crystallization of the drug within the Pheroids™, leading to retardation of diffusion of ketoconazole across the skin.
- The Pheroids™ proved to be advantageous for transdermal diffusion of acyclovir but not for ketoconazole when used as delivery system. This last observation, however, is not a disadvantage, since the target site of ketoconazole is the SC.

With the object of formulating a product for future use, the following aspects need to be considered:

- The concentrations of acyclovir and ketoconazole within the Pheroid™ microsuspension should be reduced to avoid oversaturation and crystallization of ketoconazole within the Pheroids™.
- The HPLC method should be validated over a wider range of concentrations to ensure selectivity and sensitivity during analysis of *in vitro* diffusion studies. Standards of the Pheroids™ and skin components within the appropriate receptor solution obtained after a diffusion study should be included in the HPLC validation to ensure that no interference with these components and the investigated drugs occur.
- Tape-stripping of skin can be used to determine the degree of ketoconazole distribution within the SC.
- *In vivo* testing of the product should be done to determine adverse reactions which can be associated with the use of the product.
- *In vitro* efficacy testing of the proposed microsuspension using reference strains of herpes viruses, *Candida albicans*, *Trichophyton rubrum*, *Trichophyton mentagrophytes* and *Epidermophyton floccosum*. For example, cytopathic effect inhibition assays (CPE-inhibition assays) and plaque inhibition assays can be undertaken to determine whether the concentration of acyclovir correlates with the recommended concentrations required in order to produce 50% inhibition of viral cytopathic effect or plaque formation.

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RAMDIAL, P.K. 2000. Selected topics in HIV-associated skin pathology. *Current diagnostic pathology*, 6:113-124.

RIGOPOULOS, D., PAPARIZOS, V. & KATSAMBAS, A. 2004. Cutaneous markers of HIV infection. *Clinics in dermatology*, 22:487-498.

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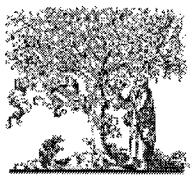
APPENDIX 1: GUIDE FOR AUTHORS EUROPEAN JOURNAL OF PHARMACEUTICAL SCIENCES



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Manuscripts should be neatly typed, double-spaced throughout, including tables, with at least 2.5 cm margins on all sides. Use one font type and size throughout the manuscript. Author(s)

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Webster's New International Dictionary or the Oxford English Dictionary should be consulted for spelling. Latin plurals should not be used if the English equivalent has become the accepted form, e.g., formulas not formulae. Use of hyphens, capital letters, numbers written or spelled out (e.g., 8 or eight) should be consistent throughout the manuscript. Words at the end of a line should not be divided.

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Some abbreviations may be used without definition:

ADP,CDP, GDP, IDP	5'-pyrophosphates of adenosine
UDP	cytidine, guanosine, inosine, uridine
AMP etc.	adenosine 5'-monophosphate etc.
ADP etc.	adenosine 5'-diphosphate etc.
ATP etc.	adenosine 5'-triphosphate etc.
CM-cellulose	carboxymethylcellulose
CoA and acetyl-CoA	coenzyme A and its acyl derivatives
DEAE-cellulose	*O*-(diethylaminoethyl)-cellulose
DNA	deoxyribonucleic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid

FAD	flavin-adenine dinucleotide
FMN	flavin mononucleotide
GSH, GSSG	glutathione, reduced and oxidized
Hepes	4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid
NAD	nicotinamide-adenine dinucleotide
NADP	nicotinamide-adenine dinucleotide phosphate
NMN	nicotinamide mononucleotide
Pi, PPi	orthophosphate, pyrophosphate
RNA	ribonucleic acid
Tris	2-amino-2-hydroxymethylpropane-1,3-diol

Two alternative conventions are currently in use in some cases. For example, for the phosphoinositides there are both the abbreviations recommended by the IUPAC-IUB and those of the Chilton Convention (e.g., PtdIns(4,5)P_2 vs. $PIP_2$for phosphatidylinositol 4,5-biphosphate). The journal will accept either of these forms but not their combination.

Abbreviations of units of measurements and other terms are as follows:

Units of mass

kilogram	kg
gram	g
milligram	mg
microgram	µg
nanogram	ng
mole (gram-molecule)	mol
millimole	mmol

micromole	µmol
nanomole	nmol
picomole	pmol
femtomole	fmol
equivalent	eq

Units of time

hour	h
minute	min
second	s
millisecond	ms
microsecond	µs

Units of volume

litre	l
millilitre	ml
microlitre	µl

Units of length

metre	m
centimetre	cm
millimetre	mm
micrometre	µm

nanometre nm

Units of concentration

molar (mol/l) M

millimolar mM

micromolar μM

nanomolar nM

picomolar pM

Units of heat, energy, electricity

joule J

degree Celsius (centigrade) °C

coulomb C

ampere A

volt V

ohm Ω

siemens S

Units of radiation

curie Ci

counts per minute cpm

disintegrations per minute dpm

becquerel Bq

Miscellaneous

gravity	g
dissociation constant	K_d
median doses	LD_{50}, ED_{50}
probability	P
routes of drug administration	i.v., i.p., s.c., i.m.
square centimetre	cm^2
standard deviation	S.D.
standard error of the mean	S.E.M.
Svedberg unit of sedimentation coefficient	S
Hill coefficient	n_H

The isotope mass number should appear before the atomic symbol, e.g., [^{3}H]noradrenaline, [^{14}C]choline. Ions should be written: Fe^{3+}, Ca^{2+}, Mg^{2+}. The term absorbance (A) is preferred to extinction or optical density. For abbreviations not included in this list consult: *Units, Symbols and Abbreviations, A Guide for Biological and Medical Authors and Editors*, 1994 (The Royal Society of Medicine, London), ISBN 0-905958-78-0, or *Scientific Style and Format. The CBE Manual for Authors, Editors, and Publishers*, 6th edn. (Cambridge University Press, Cambridge), ISBN 0-521-47154-0.

1.5. Nomenclature

Only generic and chemical names of drugs should be used, although a proprietary equivalent may be indicated once, in parentheses. *Pharmacological and Chemical Synonyms*, E.E.J. Marler, 9th edn. (Elsevier, Amsterdam, 1990) may be consulted.

The nomenclature of chemical substances should be consistent, clear and unambiguous, and should conform to the usage of the American Chemical Society and the convention recommended by the International Union of Pure and Applied Chemistry (IUPAC). When in

doubt, writers should consult the indexes of *Chemical Abstracts*; the various reports and pamphlets of the American Chemical Society Committee on Nomenclature, Spelling and Pronunciation; and from the International Union of Biochemistry and Molecular Biology (IUBMB): *Biochemical Nomenclature and Related Documents* (Portland Press, London).

When drugs, which are mixtures of stereoisomers are used, the fact that they have a composite nature and the implication of this for interpretation of the data and drawing of conclusions should be made clear. The use of the appropriate prefix is essential. Use of the generic name alone without prefix would be taken to refer to agents with no stereoisomers. The nomenclature of the various isomers and isomeric mixtures can be found in: (i) *IUPAC, Nomenclature of Organic Chemistry*, eds. J. Rigaudy and S.P. Klesney (Pergamon Press, London), 1979, p. 481; (ii) *Signs of the times: the need for a stereochemically informative generic name system*, Simonyi, M., J. Gal and B. Testa, 1989, Trends Pharmacol. Sci. 10, 349. For nomenclature of peptides, see *Neuropeptides*, Vol. 1, 1981, p. 231.

The nomenclature of receptors and their subtypes should conform to the *TIPS 1995 Receptor & Ion Channel Nomenclature Supplement (Trends Pharmacol. Sci.* Receptor Nomenclature Supplement 1995). Copies of this supplement are available from the publisher (Elsevier Trends Journals, Oxford Fulfilment Centre, P.O. Box 800, Kidlington, Oxford OX5 1DX, UK. Tel.: (44-1865) 843-699; Fax: (44-1865) 843-911).

The trivial name of the enzyme may be used in the text, but the systematic name and classification number according to *Enzyme Nomenclature*, rev. edn. (Academic Press, New York, NY, 1984) should be quoted the first time the enzyme is mentioned.

1.6. Editorial review

All manuscripts are generally submitted to 2-3 referees who are chosen for their ability to evaluate the work. Supplementary material may be included to facilitate the review process. Authors may request that certain referees should not be chosen. Members of the editorial board will usually be called upon for advice when there is disagreement among the referees or between referees and authors, or when the editors believe that the manuscript has not received adequate consideration by the referees.

All referees' comments must be responded to, and suggested changes be made. The author should detail the changes made in response to the referees' comments and suggestions in an accompanying letter. If the author disagrees with some changes, the reason, supported

by data, should be given. The editors may refuse to publish manuscripts from authors who persistently ignore referees' comments. Handwritten additions or corrections will not be accepted. Only complete retyping of the pages affected by revision is acceptable. A revised manuscript should be received by the editorial office no later than 4 months after the editorial decision was sent to the author(s); otherwise it will be processed as a new manuscript.

2. Organization and style of manuscripts

Authors should consult a current issue of the journal for the general manner of presentation. Manuscripts should be written in clear, concise English (see section 1.1), bearing in mind that English is not the native language of many of the readers. Terms that are not generally understood should be avoided; however if it is absolutely necessary to use such terms, they must be defined.

2.1. Research articles

2.1.1. General

The manuscript of a research article should be arranged as follows.

First page: title, surname(s) and full first name(s) of each author; name and address of the establishment where the work was done; name, full postal address, telephone and telefax numbers and e-mail of author to whom proofs and other correspondence should be sent. Next page: abstract and keywords (indexing terms, normally 3-6 items). Pages 3 to end: 1. Introduction; 2. Materials and methods; 3. Results; 4. Discussion; Acknowledgements; References; Tables; Figure legends and Figures. Parts 3 & 4 may be combined into one item: Results & Discussion. Subdivisions of a section should also be numbered within that section: 2.1., 2.2., 2.3., etc. All pages should be numbered consecutively, the title page being p. 1. See section 2.7 for further information.

Supplementary material for electronic publication can be published on the journal website alongside the article. In the print version, a URL reference will be made to point readers to the location of the article and supplementary material.

2.1.2. Abstract and keywords

The abstract with keywords should be typed on a separate sheet. The abstract should

include: the reason why the experiments were done, a very brief description of the experiments (including species, tissue, etc.), followed by the main results, and finally, a conclusion giving the relevance of the results to the question asked. The abstract must be completely self-explanatory. The abstract should not exceed approximately 200 words. No footnotes may be used and a reference, if cited, must be given in full. Standard terms and scientific nomenclature should be used. Abbreviations and contractions, except those for weights and measures and those explained, should not be used. Below the abstract, type 3-6 keywords or short phrases suitable for indexing. These terms will be printed at the end of the abstract. If possible, keywords should be selected from *Index Medicus* or *Excerpta Medica Index*.

2.1.3. Introduction, Materials and Methods, Results, Discussion

The introduction should not be an extensive review of the literature but should refer only to previous work which has a direct bearing on the topic to be discussed.

Materials and methods should be written clearly and in such detail that the work can be repeated by others. Procedural detail that has been published previously should be referred to by citation. When a modified procedure is used, only the author's modifications of the previously published method need to be given in detail.

Results should be described concisely. Text, tables and figures must be internally consistent.

The discussion should involve the significant findings presented. Wide digressions are unacceptable because of the limitations of space.

2.2. References

Authors are responsible for the accuracy and completeness of their references as these will not be checked by the editorial office.

References should be listed alphabetically (see sample references) according to the "Harvard" system. Articles written by the same first author with different second authors should be listed according to the second author's surname. Articles written by the same first author with more than one co-author should be listed alphabetically according to the first author's surname and then according to the year of publication. Two or more references to the same first author with the same publication year should have a, b, c, etc., suffixed to the

year indicating the alphabetical order of the second or third author, etc.

References to journals should contain the names and initials of the author(s), the year, the full title, the abbreviation of the name of the periodical according to those in the Bibliographic Guide for Editors and Authors (American Chemical Society, Washington, DC.) followed by the volume and page numbers.

References to books should include the title and name and city of the publisher.

References in the text should be cited by the author's name and the year of publication. For 3 or more authors the name of the first author followed by et al. should be used, e.g., Davis, Robinson (1990) or (Davis, Illum, 1984; de Ber et al., 1988, 1989; Borchardt et al., 1990, 1991a,b,c).

Journals:

Fagerholm, U., Lennernas, H., 1995. Experimental estimation of the effective unstirred water layer thickness in the human jejunum and its importance in oral drug absorption. Eur. J. Pharm. Sci. 3, 247-253.

Lipinski, C.A., Lombardo, F., Dominy, B.W., Feeney, P.J., 1997. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv. Drug. Deliv. Rev. 23, 3-25.

Books:

Alderborn, G., Nystrom, C., 1998. Pharmaceutical Powder Compaction Technology. Marcel Dekker, New York.

Kissel, T., Koneberg, R., 1996. Injectable biodegradable microspheres for vaccine delivery. In: Cohen, S. and Bernstein, H. (Eds.), Microparticulate systems for the delivery of proteins and vaccines. Marcel Dekker, New York, pp. 51-87.

Unpublished observations, personal communications and manuscripts in preparation or submitted for publication may be referred to in the text but should not appear in the list of references. Manuscripts in press (i.e., accepted for publication) may be included in the references citing the DOI article identifier, which enables the citation of a paper before

volume, issue and page numbers are allocated. The name of the journal in which they are to appear must be given.

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

2.3. Illustrations

The number of illustrations should be limited to the essential.

(a) It is important to allow for reduction to fit a single column, 8.4 cm wide or at most a double column, maximally 17.6 cm wide. Of preference, illustrations, especially photomicrographs, should be submitted in their final size (single or double column). When possible, all key symbols should be explained in the figures. All letters and numerals appearing in a particular illustration should be of the same size (approximately 1.4-2.0 mm height when reduced to 8.4 cm width). Comparable illustrations should carry letters, figures and numerals of the same size when reduced to 8.4 cm width.

(b) Graphs should be prepared by a skilled photographer so that the dark, cross-hatched background is eliminated, the faint portions of the graphs are intensified, and a sharp print is obtained. This process may be avoided by using blue-ruled instead of black-ruled recording paper for the originals.

(c) Drawings of chemical structures should as far as possible be produced with the use of a drawing program such as ChemDraw. Authors using the current versions of ChemDraw, ChemIntosh and ChemWindows should use the JOC format.

(d) A calibration bar should be drawn on the micrographs instead of giving a magnification factor in the figure legend.

(e) All illustrations should be referred to as figures and numbered in Arabic numerals (Fig. 1, 2, etc.).

(f) Legends to figures should make the figures comprehensible without reference to the text.

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

2.4. Tables

Tables should be prepared for use in a single column (8.4 cm wide) or be of page width (17.6 cm).

(a) Each table should have a brief explanatory heading and sufficient experimental detail (following the table body as a footnote) so as to be intelligible without reference to the text.

(b) Tables should not duplicate material in text or illustrations.

(c) Short or abbreviated column headings should be used and, if necessary, explained in footnotes, and indicated as [a], [b], [c], etc.

(d) Statistical measures of variation, S.D., S.E., etc. should be identified.

(e) Tables should be numbered separately in Arabic numerals (Table 1, 2, etc.).

2.5. Formulas and equations

Structural chemical formulas, process flow diagrams and complicated mathematical expressions should be very clearly presented. All subscripts, superscripts, Greek letters and unusual characters must be identified. Structural chemical formulas and process flow diagrams should be prepared in the same way as graphs.

2.6. GenBank accession numbers

Gene accession numbers refer to genes or DNA sequences about which further information can be found in the databases at the National Center for Biotechnical Information (NCBI) at the National Library of Medicine. Authors wishing to enable other scientists to use the accession numbers cited in their papers via links to these sources, should reference this information in the following manner:

For each and every accession number cited in an article, authors should type the accession number in **bold, underlined text.** Letters in the accession number should always be capitalised. (See Example 1 below.) This combination of letters and format will enable Elsevier's typesetters to recognize the relevant texts as accession numbers and add the required link to GenBank's sequences.

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In the final version of the ***printed* article**, the accession number text will not appear bold or underlined (see Example 2 below).

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In the final version of the ***electronic* copy**, the accession number text will be linked to the appropriate source in the NCBI databases enabling readers to go directly to that source from the article (see Example 3 below).

Example 3: "GenBank accession nos. AI631510, AI631511, AI632198, and BF223228), a B-cell tumor from a chronic lymphatic leukemia (GenBank accession no. BE675048), and a T-cell lymphoma (GenBank accession no. AA361117)".

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2.9. Commentaries and Mini-reviews

One page suggestions for commentaries and mini-reviews should be sent directly to the Editor-in-Chief at ejps-journal@helsinki.fi for consideration. Please see detailed information on commentaries and mini-reviews below.

2.9.1 Commentaries (Guidance)

The definition of a Commentary for EJPS is three-fold. Firstly, it can be an argued piece of provocative scientific writing purporting to take a balanced position on a controversial pharmaceutical science topic. A second option is for the author to approach the topic from a

particular viewpoint on one side of an argument. A third option is to provide a topical update on a hot topic in Pharmaceutical Sciences and this can be more informative than controversial.

Commentaries will be commissioned by the editors in advance or invited from non-commissioned authors if they wish to initially submit a one page summary of the intended Commentary to the editors in advance. All manuscripts will be assessed by 2-3 independent referees.

The journal is looking for a stimulating and provoking essay, with referenced material, but without an extensive reference list. Commentaries can contain one summary figure and/or table and should have no more than 30 references to prefereably recent peer-reviewed material. The word count should be approximately 2,000 words maximum.
The commentary should have a short abstract summary of 150 to 200 words and 4-5 key words should be included. The text should be broken down into 4-5 numbered sections beginning with an Introduction and ending with a Conclusions section. A model of the structures is to be found in Eur. J. Pharm. Sci. 19, 1-11 by R.D. Combes

2.9.2 Mini-review (Guidance)

Mini-reviews are thought provoking reviews of contemporary pharmaceutical research. Themes are as described in the Scope of the Journal section.

Mini-reviews will usually be commissioned by the editors in advance, but contributions are invited from non-commissioned authors if they wish to initially submit a one page summary of the intended review to the editors in advance. All manuscripts will be assessed by 2-3 independent referees.

The structure of the mini-review is as follows: a title page followed by a 200-300 word abstract with 4-5 key words. The text is then divided into numbered sections finishing with a Summary section. References should be kept to a maximum of 60 and should be mostly to recent peer-reviewed material. There is a combined maximum of 5 figures / tables. Authors are encouraged to submit their original unpublished work as part of the review if appropriate. The total length of the review should be a maximum of 4,000 words.

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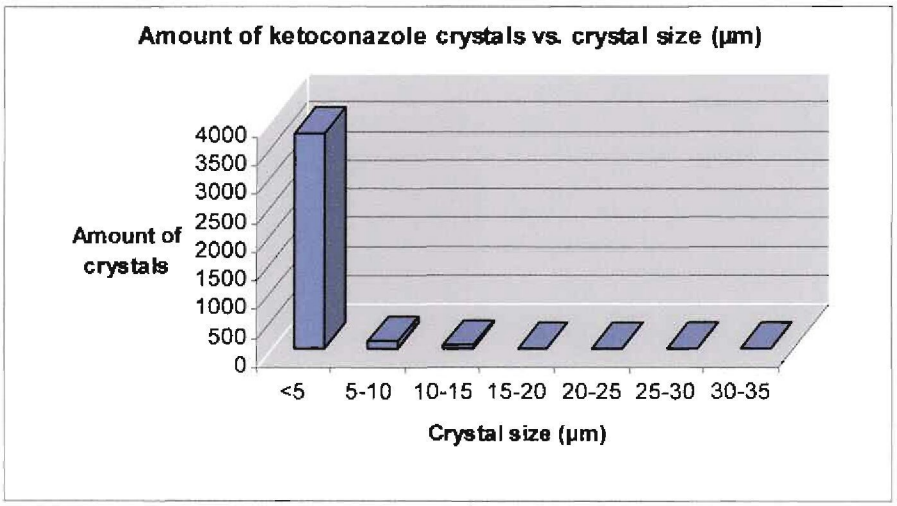
APPENDIX 2:

DETERMINATION OF KETOCONAZOLE CRYSTAL SIZE

AMOUNT OF KETOCONAZOLE CRYSTALS IN VARIOUS SIZE RANGES

The amount of ketoconazole crystals in the various size ranges was determined, and plotted against crystal size. The results are shown below.



PERCENTAGE OF KETOCONAZOLE CRYSTALS IN VARIOUS SIZE RANGES

The percentage of ketoconazole crystals in the various size ranges was calculated. The table below indicates the percentage of ketoconazole crystals within the various size ranges.

Percentage of ketoconazole crystals	
Crystal size (µm)	**Percentage**
<5	95.55
5-10	2.84
10-15	1.18
15-20	0.10
20-25	0.10
25-30	0.03
30-35	0.20

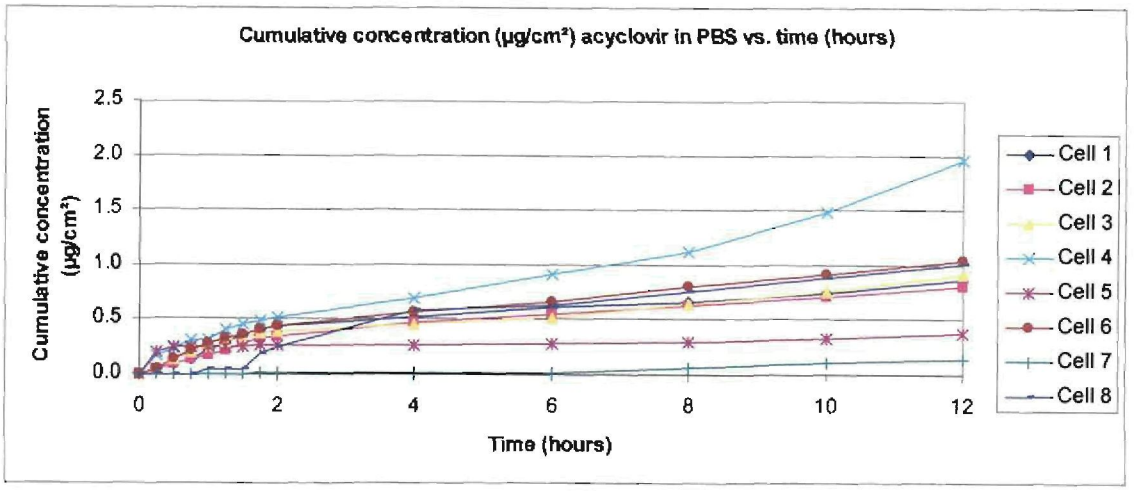
APPENDIX 3:

DATA OF FRANZ CELL DIFFUSION STUDIES

ACYCLOVIR IN PHOSPHATE BUFFERED SOLUTION (PBS) (POSITIVE CONTROL)

Acyclovir was dissolved in PBS. The data for acyclovir reported here serves as the control.

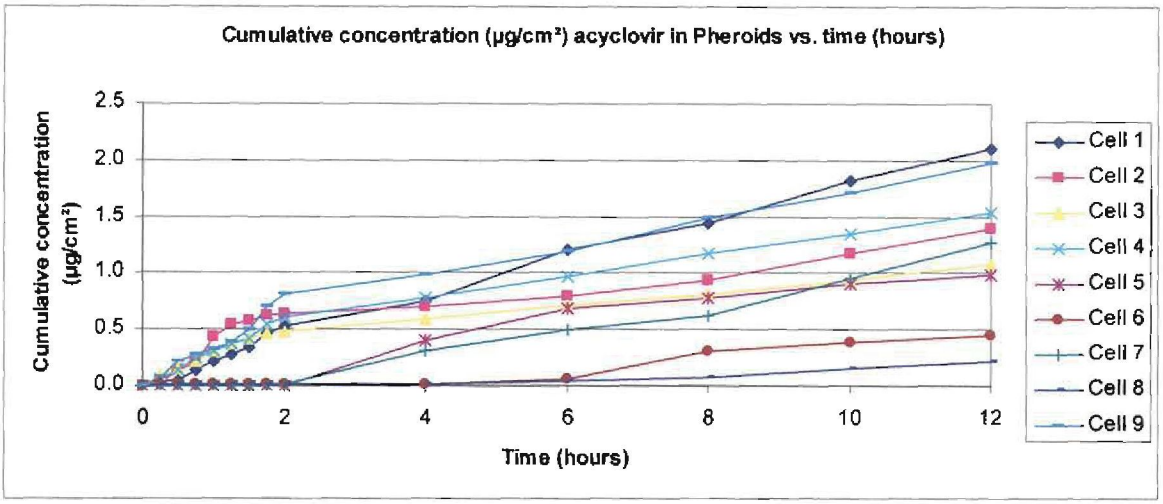
Cell number	Cumulative concentration (µg/ml)	% yield 0 – 2 hours	% yield 2 – 12 hours	Flux 0 – 2 h (µg/cm²/h)	Flux 2 – 12 h (µg/cm²/h)
1	0.869	0.00087	0.00086	0.2329	0.0409
2	0.808	0.00070	0.00092	0.1833	0.0443
3	0.914	0.00080	0.00103	0.2042	0.0511
4	1.967	0.00103	0.00291	0.2385	0.1403
5	0.387	0.00052	0.00025	0.0834	0.0123
6	1.042	0.00089	0.00120	0.2276	0.0599
7	0.140	0.00003	0.00025	0.0059	0.0136
8	1.008	0.00049	0.00152	0.1141	0.0692
	Mean	0.00067	0.00112	0.1612	0.0540
	SD	0.0003	0.0008	0.0847	0.0403
	% RSD	44.269	70.706	52.5486	74.6661



ACYCLOVIR IN THE PHEROID™ DELIVERY SYSTEM

Acyclovir was incorporated in the Pheroid™ delivery system. The data obtained for acyclovir is reported here.

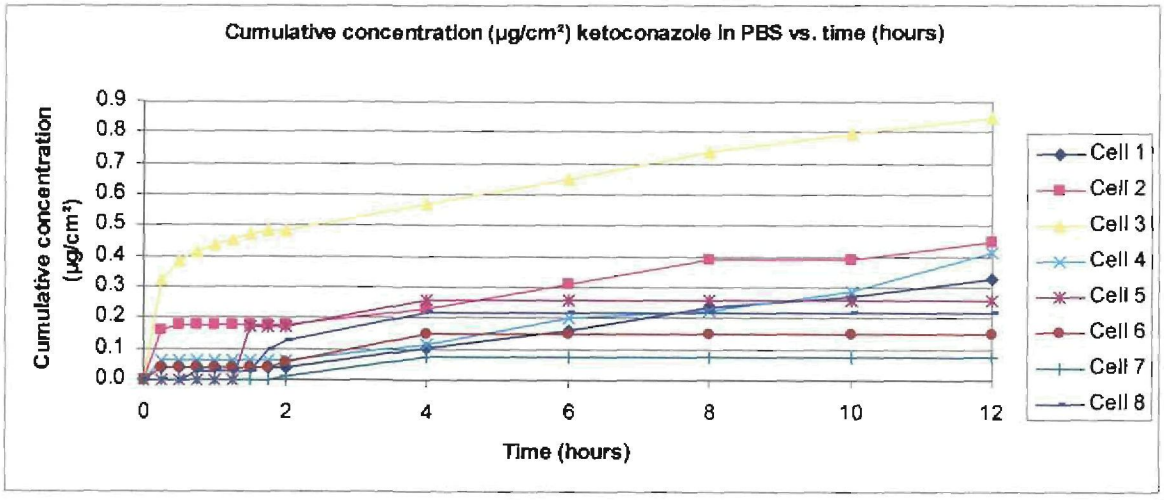
Cell number	Cumulative concentration (µg/ml)	% yield 0 – 2 hours	% yield 2 – 12 hours	Flux 0 – 2 h (µg/cm²/h)	Flux 2 – 12 h (µg/cm²/h)
1	2.112	0.00107	0.00315	0.2755	0.1613
2	1.400	0.00129	0.00151	0.3736	0.0760
3	1.080	0.00097	0.00119	0.2487	0.0588
4	1.535	0.00123	0.00184	0.3086	0.0925
5	0.994	0.00001	0.00198	0.0021	0.0933
6	0.460	0.00003	0.00089	0.0036	0.0519
7	1.270	0.00004	0.00250	0.0057	0.1192
8	0.217	0.00004	0.00039	0.0076	0.0202
9	1.978	0.00164	0.00231	0.3904	0.1174
	Mean	0.00070	0.00175	0.1795	0.0878
	SD	0.00063	0.00081	0.1616	0.0397
	% RSD	89.18790	46.01961	90.0038	45.1468



KETOCONAZOLE IN PHOSPHATE BUFFERED SOLUTION (PBS) (POSITIVE CONTROL)

Ketoconazole was dissolved in PBS. The data for ketoconazole reported here serves as the control.

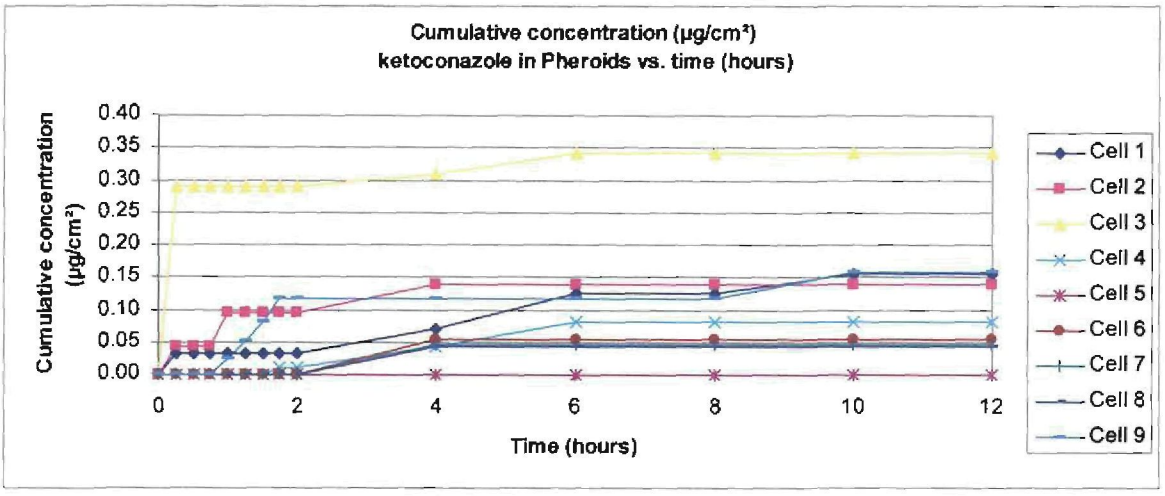
Cell number	Cumulative concentration (µg/ml)	% yield	Flux (µg/cm^2/h)
1	0.327	0.00164	0.026
2	0.449	0.00225	0.026
3	0.848	0.00425	0.041
4	0.415	0.00208	0.028
5	0.258	0.00129	0.024
6	0.151	0.00076	0.012
7	0.075	0.00038	0.008
8	0.219	0.00109	0.021
	Mean	0.00171643	0.023
	SD	0.00120214	0.010
	% RSD	70.03721239	43.046

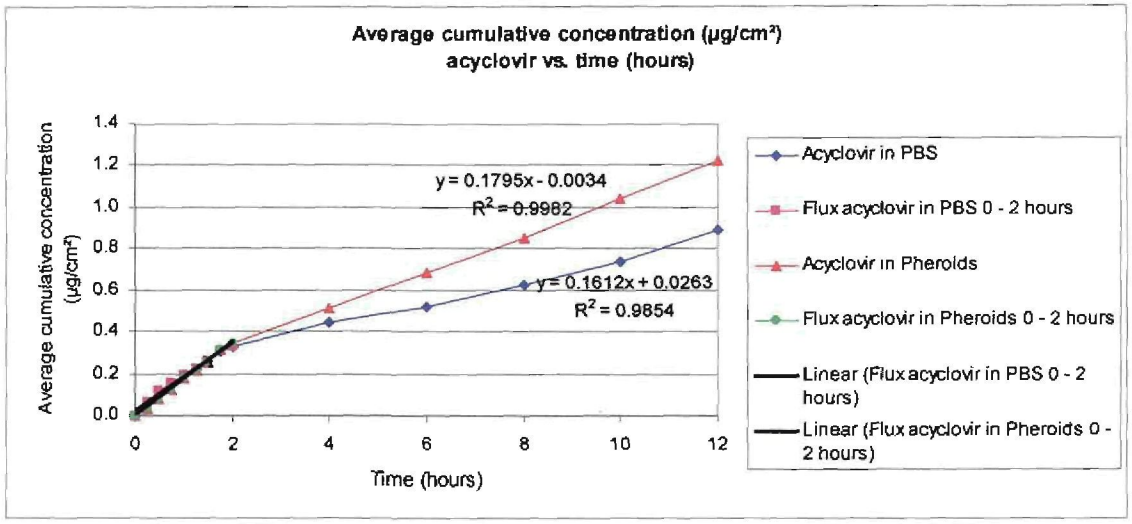


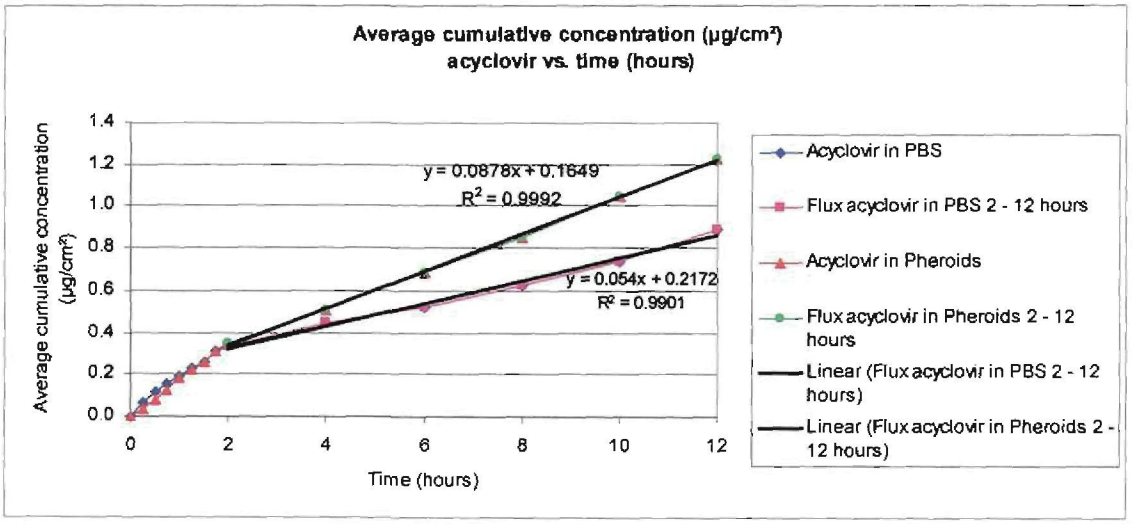
KETOCONAZOLE IN THE PHEROID™ DELIVERY SYSTEM

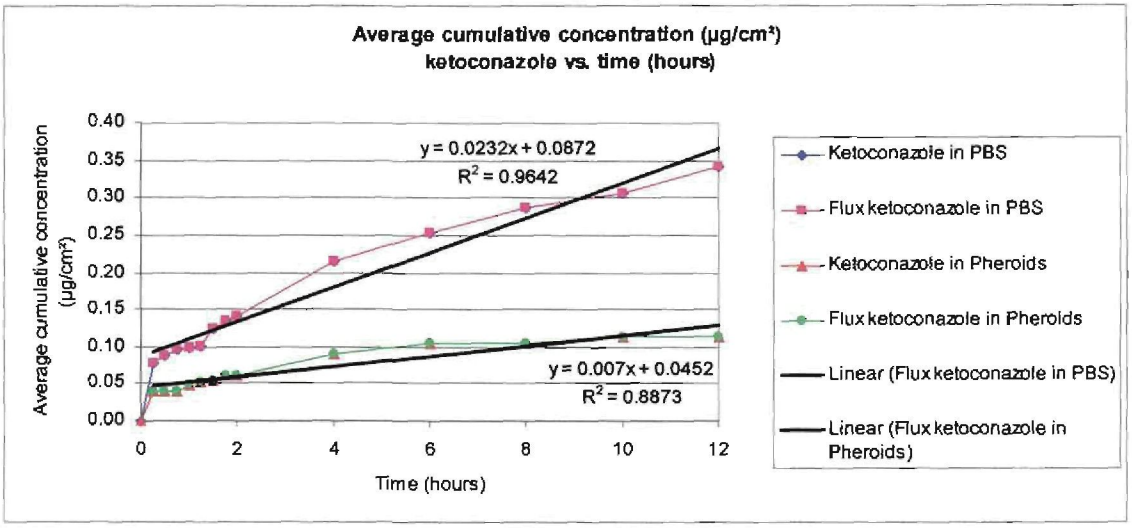
Ketoconazole was incorporated in the Pheroid™ delivery system. The data obtained for ketoconazole is reported here.

Cell number	Cumulative concentration (µg/ml)	% yield	Flux (µg/cm²/h)
1	0.156	0.00078	0.013
2	0.140	0.00070	0.008
3	0.341	0.00171	0.006
4	0.082	0.00041	0.009
5	0.000	0.00000	0.000
6	0.054	0.00027	0.006
7	0.046	0.00023	0.005
8	0.045	0.00022	0.005
9	0.159	0.00080	0.012
	Mean	0.000568601	0.007
	SD	0.000480303	0.004
	% RSD	84.47097777	52.124




Average cumulative concentration (µg/cm²) acyclovir vs. time (hours)
Average cumulative concentration (µg/cm²)
Time (hours)
y = 0.1795x - 0.0034
R² = 0.9982
y = 0.1612x + 0.0263
R² = 0.9854
Acyclovir in PBS
Flux acyclovir in PBS 0 - 2 hours
Acyclovir in Pheroids
Flux acyclovir in Pheroids 0 - 2 hours
Linear (Flux acyclovir in PBS 0 - 2 hours)
Linear (Flux acyclovir in Pheroids 0 - 2 hours)


Average cumulative concentration (µg/cm²) acyclovir vs. time (hours)
Average cumulative concentration (µg/cm²)
Time (hours)
y = 0.0878x + 0.1649
R² = 0.9992
y = 0.054x + 0.2172
R² = 0.9901
Acyclovir in PBS
Flux acyclovir in PBS 2 - 12 hours
Acyclovir in Pheroids
Flux acyclovir in Pheroids 2 - 12 hours
Linear (Flux acyclovir in PBS 2 - 12 hours)
Linear (Flux acyclovir in Pheroids 2 - 12 hours)


Average cumulative concentration (µg/cm²) ketoconazole vs. time (hours)
Average cumulative concentration (µg/cm²)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
2
4
6
8
10
12
Time (hours)
y = 0.0232x + 0.0872
R² = 0.9642
y = 0.007x + 0.0452
R² = 0.8873
Ketoconazole in PBS
Flux ketoconazole in PBS
Ketoconazole in Pheroids
Flux ketoconazole in Pheroids
Linear (Flux ketoconazole in PBS)
Linear (Flux ketoconazole in Pheroids)

APPENDIX 4: VALIDATION OF EXPERIMENTAL METHODS

VALIDATION OF THE HPLC ANALYTICAL METHOD

1 PURPOSE OF THE VALIDATION

The purpose of validation of the HPLC method was to ensure that the analytical method was sensitive and reliable in the determination of the amount of drug that permeated the skin.

2 CHROMATOGRAPHIC CONDITIONS

Analytical instrument: The HPLC analysis of acyclovir and ketoconazole was performed by using an Agilent 1200 Series HPLC, equipped with an Agilent 1200 pump, autosampler, UV detector and Chemstation Rev. A.06.02 data acquisition and analysis software.

Column: A Restek Ultra C18, 150 x 4.6mm, 5µm column was used during the HPLC analysis.

Mobile phase: Initially the mobile phase consisted of a mixture of 0.5 volumes of acetonitrile and 9.5 volumes of a 0.02M ammoniumphosphate buffer solution which changed by linear-gradient elution to a mixture of 8 volumes of acetonitrile and 2 volumes of a 0.02M ammoniumphosphate buffer solution over 9 minutes, followed by the final elution mixture for 5 minutes. Table 1 illustrates the composition of the mobile phase and the timetable which was used during the gradient elution, where A indicates the percentage ammoniumphosphate buffer solution and B indicates the percentage acetonitrile.

Table 1: Timetable for the composition of the mobile phase during gradient elution

Time (minutes)	A (%)	B (%)
2.00	95.0	5.0
4.00	20.0	80.0
9.00	20.0	80.0
9.20	95.0	5.0

The ammoniumphosphate buffer solution was prepared by accurately weighing and dissolving 2.3g ammoniumphosphate monobasic [$(NH_4)H_2PO_4$] in 950ml of HPLC water and adjusting pH to 7.20 ± 0.01 with a 10% ammonium hydroxide solution, diluted to 1000ml, filtered through a 0.45 µm Millipore filter and degassed. However, it was later found that the ammoniumphosphate buffer solution precipitated in the HPLC system during long analysis peroids, which caused the HPLC to stop functioning effectively. A 0.01M ammoniumphoshate buffer solution, rather than a 0.02M solution was then prepared similar to the 0.02M solution. This overcame the abovementioned problem and there was no difference in the data obtained with the 0.01M and 0.02M ammoniumphosphate buffer component of the mobile phase.

Flow rate: The flowrate was set to 1.0 ml/min.

Injection volume: The injection volume was 100 µl.

Detection: The UV-detector was set at 252nm for the first 6 minutes to detect the acyclovir, whereafter it was set at 243nm to detect the ketoconazole.

Retention time: The retention times of acyclovir and ketoconazole was approximately 3.4 - 3.9 minutes and 6.95 – 7.02 minutes respectively.

Runtime: The runtime was set to 14 minutes.

Solvent: The solvent was composed of 20:80 v/v of methanol and phosphate buffer solution (PBS). The PBS was prepared by accurately weighing and dissolving 0.250g potassium dihydrogen orthophosphate (KH_2PO_4), 1.50g disodium hydrogen orthophosphate dihydrate ($Na_2HPO_4.2H_2O$) and 7.333g sodium chloride (NaCl) in 1000ml HPLC water. The pH of the PBS was approximately 7.40 ± 0.05.

3 CALIBRATION CURVE

A calibration curve for acyclovir and ketoconazole was established by using the standard solutions with the following concentrations: 0.125, 0.25, 1.25, 2.5, 12.5, and 25 µg/ml acyclovir and 0.05, 0.1, 0.5, 1.0, 5.0 and 10 µg/ml ketoconazole. The solutions were prepared as follows:

1. Weighed approximately 25mg acyclovir and 10mg of ketoconazole accurately and dissolved in a 100ml volumetric flask. Made up to volume with 50:50 v/v methanol/PBS and dissolved by means of sonification for a few minutes (250 µg/ml acyclovir and 100 µg/ml ketoconazole – mother solution);

2. Diluted 5ml of the mother solution to 10ml with 20:80 v/v methanol/PBS (125 µg/ml acyclovir and 50 µg/ml ketoconazole);

3. Diluted 5ml of the 125 µg/ml acyclovir and 50 µg/ml ketoconazole solution to 25ml with 20:80 v/v methanol/PBS (25 µg/ml acyclovir and 10 µg/ml ketoconazole);

4. Diluted 5ml of the 25 µg/ml acyclovir and 10 µg/ml ketoconazole solution to 10ml with 20:80 v/v methanol/PBS (12.5 µg/ml acyclovir and 5 µg/ml ketoconazole);

5. Diluted 5ml of the 12.5 µg/ml acyclovir and 5 µg/ml ketoconazole solution to 25ml with 20:80 v/v methanol/PBS (2.5 µg/ml acyclovir and 1.0 µg/ml ketoconazole);

6. Diluted 5ml of the 2.5 µg/ml acyclovir and 1.0 µg/ml ketoconazole solution to 10ml with 20:80 v/v methanol/PBS (1.25 µg/ml acyclovir and 0.5 µg/ml ketoconazole);

7. Diluted 5ml of the 1.25 µg/ml acyclovir and 0.5 µg/ml ketoconazole solution to 25 ml with 20:80 v/v methanol/PBS (0.25 µg/ml acyclovir and 0.1 µg/ml ketoconazole);

8. Diluted 5ml of the 0.25 µg/ml acyclovir and 0.1 µg/ml ketoconazole solution to 10ml with 20:80 v/v methanol/PBS (0.125 µg/ml acyclovir and 0.05 µg/ml ketoconazole).

4 VALIDATION PARAMETERS

4.1 LINEARITY

The linearity of an analytical method is described as its ability (within a given range) to obtain results that are directly proportional to the concentration (amount) of analyte in the sample. The linearity of acyclovir and ketoconazole was determined by performing linear regression analysis on the plot of the peak area ratios versus concentration (µg/ml). The standards were prepared as described. Figure 1 and Figure 2 illustrates the linear regression curves obtained for acyclovir and ketoconazole respectively. The regression value (r^2) obtained indicates a high degree of linearity and therefore demonstrates the good stability of the analysis system.

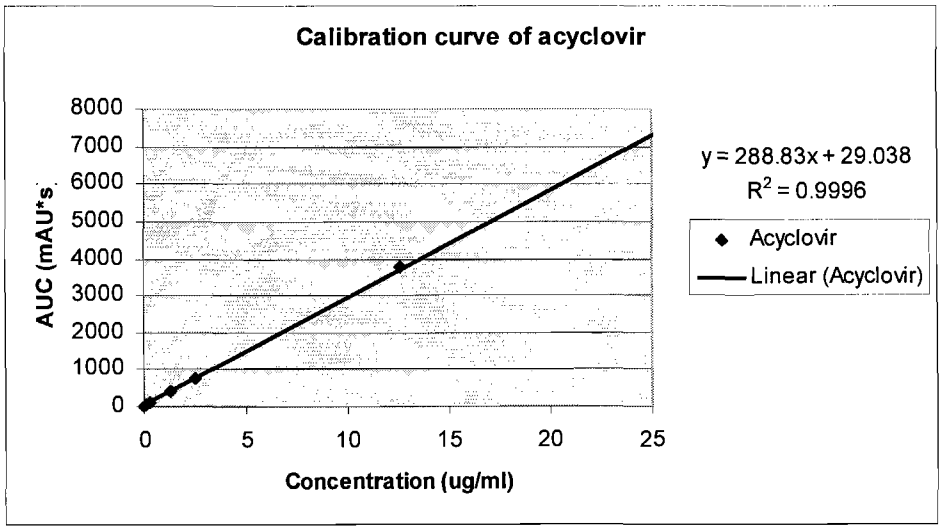


Fig. 1: Linear regression curve of acyclovir standards

Table 2: Peak area ratio values of acyclovir standards

Standard (µg/ml)	Peak area ratio
0.0000	0.0000
0.1262	37.5085
0.2523	80.6000
1.2620	389.2260
2.5230	787.5900
12.6150	3783.0485
25.2300	7258.5470
r^2	0.9996
Intercept	29.0383
Slope	288.8285

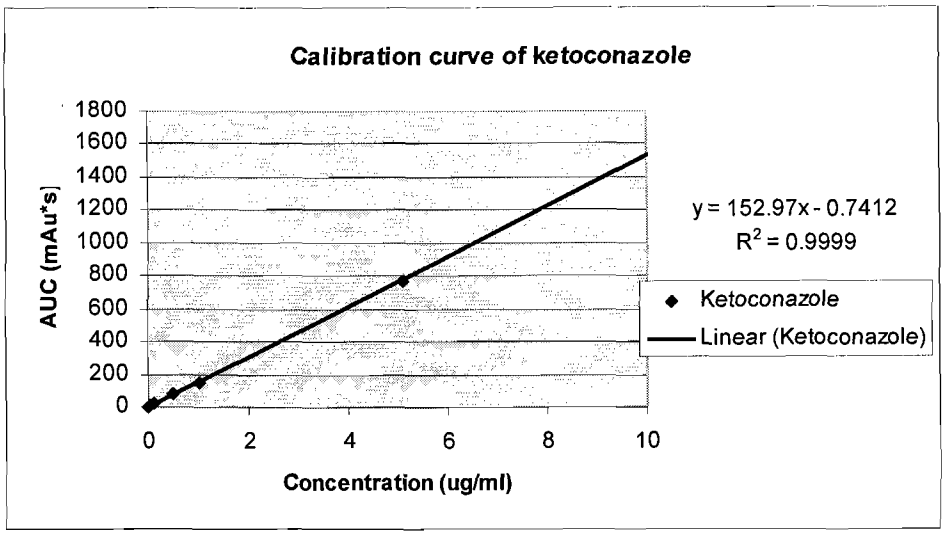


Fig. 2: Linear regression curve of ketoconazole standards

Table 3: Peak area ratio values of ketoconazole standards

Concentration (µg/ml)	Peak area ratio
0.0000	0.0000
0.0509	10.3465
0.1020	18.2730
0.5090	76.4070
1.0180	154.0265
5.0900	766.1120
10.1800	1562.4035
r^2	0.9999
Intercept	-0.7412
Slope	152.9659

4.2 ACCURACY AND PRECISION

The precision of an analytical procedure expresses the proximity of agreement between a series of measurements obtained from multiple sampling of the same homogenous substance under the prescribed conditions. Precision was investigated in terms of intraday (repeatability) variation and inter-day (reproducibility) variation.

4.2.1 Accuracy and Intraday precision

The intraday precision was determined by performing HPLC analysis (n = 3) on three different samples of known concentrations of both drugs (25, 125, 250 µg/ml of acyclovir and 10, 50, 100 µg/ml ketoconazole) three times during the same day. The results can be seen in Table 3 and Table 4, with all the results complying with acceptible pharmaceutical

standards/meeting the requirements of the current USP. The recovery was found to be ranging between 97.81% and 100.29% for acyclovir and 100.16% and 101.19% for ketoconazole.

The percentage recovery is an indication of the accuracy of the system.

Table 4: Accuracy and intraday precision parameters of acyclovir standards

Standards (µg/ml)	% Recovery			Mean	SD	%RSD
	t = 1	t = 2	t = 3			
25	100.29	100.17	99.98	100.15	0.12	0.12
125	97.81	98.11	98.09	98.00	0.14	0.14
250	98.43	98.56	98.91	98.63	0.20	0.21

Table 5: Accuracy and intraday precision parameters of ketoconazole standards

Standards (µg/ml)	% Recovery			Mean	SD	%RSD
	t = 1	t = 2	t = 3			
10	101.09	100.70	100.25	100.68	0.35	0.34
50	100.31	100.28	100.16	100.25	0.06	0.06
100	101.19	101.10	101.17	101.15	0.04	0.04

4.2.2 Inter-day precision

The inter-day precision was determined by performing HPLC analysis (n = 3) on 3 different samples of known concentrations of both drugs (250 µg/ml of acyclovir and 100 µg/ml ketoconazole) on three different days. The results can be seen in Table 5 and Table 6, with all the results complying with acceptible pharmaceutical standards/meeting the requirements of the USP. The recovery was found to be ranging between 98.43% and 102.59% for acyclovir and 94.97% and 104.57% for ketoconazole.

Table 5: Interday precision parameters of acyclovir standards

Concentration (µg/ml)	Day 1	Day 2	Day 3	Between days
250	98.43	100.45	102.72	
250	98.56	100.34	101.55	
250	98.91	102.59	101.32	
Mean	98.63	101.13	101.86	100.54
SD	0.18	0.90	0.53	1.38
%RSD	0.18	0.89	0.52	1.38

Table 6: Interday precision parameters of ketoconazole standards

Concentration (µg/ml)	Day 1	Day 2	Day 3	Between days
100	101.19	104.57	95.58	
100	101.10	103.49	95.17	
100	101.17	103.93	94.97	
Mean	101.15	104.00	95.24	100.13
SD	0.03	0.39	0.22	3.65
%RSD	0.03	0.37	0.23	3.64

4.3 SENSITIVITY

The sensitivity of the analytical method can be assessed by determining the limit of detection, as well as the lowest limit of quantification. The %RSD for both measurements should be <15%.

4.3.1 Limit of detection (LOD)

Limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected, but not necessarily be quantified as an exact value. The LOD for acyclovir and ketoconazole was determined to be 0.05 µg/ml and 0.02 µg/ml respectively.

4.3.2 Limit of quantification (LOQ)

The limit of quantification (LOQ) is defined as the lowest concentration of an analyte in a sample that can be quantitatively determined with acceptible precision and accuracy (%RSD <15%). The LOQ of acyclovir and ketoconazole was determined to be 0.025 µg/ml and 0.01 µg/ml respectively.

4.4 SYSTEM REPEATABILITY

In an attempt to evaluate the repeatability of the peak area and retention time, samples of acyclovir and ketoconazole with a known concentration (25 µg/ml acyclovir and 10 µg/ml ketoconazole) were injected six times. The variations in response (%RSD) of the detection system when six determinations of acyclovir and ketoconazole were made on the same day, and under the same conditions, were as follows:

Table 7: Variations in response (%RSD) of the detection system regarding peak area and retention time for acyclovir

Concentration (µg/ml)	Injection nr	Peak area (mAU*s)	Retention time (min)
25	1	8870.446	3.701
25	2	8858.743	3.686
25	3	8870.537	3.666
25	4	8860.308	3.649
25	5	8850.843	3.648
25	6	8858.715	3.643
	Mean	8861.599	3.666
	SD	6.976	0.021
	%RSD	0.079	0.586

Table 8: Variations in response (%RSD) of the detection system regarding peak area and retention time for ketoconazole

Concentration (µg/ml)	Injection nr	Peak area (mAU*s)	Retention time (min)
10	1	1723.174	7.008
10	2	1723.432	7.005
10	3	1714.448	7.008
10	4	1709.235	7.003
10	5	1705.127	7.004
10	6	1705.234	7.001
	Mean	1713.442	7.005
	SD	7.635	0.003
	%RSD	0.446	0.036

4.5 SELECTIVITY

Selectivity is the ability of the analytical method to selectively detect the analyte in the presence of other compounds that may interfere with analyte detection. The method is selective when no interfering peaks with the same retention time as the drug are detected. A blank injection, a sample of the 20:80 v/v of methanol and PBS was analyzed and illustrated no interference.

Figure 3, Figure 4 and Figure 5 depict HPLC chromatograms emphasizing the above facts. Number 1 and Number 2 in Figure 3 illustrate the peaks of acyclovir and ketoconazole with retention times of 3.701 and 7.008 minutes respectively. Figure 4 illustrates the peaks obtained with a blank injection. Figure 5 illustrates the peaks obtained when a sample of 20:80 v/v of methanol and PBS was analyzed. Neither of the peaks obtained with the blank

injection or 20:80 v/v of methanol and PBS demonstrated interference, since their retention times differed from that of acyclovir and ketoconazole.

Methanol can be replaced by ethanol as solvent in the preparation of standards and test samples. The ethanol is a more appropriate option for transdermal diffusion studies, since methanol can act as penetration enhancer.

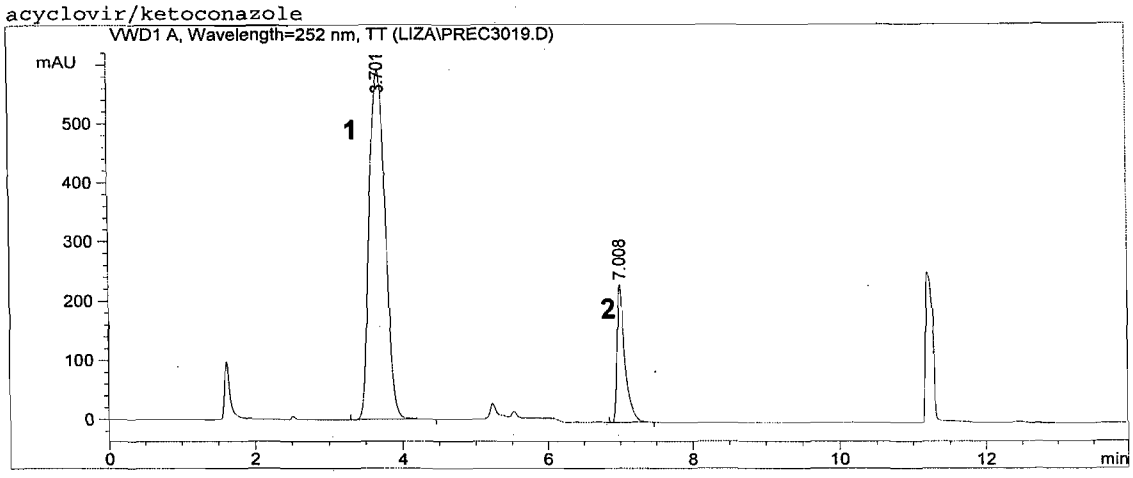


Fig. 3: HPLC chromatogram illustrating the retention times of acyclovir and ketoconazole

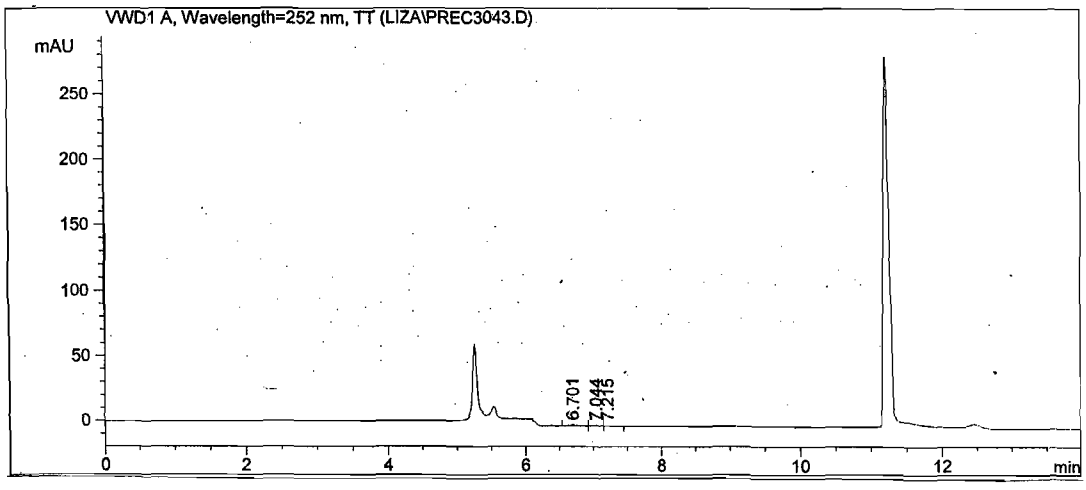


Fig. 4: HPLC chromatogram illustrating the peaks obtained with a blank injection

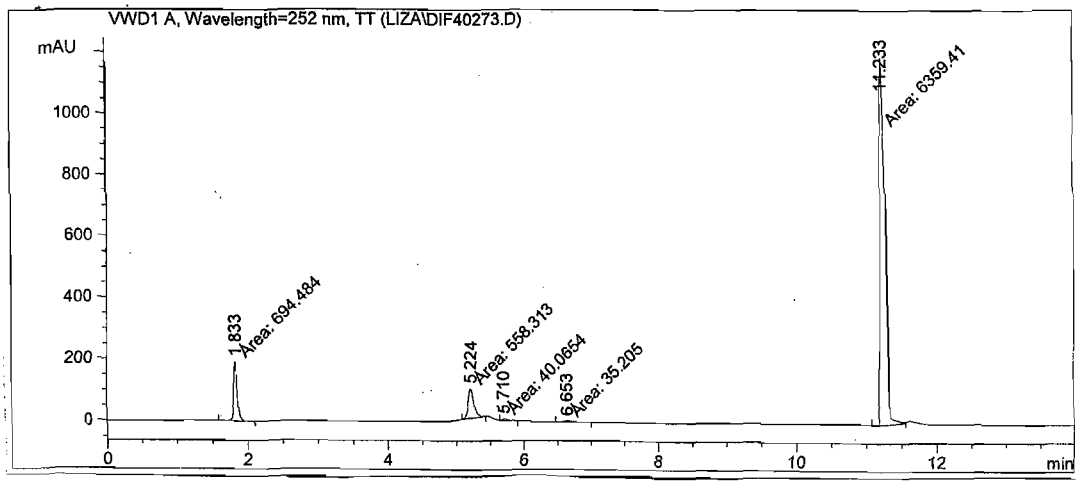


Fig. 5: HPLC chromatogram illustrating the peaks obtained with 20:80 v/v of methanol and PBS

APPENDIX 5: PHOTOS OF APPARATUS USED DURING DIFFUSION STUDIES AND HPLC ANALYSIS

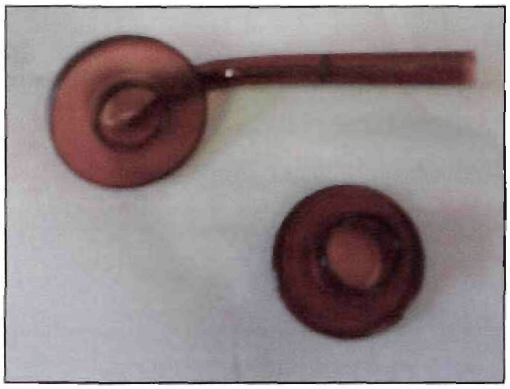


Fig. 1: Donor and receptor compartment of Franz diffusion cell

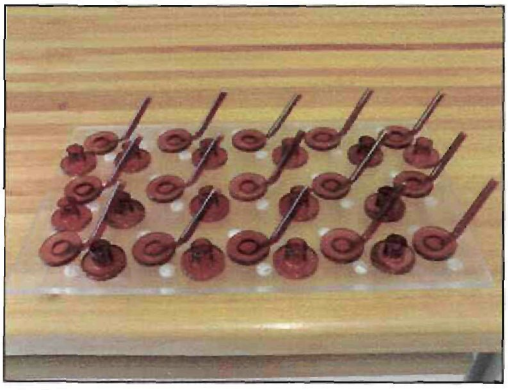


Fig. 2: Franz diffusion cells in stand

Fig. 3: Franz diffusion cell in metal clamp

Fig. 4: Grant water bath

Fig. 5: Variomag® magnetic stirring plate

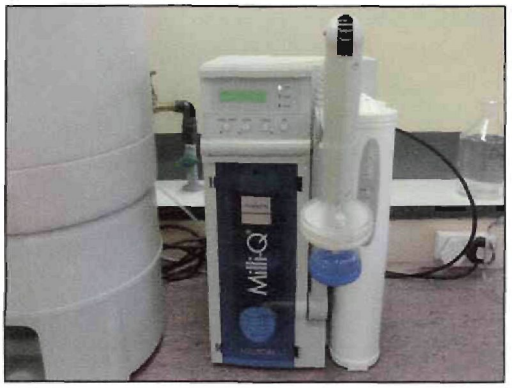


Fig. 6: Milli-Q water purifying system

Fig. 7: Agilent Series 1200 HPLC

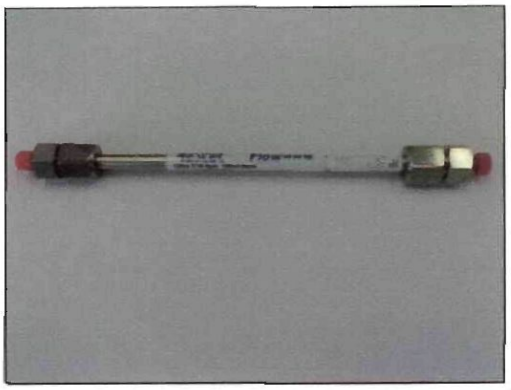


Fig. 8: Restek Ultra C18 HPLC column

Fig. 9: Diffusion study samples in HPLC vials