THE PREFORMULATION AND FORMULATION DEVELOPMENT FOR TRANSUNGUAL DELIVERY OF ANTIFUNGAL DRUG CICLOPIROX OLAMINE

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ABSTRACT

Onychomycosis also known as dermatophytic onychomycosis is the fungal infection of the toenails and fingernails, characterized by discoloration and thickening of the nail and involves the nail plate, nail bed and nail folds. The disease is more than a cosmetic problem, as it severely impacts the patient's quality of life. Onychomycosis is an opportunistic infection in special subpopulations of patients suffering from diabetes, psoriasis, HIV/AIDS etc. The current treatment strategies involve systemic delivery of oral antifungal agents including azoles (e.g. itraconazole) and allylamines (e.g. terbinafine hydrochloride) which are delivered to the nail plate from the nail bed. More recently, topical delivery of drugs including amorolfine and bifonazole/urea (available outside the United States) and Penlac® nail lacquer (ciclopirox) topical solution, 8%, available in the US are an alternative treatment option to the oral antifungal agents. Topical delivery of antifungal agents through the human nail offer several advantages over oral therapy including lower incidence of adverse events and lower potential for drug-drug interaction with drugs used to treat diabetes, HIV/AIDS and psoriasis.

The objectives of this project were to: 1) To determine the critical factors affecting the delivery of ciclopirox olamine across the human nail, 2) To screen and select penetration enhancer(s) specific for ciclopirox olamine delivery into the target tissue(s) and 3) To develop a novel transungual formulation containing ciclopirox olamine (CPO) and penetration enhancer(s) for transungual delivery. Ciclopirox olamine, the salt form of the free acid of ciclopirox was used in the study to develop a novel transungual patch formulation and skin and nail permeation from the patch formulation was compared to Penlac® nail lacquer.

Various factors such as drug partitioning into the healthy and infected toenail, drug-keratin binding, lateral diffusion, drug-epidermal binding and the formulation components, all play a role in achieving optimum drug penetration and permeation through the nail. Understanding the interplay of these factors helped in the development of an effective topical formulation which was observed to be superior to Penlac® nail lacquer in the *in vitro* studies. Most cases of onychomycosis show infection and inflammation of the nail folds (skin surrounding the nails). Therefore for an efficient treatment of OM, the antifungal drugs must be delivered to two target tissues – human nail and the nail folds. The major challenges in developing a topical formulation for treatment on OM are:

a) Achieving antifungal drug minimum inhibitory concentration (MIC) in the epidermis of the nail folds.
b) Enhancing penetration and permeation of the antifungal drug across the human nail to reach the nail bed and achieve the necessary MIC (tissue underneath the nail).

Twelve chemical penetration enhancers (PEs) were screened for their ability to enhance ciclopirox olamine accumulation into the nail folds and permeation through the nail. Propylene glycol (PG) enhanced the levels of the drug in the epidermis of the skin while limiting its permeation across the skin. Thiourea (TU) was selected as the best enhancer to increase ciclopirox olamine penetration into the nail. The diffusion of the antifungal drug across the human nail was studied *in vitro* using human cadaver toenails mounted in Franz diffusion cells. Pressure sensitive adhesives (PSA) belonging to the polyisobutylene, polysiloxane and polyacrylate classes of adhesives were screened to

develop a monolithic drug-in-adhesive-type nail patch. The *in vitro* release of CPO from the PSA patches were limited and did not improve in presence of hydrophilic plasticizer (propylene glycol) and hydrophobic plasticizers (triacetin and triethyl citrate). Increasing the concentration of TU from 1 % to 10 %, lead to its crystallization in the dry patches. Therefore a change in the patch design was recommended. Other hydrophilic polymers including Polyoxyethylene (POLYOX®) and hydroxyl propyl methyl cellulose (HPMC) were also screened to develop a modified drug-in-hydrophilic matrix patch design. The patch was designed to incorporate CPO, PG and TU in the polymer matrix overlaid on a non-occlusive backing membrane cast with polyacrylate PSA. The HPMC films showed the best drug release profile with 80 % release in 2 to 4 hours using a USP apparatus 5. These patches were characterized for drug penetration into the skin and nail permeation. Penlac® nail lacquer was used as the comparator control product. The prototype HPMC K15M patch containing 10 %w/w each of the drug and TU and 150 % w/w of PG showed 2.8 fold increase in CPO accumulation in epidermis compared to Penlac® nail lacquer in 24 hours. The skin permeation was found to be similar to that of Penlac®. The HPMC K15M patch formulation showed 2.7 fold increase in CPO concentration within the nail and 4.2 fold increase in transungual flux compared to Penlac®. The patch delivered higher levels of ciclopirox olamine into the target tissues with a lower permeation lag-time. The novel nail patch delivery system had the following properties: a) Ease of application, b) Contact with the nail surface, c) Increased concentration of drug in dissolved form within the patch, d) Presence of enhancers. The novel nail patch formulation has shown increased efficiency in topical and transungual drug delivery for treatment of OM, when compared to the commercial formulation, Penlac® nail lacquer in

the *in vitro* studies. The physical characterization of the patch using Scanning Electron Microscopy, Polarized Light Microscopy, Optical Light Microscopy, Differential Scanning Calorimetry, X-Ray Diffractometer and Fourier Transform Infrared Spectroscopy show that ciclopirox olamine exists at a sub-saturation level in a non-crystalline form in the patch without any significant drug-polymer interaction. In conclusion, all the objectives of the study were met by successfully selecting penetration enhancers for CPO delivery into the nail folds and across the nail plate, evaluating the interaction between CPO and target tissues, developing a transungual patch system and characterizing the novel transungual patch.

ACKNOWLEDGEMENT

"*The road of life twists and turns and no two directions are ever the same. Yet our lessons come from the journey, not the destination*"

- Don Williams Jr. (American Novelist and Poet)

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This Dissertation is dedicated to

My Loving Parents, Girija and Baburajan Palliyil

My Sister Sneha Palliyil

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INTRODUCTION

1.1 Abbreviations

CPO: Ciclopirox olamine

CP: Ciclopirox

DLSO: Distal and Lateral Subungual Onychomycosis

EO: Endonyx Onychomycosis

OM: Onychomycosis

SO: Proximal Subungual Onychomycosis

TDO: Total Dystrophic Onychomycosis

1.2 Introduction

Over the years, the human nail has become a fashion accessory, and even with a slacking economy, people are willing to spend money for inexpensive ways to make their nails look good. An estimation of the total money spent on nail salon services alone, during the economic recession was $ 5.46 billion, $6.3 billion and $6.06 in the years 2007, 2008 and 2009 respectively (http://files.nailsmag.com/Market-Research/bb2010-11stats-reprints.pdf). This only highlights the current perceived importance of well groomed, healthy nails. A study conducted in 2011-2012, has

shown the growth of the nail care industry (nail salon services in particular) from $ 6.0 billion to $ 6.6 billion from 2009 to 2011 and a projected increase to $ 7.3 billion in 2012 (http://files.nailsmag.com/Market-Research/NAILSstats11-12.pdf).

The booming nail care product market clearly underscores the willingness of people to have beautiful looking nails. This economic trend helps to set a foundation to appreciate one aspect of the disease onychomychosis – its unsightliness.

1.3 Onychomycosis

1.3.1 Definition, prevalence and predisposing factors

OM is the chronic fungal infection of toenails and fingernails, characterized by discoloration, deformation and thickening of the nail and involving the nail plate, the nail bed and the nail folds. The global prevalence of OM is estimated to be 10-30 % (Gupta AK and Simpson FC. 2012). It accounts for up to 50 % of all nail disorders and 30% of cutaneous fungal infections (Elewski, B. 2000 and J.Thomas *et al* 2010). The probability of toe nail infection is up to tenfold higher than finger nail infection (Baran R. and Kaoukhov, A. 2005). The prevalence of OM is increasing because the predisposed population is increasing. The predisposing factors for OM are immunosuppression, genetic tendencies, family history, HIV/AIDS, diabetes, occlusive clothing and footwear, male gender, increased age, psoriasis (Zisova L. *et al* 2011), smoking (Gupta AK *et al*, 2000) and tinea pedis (Gupta AK *et al* 2004, Baran R. and Kaoukhov A. 2005).

1.3.2 Causative organisms

Ninety percent of the toe nail infections are caused by dermatophytes of the *Trichophyton*, *Epidermophyton* and *Microsporum* spp. Among the dermatophytes, *Trichophyton rubrum and Trichophyton mentagrophytes* are responsible for 90 % of the cases of OM (Schlefman, B.S. 1999). The non-dermatophytic molds (NDMs) responsible for 2-11 % of OM cases are *Scytalidium*, *Acremonium*, *Fusarium* spp. and *Aspergillus* spp. (Thomas *et al*, 2010). Fingernails are infected more frequently by yeasts like *Candida albicans*. *Candida albicans* is responsible for 2-10 % of OM (J. Thomas *et al* 2010).

1.3.3 Types of Onychomycosis

OM can be classified into five subtypes, as shown in table 1.1 and figure 1.1 (Baran R *et al* 1998 and Schlefman BS 1999).

1) Distal and Lateral Subungual Onychomycosis (DLSO): The fungi penetrate the stratum corneum of either the hyponychium or the lateral nail folds. About 90 % of OM cases belong to this category.
2) Proximal Subungual Onychomycosis (PSO): The infection starts at the proximal end of the nail and leads to hyperkeratosis and onycholysis (separation of nail plate from nail bed). This type of OM is least common accounting for only 1% of all OM cases; however, it is more common in HIV/AIDS patients.

3) Superficial Onychomycosis (SO): In SO, the fungi directly infect the dorsal layer of the nail plate with the lesions dispersed over the nail. If left untreated, these lesions can encompass the entire nail plate. In SO, superficial white onychomychosis (SWO) accounts for 10% of all OM cases.

4) Endonyx Onychomycosis (EO): In EO, the entire nail plate is infected specifically by *T. soudanense* with intact hyponychium and nail bed (Tosti, A. *et al* 1999). There are no signs of hyperkeratosis of nail bed and onycholysis. The nail plate thickness also remains unaffected.

5) Total dystrophic Onychomycosis (TDO): The primary TSO is a result of chronic mucocutaneous candidiasis involving all the components of the nail apparatus (Baran, R. *et al* 1998). The secondary TSO is the end point of all the above four types of OM.

Table 1.1: Causative organisms and symptoms in Onychomycosis (Baran R et al 1998 and Schlefman BS 1999)

Type	Causative organism	Symptoms
Distal Lateral Subungual OM (DLSO)	*Trichophyton rubrum, Scytalidium dimidiatum, Candida spp.*	Yellow brown discoloration, Subungual hyperkeratosis without thickening of the nail plate, Onycholysis, Paronychia, Splintering
Proximal Subungual OM (PSO)	*T.rubrum, Candida species Fusarium and Scopulariopsis brevicaulis*	Onycholysis along lateral edge of nail plate, Paronychia, discoloration of nail
Superficial OM • *Black OM due to non-dermatophytes* • *White OM due to dermatophytes*	*Acremonium* sp., *Aspergillus* sp. *Fusarium* spp. *T. mentagrophytes and T.rubrum*	Opaque,well demarcated islands on nail plate, crumbly appearance on the nail plate.
Endonyx OM	*T. soudanense* *T. violaceum*	Nail splitting with infection of both surface and deeper layers of nail plate. No nail bed hyperkeratosis or onycholysis
Total dystrophic OM • Primary total dystrophic OM • Secondary dystrophic OM	*Candida species*	Swelling and erythema of proximal and lateral nail folds and secondary involvement of the nail plate.

Figure 1.1: Types of Onychomycosis (Baran R et al 1998 and Schlefman BS 1999).

1.3.4 Impact of Onychomycosis

The symptoms and the images shown in table 1.1 and figure 1.1 clearly show that the greatest impact of the disease is visual. However, OM is not just a cosmetic problem as perceived in general. OM often causes complications for people belonging to the special patient population (children, elderly, immunocompromised, patients with psoriasis, diabetics). The treatment of OM with systemic agents becomes a challenge

in these special patient populations because of the high risk of adverse drug reactions and drug-drug interactions. The disease is more wide-spread in the immunocompromised patients, requiring systemic therapy in most cases (Gupta, A. *et al* 2004).

The prevalence of OM in children (8 years of age) and the elderly (60-79 years of age) are 0.16 % and 18.2 % respectively (Gupta, A.K. et al 2004).

OM is seen in HIV/AIDS patients when their CD4 count falls to 450 (normal CD4 count is 1200-1400) (Gregory, N.1996). Proximal Subungual Onychomycosis (PSO) is clinically presented in association with HIV/AIDS (Rongioletti, F. *et al* 1994 and Aly, R. and Berger, T. 1996). Use of systemic antifungal agents in treatment of HIV/AIDS patients must be performed with caution because of the lower WBC counts, the risk of drug-drug interactions, and the effect on liver function (Gupta, A.K. *et al* 2004). In immune compromised patients with low neutrophil counts, invasion to adjacent tissue and consequent systemic infection by *Fusarium* spp from infected nails has been reported (Arrese, J.E. *et al* 1996, Nucci, M. and Anaissie, E. 2002 and Piérard, G.E. *et al* 2007).

The prevalence of OM in diabetic patients is one in every three diabetic patients (Winston, J.A. and Miller, J.L. 2006). Neuropathy, impaired blood circulation, retinopathy, renal disease, obesity and cardiovascular disease are some of the complications associated with diabetes mellitus (Mayser, P. *et al* 2009). Diabetics who are obese or who have retinopathy find it difficult to examine their toenails for any of the symptoms of OM. One of the leading causes of non-traumatic limb

amputations in the United States is diabetes. Most of these amputations are as a result of diabetic foot ulcers. In the presence of OM in diabetics, there is increased possibility of gangrene and foot ulcers by three folds compared to diabetics without OM (Mayser, P. *et al* 2009). OM is one of the leading causes of terminal limb amputations in diabetics and patients with peripheral neuropathy. The blood circulation to the lower extremities is impaired in these patients. The fungal infection thickens and deforms the toenails, making them sharp and pointed (Gupta A. *et al* 2004). These nails can injure the adjacent skin and if left untreated, these cuts become the port of entry for bacteria. The secondary bacterial infections can be minor or may advance to serious conditions like paronychia, cellulitis and even limb amputation (Gupta A. *et al* 2004). OM can also spread to the adjacent skin and cause tinea pedis, which further exacerbates the condition by forming cuts in the skin between the digits and paving way for secondary bacterial infection (Winston, J.A. and Miller, J.L. 2006).

Nail deformation most often accompanies psoriasis of skin (Kacar, N. *et al* 2006).The symptoms of psoriatic nail are very similar to that of onychomychosis. The dystrophy in the psoriatic nail predisposes it to OM.

Additionally OM shows severe physical, psychosocial and financial implications, thereby reducing the quality of life of the patients (Scher, R.K. 1994, Elewski, B. 1997, Drake, L.A. *et al* 1998, Elewski, B. 2000 and Turner, R.R. and Testa, M.A., 2000). The nail plays important functions: a) protect tips of toes and fingers, b) improve sensitivity, c) increase sense of touch, d) mechanical (help in picking fine objects) (Scher, R.K. 1994). Infected nails are associated with inflammation,

discomfort and pain, which lead to loss of some of these functions. If the toenails are involved, patients experience difficulty in walking and standing. All these may negatively impact the ability of the person to perform physical work and to socialize (Elewski, B. 2000).

OM has a significant impact on the quality of life, as patients suffer from embarrassment, reduced self-esteem, reduced social interaction and concern about the appearance of their nail (Scher, R.K. 1994 and Drake, L.A. *et al* 1998). Figure 1.2 shows a plot developed from data reported in Drake, L.A. *et al* 1998 to substantiate some of the implications of OM. Though the data is obtained from the small sample pool, it highlights the problems associated with OM which was initially recognized merely as a cosmetic problem.

The financial implication of OM has been calculated as the cost per complete cure (Warshaw, E., 2006). A software program called Toenail Onychomycosis Economic (TOE) Model was used to determine the cost per complete cure, taking into consideration the cost of actual medication, duration of treatment, the dose and regimen of therapy, complete cure rate, frequency and severity of adverse drug reactions and miscellaneous costs (blood tests, initial and follow-up visit costs, nail debridements etc). Figure 1.3 shows the compilation of data from the calculated amounts (TOE generated) of money reported for the antifungal agents (Warshaw, E., 2006).

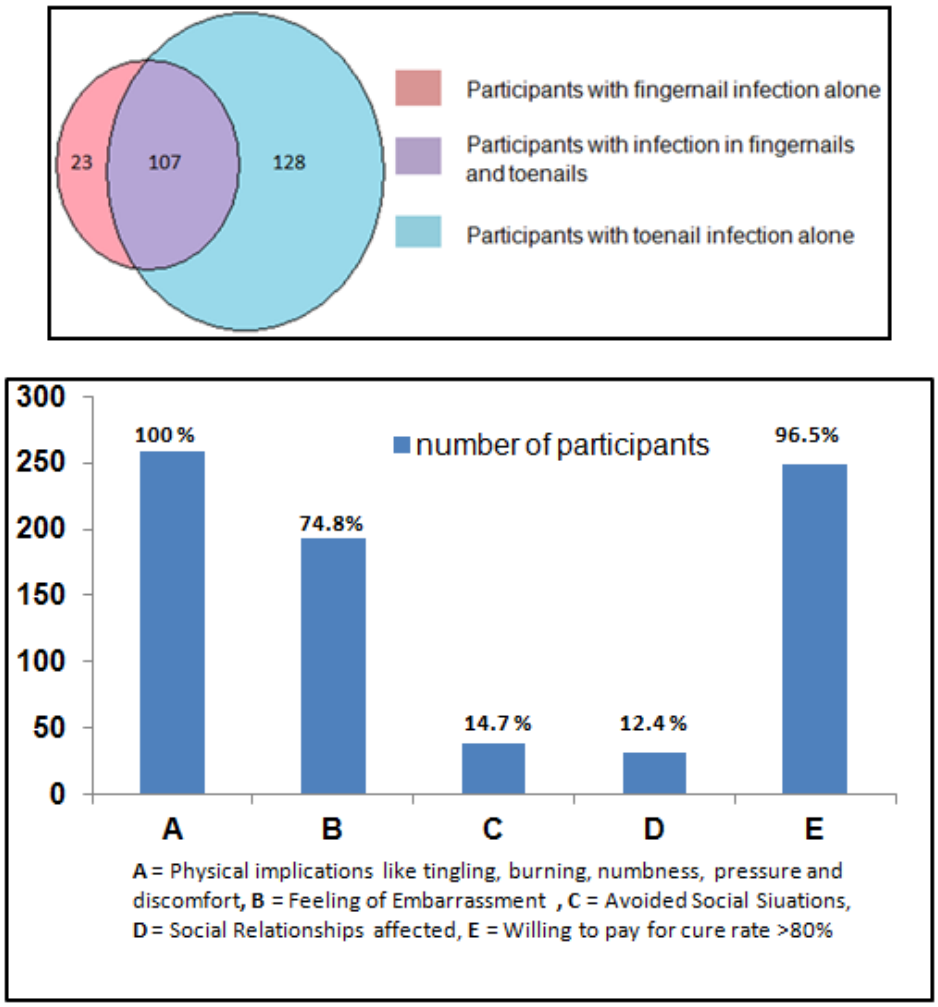


Figure 1.2: Total number of participants in the survey with fingernail and toenail oychomycosis and number of participants suffering from physical and psychosocial impact of onycomycosis

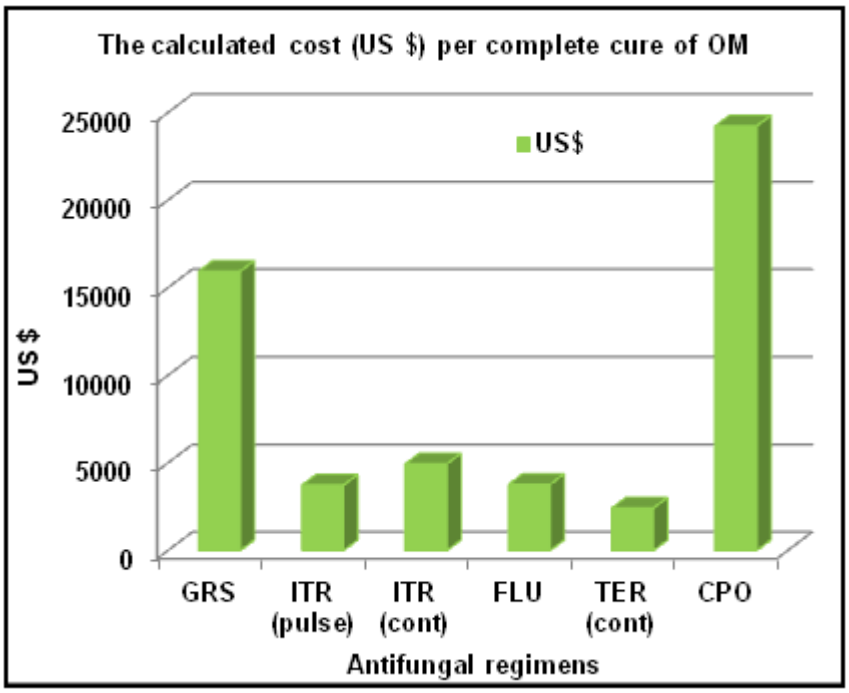


Figure 1.3: Cost per complete cure of onychomycosis calculated using the Toenail Onychomycosis Economic (TOE) Model

In the figure 1.3, the abbreviations are griseofulvin 500 mg/day for 52 wk (GRS), itraconazole pulse therapy with 200 mg twice daily for 1 week per month for 4 months (ITR pulse), itraconazole continuous therapy with 200 mg daily for 12 wk (ITR cont), fluconazole therapy with 450 mg once weekly for 9 months (FLU), continuous terbinafine regimen with 250 mg/day for 12 wk (TER cont) and 8% ciclopirox nail lacquer applied daily for 48 wk with nail debridement (CPO). The study concluded that the commercial cost of medication for the duration of therapy was lowest for CPO ($126) compared to ITR pulse ($3810), ITR cont ($6074), TER cont ($ 2001), FLU ($979) and GRS ($12376) (Warshaw,E., 2006). However,

considering the long duration of therapy and lower cure rates, the total cost per complete cure using the nail lacquer formulation was the highest. These numbers emphasize the financial impact of OM.

1.3.5 Diagnosis and Cure rate of Onychomycosis

OM is diagnosed by various methods like clinical observation, dermatophyte test medium (DTM) culture (Scherer, W.P. and Kinmon K. 2000), fluorescent potassium hydroxide (KOH) preparation, toenail biopsy, periodic acid-Schiff reaction, mycological laboratory analysis on fungal culture (Scherer, W.P. and Kinmon K. 2000), immunohistochemistry, flow cytometry, histopathologic examination and light microscopy (Scherer, W.P and Scherer, M.D. 2004).

It is important to consider both clinical and mycological criteria to ensure proper diagnosis of the disease (Scher, R.K. *et al* 2007).

- Primary clinical criteria: White/yellow or orange/brown patches or streaks
- Secondary clinical criteria: It includes some of the nail changes which are not specific to OM. Onycholysis, subungual hyperkeratosis and nail-plate thickening can also be the result of trauma or psoriasis.

It is therefore important to consider the mycological evidence to ascertain the presence of OM.

- Positive microscopic evidence of septae/hyphae and/or arthroconidia (Scher et al. 2007)

- Positive culture of Dermatophytes like *Trichophyton*, *Epidermophyton* or *Microsporum* species.

The clinical symptoms of OM overlap with other nail disorders and are not specific to OM, e.g. onycholysis (separation of nail plate from nail bed) and hyperkeratosis are also seen due to trauma or psoriasis. It is therefore important to have both clinical and mycological data to establish OM in the nails.

The criteria for cure of OM are (Scher, R.K. *et al* 2007):

- 100% absence of clinical signs of OM (mycology not required)

  OR

- Distal subungual hyperkeratosis or onycholysis with less than 10% nail affected, with negative mycological laboratory results
- Nail-plate thickening which does not improve with treatment because of a pre-existing condition, with negative mycological laboratory results.

After treatment it is very difficult to attain a normal looking nail. Due to the chronic nature of this disease; a cured nail will still show some signs of onycholysis (separation of nail from nail bed) or onychoschizia (nail splitting at distal end). The patients expect normal looking nails at the end of treatment of OM. However, this clinical outcome is unachievable in most cases, because the chronic nature of the disease causes some permanent change in the appearance of the nail. 'Mycological cure' implies complete treatment of fungal infection but not 100% normal nail.

1.3.6 Challenges in treatment of OM

The following are the challenges currently faced in effective treatment of OM:

1) Frequent relapse or reinfection: Recurrence of OM ranges from 10-53% (Scher R K *et al* 2007; Sigurgeirsson, B. *et al* 2002; De Doncker, P.D. *et al* 1997 and Villars, V.V. and Jones, T.C. 1992). Recurrence entails a case where the nail apparatus is devoid of the clinical symptoms but mycological cure was not attained or when a new infection develops during or after termination of treatment. This often occurs
2) Slower growth rate of nails: The healthy fingernails and toenails grow at the rate of 3.47 mm/month and 1.62 mm/month respectively (Yaemsiri, S. *et al* 2010). This means that the fingernail requires 4 to 6 months to completely grow out and for a toenail this period is 12-18 months (Geyer, A.S. *et al* 2004). The nails that are severely infected with OM show a relatively slower growth compared to healthy nails. The growth rate also diminishes by 0.5 % per year after 25 yr of age (Geyer, A.S. *et al* 2004).
3) Increasing population of predisposed patient population
4) Lack of antifungal drugs designed specifically for OM which have increased spectrum of action, decreased drug-drug interactions, decreased relapse and reinfection.
5) Low patient compliance due to longer duration of therapy (Gupta, A.K. and Simpson, F.C. 2012)

1.4 Tissues affected by Onychomycosis

1.4.1 Human Nail

The nail is a highly differentiated appendage of the skin serving protective functions (Cashman, M.W. and Sloan, S.B. 2010 and Stenn K. and Fleckman, P., 2000). Apart from their protective functions, the nails also play a role in amplifying the sense of touch in the fingertips (Conejo-Mir, J. and Requena, L. 2007).The nail apparatus consists of the nail plate, the nail bed, nail folds, the nail matrix, and the hyponychium, as shown in figure 1.4 (Murdan S. 2008). The human nail is composed on 80-90 layers of dead, highly keratinized flattened tightly bound cells which lack cell organelles and nucleus (Murdan S. 2008 and Conejo-Mir, J. and Requena, L. 2007) (figure 1.5). The nail plate is transculent, but appears pink is color due to the rich vasculature in the nail bed underneath (Cashman, M.W. and Sloan, S.B., 2010). *The nail matrix* is thick, specialized germinative epithelial structure responsible for generation of bulk of the cornified cells of the nail plate (Picardo, M. *et al* 1994). The process of keratinization is different in the nail matrix relative to that in the epidermis because; the onychocytes in the nail matrix undergo complete keratinization without formation of keratohyalin granules (Picardo, M *et al* 1994). *The proximal nail fold* covers and protects the nail matrix. The newly formed nail emerges out of the proximal nail fold and is supported by the nail bed. *The nail bed* extends from the lunula to the hyponychium (Figure 1.4). About 20% of the thickness and the mass of the ventral layer of the nail plate is produced by the nail bed (Cashman, M.W. and Sloan, S.B. 2010). The nail bed is viable epidermis (Kobayashi et al, 1999). The interdigitations on the ventral side of the nail plate hold the nail plate firmly in place

with the nail bed (Rice, R.H. *et al* 2010). *The lateral nail folds* help to hold the nail plate in place. *The hyponychium* is epidermis between the distal nail groove and the nail bed (Murdan S. 2002). The nail bed, the nail matrix and the nail folds are well perfused with blood and lymphatic vessels (Murdan S. 2002).

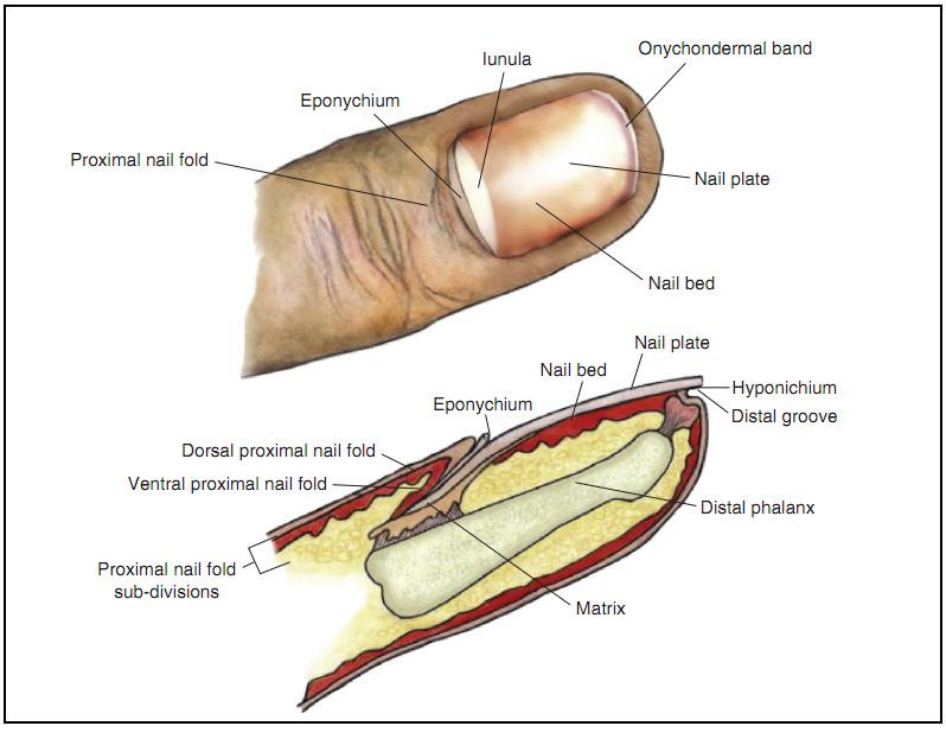


Figure 1.4: Components of the nail apparatus (Murdan S. 2008)

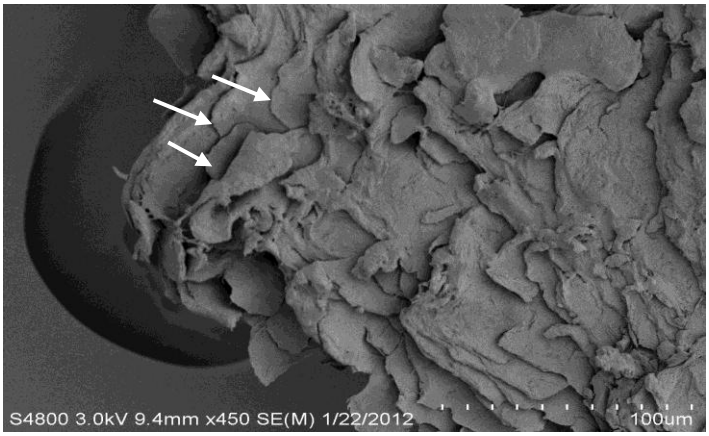


Figure 1.5: Scanning Electron Microscopy image of corneocytes in the human toe nail. The arrows show the corneocytes.

The nail consists of keratin which is a combination of 'hair like' keratins (hard keratin, 80%) and 'skin like' keratins (soft keratins, 20%) (Lynch, M. *et al* 1986). The nail is composed of high sulphur (10% by weight) and high cystine, glycine and tyrosine proteins which form inter-filamentous matrix. The disulphide bonds formed by cystine in the matrix proteins help to hold the keratin fibers together and provide the tensile strength to the nail plate (Cashman, M.W. and Sloan, S.B. 2010). Calcium is present at a low level of 0.2% by weight of the nail plate. The nail plate contains 10-30% of water and only 0.1-1% lipids (Murdan S. 2008). Glycolic acids and stearic acids are the lipids found in the nail plate (Cashman, M.W. and Sloan, S.B. 2010). Lipid bilayers are present only in the dorsal and the ventral layers (Garson et al 2000). The nail behaves like a hydrogel (Walters, K.A. and Flynn, G.L. 1983); this is opposite to the hydrophobic nature of the stratum corneum of the skin. Hence it is difficult to maintain high concentrations of lipophilic drugs in the nail plate

(Kobayashi, Y. *et al* 1999). The nail comprises of a dense network of keratin fibres. Pores are created along the nail keratin matrix by the thermal movement of these keratin fibres. There is extensive pore tortuosity and as the radius of the dissolved molecule gets closer to the pore size, the penetration decreases (Mertin, D. and Lippold, B, 1997).

The human nail plate has three distinct layers with a thickness ratio of 3:5:2 namely the dorsal layer, the intermediate layer and the ventral layer as shown in figure 1.6 (Kobayashi, Y. *et al* 1999; Repka, M.A. *et al* 2002). Each of these three layers possesses different drug permeabilities because of their varied structure. The keratins, their unique orientations in the three layers and the disulphide bonds impart the mechanical strength and rigidity to the nail (Cashman, M.W. and Sloan, S. B. 2010).

- The dorsal layer is hard and brittle and shows overlapping cells. The drugs have lowest diffusivity in the dorsal layer of the nail plate (Kobayashi, Y. *et al* 1999). It consists of the 'skin type' soft keratins which are arranged parallel and perpendicular to the growth axis. (Garson, J. *et al* 2000)
- The intermediate layer is the most hydrophilic layer of the three layers. It is soft, thick and more flexible (Murdan S., 2002). The 'hair like' α-keratin filaments (81 A^o diameter) constitute the entire intermediate layer (Garson, J.C. et al., 2000). These keratins are arranged perpendicular to the growth axis of the nail plate. As the thickness ratio shows, the intermediate layer is the thickest layer of all.
- The ventral layer is only a few layers of cells which are more plastic and help to connect the nail plate to the nail bed. The ventral layer is reported to show high

lipophilicity (Kobayashi, Y. *et al* 1999). The keratins and their orientation are similar to the dorsal layer.

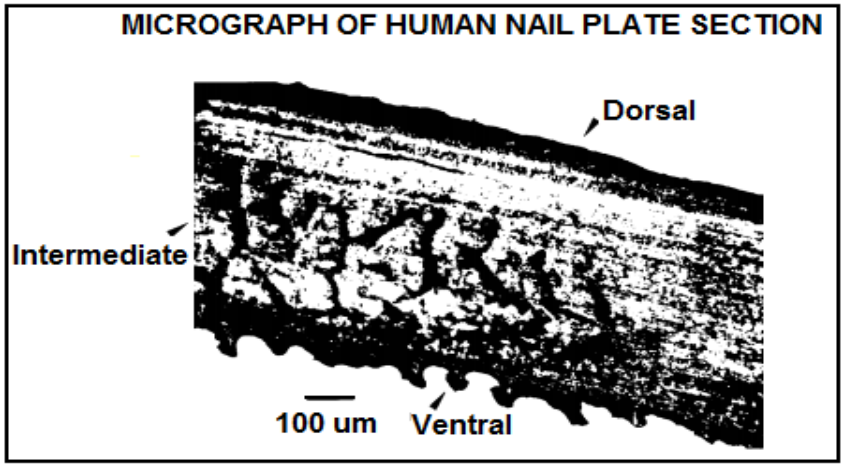


Figure 1.6: Layers of human nail plate (Kobayashi, Y. et al 1999)

Thus the inherent structure and the compact nature of the nail provide the utmost resistance to drug penetration and permeation through the nail.

The fungal infection occurs preferentially in the loose layers of the nail especially when they migrate from the adjacent infected skin into the nail (Richardson, M. and Edward, M. 2000).The nail plate acts as a growth substrate for dermatophytes. The non-dermatophytes show different degrees of growth and invasion of the nail plate, with *Fusarium* being the most invasive.

There are number of studies done using hooves as the substitute of human nail in the *in vitro* studies; but human nail has a denser network of keratin than hoof. A recent study shows that although, the hoof may over predict the transungual permeation of antifungal drugs, but it is a validated model for transungual permeation in infected toenails (Monti, D. *et al* 2011). In the present study whole human cadaver nails (Anatomy Gifts Registry, MD) were used for the *in vitro* studies to mimic the target tissue (human nail).

Visually, the infected nails are discolored and thicker than the healthy nails (figure 1.7). It is reported that the water loss from dystrophic nail (nail affected by psoriasis and OM) is lower than that in healthy nail (Kronauer, C. *et al* 2001). This is speculated to be due to the presence of a granular layer in the dystrophic nail which imparts the water barrier to diseased nails (Kronauer, C. *et al* 2001). There is no extensive study reported in literature showing the marked difference in drug permeabilities in diseased and normal nails (Murdan, S. 2008). Moreover, it is difficult to obtain infected nails showing the same type, stage and causative organism of OM, leading to higher variation in the experimental data. No two infected nails from the same donor will show similar characteristics. Therefore there is high potential for inter and intra donor variability upon using infected cadaver nails in the *in vitro* experiments.

Figure 1.7: Cadaver toenails obtained from Anatomy Gifts Registry: Healthy Cadaver toenails (left panel) and Infected Cadaver toenails (right panel)

Severely infected nails are much thicker than the healthy nails, which can result in low drug permeability and increased lag-time (Murdan, S 2008). The diseased nails have dense focus of fungal hyphae which can alter the drug diffusion through the nail (Murdan S. 2008). Additionally, the severely infected nails can collapse when placed for hydration (Kobayashi, Y. *et al* 2004), are more fragile and show greater porosity which can increase the permeation of drugs (Murdan, S. 2008). It is also reported that the drug permeability in infected nails can be estimated from the drug permeation in healthy nails (Kobayshi, Y. *et al* 2004). Due to the limited availability of diseased nails and lack of literature reports showing significant difference in drug permeation in infected and healthy nails, the initial screening studies were done using nail clippings from healthy volunteers and transungual permeation using healthy human cadaver nails. Infected nails were used for comparative study of drug partitioning into healthy and infected nails. The human nail is a very compact, hard and tough barrier. The limited permeability of drugs through this barrier is the major challenge in developing topical formulations for treatment of OM. Therefore presence of

appropriately selected penetration enhancers in the formulation is critical for efficient drug delivery.

1.4.2 Human Skin

The skin is the largest organ of the human body, accounting for 15% of total body weight (Li, M. and Urmacher, C.D. 2007). The skin is the major barrier to prevent entrance of any foreign material (chemicals, microorganisms) from the external environment and the loss of any endogenous substance (electrolytes) (Brown, M.B. *et al* 2006). It helps to maintain the body temperature and electrolyte balance (Li, M. and Urmacher, C.D. 2007).

The skin comprises of three layers epidermis, the dermis and the subcutaneous fat layer (Figure 1.8). The epidermis consists of the basal layer, the spinous layer, the granular layer and stratum corneum. The cells formed in the basal layer move towards the surface of the skin, and during the migration undergo a series of differentiation processes. The terminal differentiation occurs, as the cells migrate from the granular layer to stratum corneum, and the cells are converted to keratin filled dead cells called corneocytes (Bouwstra, J. and Ponec, M. 2006). Cross-linked proteins and lipids form an envelope around the corneocytes. The major barrier to permeability of molecules across the skin is the stratum corneum. The viable epidermis is an avascular layer. The dermis contains blood capillaries, sebaceous glands, hair follicles, sweat glands and nerves. The epidermis and the dermis are separated by the basement membrane. The heat separation methods described in literature produce quick and clear

separation of epidermis from the dermis at this basement membrane (Frank, J.D. 1995; Kassis, V. and SØndergaard, J. 1982 and Wadskov, S. and SØndergaard, J.1978).

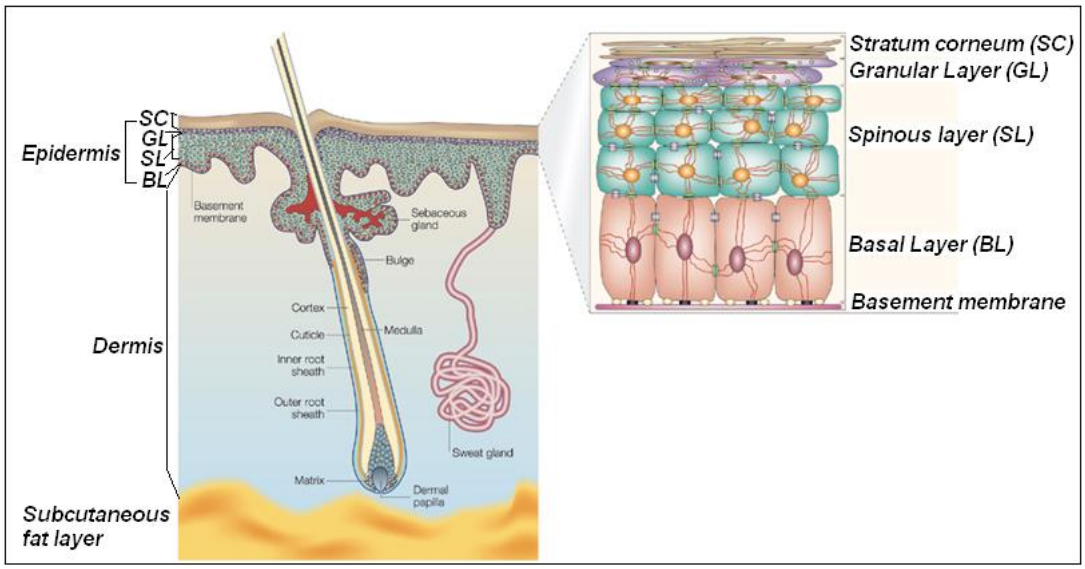


Figure 1.8: Layers of the skin (Fuchs, E. and Raghavan, S. 2002)

There are three routes of drug penetration into and across the skin (Thong, H.Y. *et al* 2007)

1. Appendageal (through the sweat ducts, hair follicles and sebaceous glands)
2. Transcellular (through the corneocytes of stratum corneum)
3. Intercellular (extracellular domain of the stratum corneum)

The nail folds (lateral and proximal) are the skin surrounding the nail on three sides and holding it in place. Hyponychium is an epidermal tissue which provides a tight

seal between the distal end of the nail and the nail bed and provides protection against microorganisms under the distal end of the nail. In case of DLSO, the lateral nail folds and hyponychium are the ports of entry for the causative microorganisms before it infects the nail bed (Table 1.1). The initial infection of the nail matrix occurs through the proximal nail fold in case of PSO (Table 1.1). The nail bed comprises of viable epidermis and upon infection may show hyperkeratosis, leading to onycholysis .In order to tackle the nail fungal infections from all possible routes, the nail folds were also selected as a target tissue for topical drug delivery.

In OM, the nail folds get infected and inflamed (paronychia). The main causative organism, dermatophytes are not part of the natural flora of the skin. These organisms use keratin in the stratum corneum as a nutrient and rarely infect beyond the stratum corneum in the epidermis (Johnson, L. 2003). It is reported that these organisms show hyphae penetrating in longitudinal and perpendicular directions through the stratum corneum.

There are various conditions in skin which favor the growth of dermatophytes (Richardson, M. and Edward, M. 2000):

- The stratum corneum of the skin is distant from the defense mechanism of the body because it is avascular tissue consisting of dead cells.
- The temperature of the skin (32 ºC) and its pH (5.5-6.7) are also on the lower side, compared to that of the body.
- The stratum corneum is exposed to the aerobic conditions of the environment and is always well hydrated.

- The composition of stratum corneum (lipids, proteins, amino acids and carbohydrates) makes it conducive for the growth of dermatophytes.
- The anatomy of certain parts like the hair, the hyponychium of the nail and the interdigital space between the toes makes them trapping devices for the dermatophytes.

The non-dermatophytic molds like *Scopulariopsis* remain confined to the stratum corneum of the skin, while *Fusarium* and *Acremonium* penetrate into the deeper layers of the epidermis and the dermis (Richardson, M. and Edward, M. 2000).

For an optimum treatment of OM, the antifungal drug has to be delivered to the nail plate, nail bed and the nail folds. In case of the topical delivery into the skin, it has to be ascertained that the topically applied drug remains within the upper layers of the skin, especially the stratum corneum of the epidermis and does not permeate into the systemic circulation. The epidermal thickness varies based on the site on the body (Sandby-MØller, J. *et al* 2003). Human cadaver skin (Allosource, OH) is used in the current work to simulate the nail folds and hyponychium in the nail apparatus. Hence the thickness of the skin used in the *in vitro* studies is very different from that of the nail folds. The thickness of the epidermis is measured during the penetration studies, for the calculation of drug concentration in the epidermis. Nevertheless, use of cadaver skin gives a good estimate of the drug concentrations within the epidermis. There are no literature reports of development of a transungual formulation which delivers the drug to both skin and the nail. The major challenge in topical delivery of drugs for fungal infection of the skin is to enhance penetration of the drug into the epidermis while minimizing any drug permeation into the systemic circulation. This is

contradictory to the objective in transdermal drug delivery. In case of topical drug delivery, stratum corneum is still the major barrier to drug penetration into epidermis. Therefore penetration enhancers were screened in the current project to select the enhancer which would minimize the drug levels permeating the skin while enhancing drug penetration into the epidermis.

1.5 Treatment modalities for OM

The treatment modalities for OM can be broadly classified into, systemic, topical and mechanical therapies (Table 1.2). The systemic therapy of OM involves the use of antifungal agents like fluconazole, itraconazole, and terbinafine. In patients with reduced peripheral blood flow, higher doses of these systemic agents are required. Higher doses of these drugs can have serious side effects and drug-drug interactions (especially for patients undergoing treatment for other co-morbid conditions) (Table 1.2). Other factors which impact the systemic therapy are limited success rate, toxicity and higher cost of medication and increase in number of resistant strains of microorganisms (Repka, M.A. *et al* 2002). These systemic antifungal agents do not efficiently penetrate the nail plate from the nail bed and nail matrix (Gupta, A.K. and Simpson, F.P. 2012 and Murdan,S. 2007).

The use of topical agents is limited to mild to moderate cases of infection, in conjunction with systemic therapy or as an alternative to systemic therapy (Gupta, A.K. *et al* 2004). These agents are also used to prevent reinfection or relapse of OM. Topical therapy is the best alternative for children and elderly populations. Compared

to the systemic agents, topical agents have reduced side effects and are cost effective (Gupta, A.K. 2000). However, the period of topical therapy lasts longer than the systemic therapy. The major limitation is the poor penetration of the topical agents through the nail to reach the nail bed. The first topical agent approved in the United States for treatment of OM is ciclopirox 8% topical solution nail lacquer (Penlac®, Sanofi Aventis). In order to treat OM topically, there is a need to develop a more effective topical formulation – a formulation which is easy to apply and remove, and which is capable of delivering the drug into the deeper layers of the nail at concentration above the MIC of the antifungal drug. Moving towards this goal, there are a number of newer treatment approaches under development (Table 1.3). One of the newer approaches is to reformulate the approved drugs to increase the efficiency of drug delivery into the nail folds, the nail bed and nail plate.

Table 1.2: The Current Treatment Regimens for Onychomycosis

Antifungal agents	**Dosage regimen (for toenail OM) (Gupta,A. *et al* 2004)**	**Adverse effects (Baran,R. 2008)**
Itraconazole (Pulse)	200 mg twice daily for 1 wk each month for 3 months	Hepatitis after 4 wk of continuous regimen; frequent liver function tests required during therapy. Contraindicated if patient has ventricular dysfunction. Inhibits CYP450 enzymes and therefore affects the metabolism and clearance of cisapride, simvastatin, cyclosporine, tacrolimus, oral hypoglycemics etc.
Itraconazole (Continuous)	200 mg /day for 12 wk	
Terbinafine	250 mg/day for 12 wk	Hepatitis may occur without prior liver conditions, Abnormal liver enzyme levels. Baseline liver function tests and blood count recommended during therapy, Inhibits CYP2D6 enzyme.
Fluconazole	150 mg once weekly until growth of the infected nail	Complete drug history of patient required prior to use of Fluconazole. Inhibits human CYP3A4 and CYP2C9 enzymes. Contradindications not as severe as for Itraconazole.
Ciclopirox 8 % nail lacquer	Applied once daily to infected nails for 48 wk	Erythema of the surrounding nail folds and irritation of the nail bed which subside on their own
Amorolfine 5 % nail lacquer	Applied once or twice weekly to all infected nails	Chromonychia (bluish or yellowish brown discoloration), onycholysis, contact dermatitis, burning sensation
Debridement	Used in combination of topical and oral therapy	N/A

Table 1.3: The newer Therapeutic options for treatment of OM (information compiled from Gupta, A.K. and Simpson,F.C. 2012)

Chemical Class /Agents	**Mechanism of Action**	**Novel formulations in development**
Allylamines e.g. Terbinafine, Naftifine and Butenafine	Inhibition of squalene epoxidase	• ***Terbinafine Nail Lacquer (TNL)*** : consists of 10% terbinafine and 0.5% docecyl-2-N,N-dimethylaminoproprionate hydrochloride (penetration enhancer). Tested in three clinical trials. TCL was found to be less effective than amorolfine in mycological cure. • ***TDT-067:*** Lipid-vesicle based topical formulation of terbinfine. Clinical trial (51patients) showed mycological cure rate of 90.1% at 12 wk and 38.5 % at 48 wk. • ***NB*-002:** Nanoemulsion based topical formulation of terbinafine. *In vitro* MIC value was much lower than ciclopirox, terbinafine, itraconazole, naftifine, griseofulvin. Currently in Phase II clinical trial.
Azoles e.g. Posaconazole Ravuconazole	Inhibits conversion of lanosterol to ergosterol and inhibits fungal cell growth	• ***Posaconasole*:** Phase II clinical trials (for 4 oral dosage regimens), compared to oral terbinafine completed. 200mg/day for 24 wk gave complete cure rate of 54.1% compared to 37.1% by terbinafine. Same regimen gave mycological cure rate of 70.3 % which is comparable to that of terbinafine • ***Ravuconazole:*** Three oral dose regimens were compared to placebo in clinical trial. Mycological cure rate of 59% and complete cure rate of 46% was reported for 200mg/day of ravuconazole. Currently no clinical trial in the US.

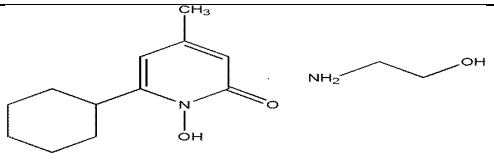
Table 1.3 continued

Chemical Class /Agents	**Mechanism of Action**	**Novel formulations in development**
Benzoxaboroles e.g. Tavaborole (formerly AN-2690) AN-2718	Inhibits protein synthesis by inhibiting fungal LeuRS tRNA synthetase enzyme	• ***Tavaborole (AN-2690):***Topical formulation showed better penetration into the intermediate and ventral layers of nail plate compared to ciclopirox, after 72 hr of application. Currently in two Phase III clinical trials. • ***AN-2718:*** Initial data of this topical compound shows superior results than Tavaborole. Completed Phase I clinical trial
Iontophoresis e.g. Power Paper Iontophoresis device + Terbinafine patch, NitroBio iontophoresis device	Electric current is utilized to increase the topical drug delivery into nail plate	Two devices are under development • ***Power Paper Iontophoresis device + Terbinafine patch :*** 18 patients applied 1% terbinafine patch and 20 patients applied 1% terbinafine patch with 100 $\mu A/cm^2$ of electric current. The mycological cure rate of the device group was 84% compared to 47 % in the patch group. • ***NitroBio Iontophoresis device:*** Terbinafine is the active ingredient. The device can target just the nail plate alone or with the surrounding skin. Phase I trial completed and undergoing a Phase II study in North America
Topical Barrier films e.g. Topical drug-free polymeric films	Thin polymeric film dries the water in the nail plate and nail bed, thereby altering the micro-environment in the nail plate.	After 6 months of application, mycological cure was reported in 63% of patients Currently no clinical trial for topical films

1.6 Ciclopirox olamine (CPO)

Ciclopirox olamine is the ethanolamine salt of 6-cyclohexyl-1-hydroxy-4-methyl-2(1H)-pyridone, where 1% of the salt form is equivalent to 0.77% of the free acid form ciclopirox (Gupta, A. K. 2001). Both the free acid (Ciclopirox) and the salt form (Ciclopirox olamine) have the same antifungal activity, because the antifungal activity is attributed to the hydroxypyridone group in the molecules (Schaller, M. *et al* 2009). It is a broad spectrum antifungal agent, effective against, dermatophytes, non-dermatophytes, yeast, gram-positive and gram negative bacteria. Both ciclopirox and ciclopirox olamine are well established antifungal agents and are available in a variety of topical formulations – cream, vaginal cream, lotion, solution, suspension, gel powder and nail lacquer (Bohn, M. and Kraemer, K. 2000)

1.6.1 Physicochemical Properties

Molecular weight	268.36 g/mol
Molecular formula	$C_{12}H_{17}NO_2.C_2H_7NO$
Chemical name	6-cyclohexyl-1-hydroxy-4-methyl-2-(1H)-pyridone compound with 2- aminoethanol (1:1)
Log P	2.73
Melting Point	143-145 ºC
Description	White to pale yellow crystalline powder. The details of the structure are shown in figure 1.9.
Chemical Structure	

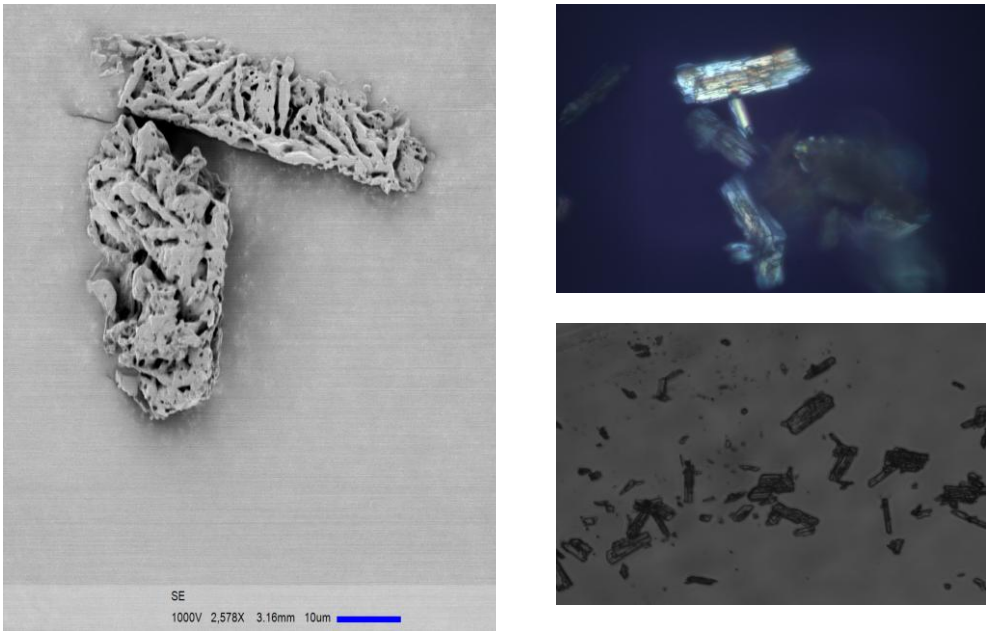


Figure 1.9: SEM image of CPO crystals (left panel), the scale in figure corresponds to 10 µm, polarized light microscope image of CPO (40X) (top right panel) and optical microscope image of CPO crystals (20X) (bottom right panel).

1.6.2 Mechanism of Action

Unlike the other antifungal agents, CPO does not inhibit the sterol synthesis in the fungal cells *(Gupta.A.K; 2001).* CPO targets different metabolic processes in the microbial cell. It has an affinity for the trivalent metal cations like Fe^{3+}. It chelates to these multivalent cations thereby inhibiting the cytochrome in the fungal cell. As a result, the mitochondrial electron transport processes and consequently the microbial metabolism within the microbial cell are impaired (Bohn, M. and Kraemer, K. 2000 and Abrams, B. *et al* 1991). Catalase and peroxidase enzymes which play a key role in degradation of toxic peroxides in the fungal cells are reduced by CPO. It is also

reported that CPO depletes the intracellular essential amino acids and disrupts protein synthesis within the microbial cell (Bohn, M. and Kraemer, K. 2000). Transmission electron microscopy (TEM) of a freeze fractured sample showed modification of plasma membrane of fungi and yeast in presence of CPO (Del Palacio- Hernanz, A. *et al* 1990).These multiple mechanisms unique to CPO, make it very difficult for development of any resistance to CPO, compared to other antifungal agents.

1.6.3 Pharmacodynamic properties

- Antifungal Activity

CPO shows a broad spectrum of antifungal activity against dermatophytes, non-dermatophytes and yeasts. The *in vitro* minimum inhibitory concentration (MIC) values reported in literature differ based on the method used. Table 1.4 gives compiled data for the MIC of CPO from literature (Subissi, A. *et al* 2010).

Table 1.4: The in vitro Minimum Inhibitory Concentrations (MICs) of ciclopirox olamine against different strains of dermatophytes and yeasts (Subissi,A. et al 2010, Kokjohn,K. et al 2003, Gupta,A.K. and Kohli, Y. , 2003, Shehata, A.S. et al 2008 and Singh, J et al 2007)

Microorganisms	**Method**			
	Broth microdilution **CLSI M27-A**	***Broth microdilution*** **CLSI M38-A**	***Broth microdilution*** **CLSI M27-A**	***Broth microdilution*** **CLSI M38-A**
Dermatophytes				
Trichophyton	0.06-0.125 (5)	0.03-0.125 (14)	0.25-1.0 (16)	0.06-0.25 (7)
mentagrophytes	0.125 (5)	0.015-0.125 (68)	0.25-0,5 (16)	0.125-0.5 (15)
Trichophyton rubrum	---	0.06 (1)	---	---
Trichophyton verrucosum	0.3-0.6 (5)	0.03-0.06 (5)	0.25-1.0 (2)	0.06-0.5 (11)
Trichophyton tonsurans	---	---	---	---
Trichophyton quinckeanum	---	0.03 (5)	0.5 (2)	---
Other Trichophyton species	0.004-0.06 (5)	0.03 (1)	0.25-1.0 (7)	---
Microsporum canis	0.06-0.25 (5)	0.03-0.06 (3)	---	---
Microsporum gypseum	---	0.03 (3)	---	---
Other Microsporum species	0.03 (4)	0.015-0.03 (3)	0.25 (2)	0.125-1.0 (13)
Epidermophyton floccosum				

Table 1.4: Contd

Microorganisms	**Method**			
	Broth microdilution **CLSI M27-A**	***Broth microdilution*** **CLSI M38-A**	***Broth microdilution*** **CLSI M27-A**	***Broth microdilution*** **CLSI M38-A**
Yeasts				
Candida albicans	0.06-0.25 (5)	0.03 (3)	---	---
Candida tropicalis	---	0.03 (1)	---	---
Candida krusei	---	0.06 (1)	---	---
Candida parapsilosis	---	0.06 (6)	---	---
Candida pseudotropicalis	---	---	---	---
Candida glabrata	---	0.03-0.06 (3)	---	---
Other Candida species	---	0.03 (3)	---	---
Malassezia furfur	0.001-0.125 (5)	---	---	---
Criptococcus neoformans	---	---	---	---
Saccharomyces cerevisiae	---	---	---	---

Note: The numbers in parenthesis represent the number of strains tested.

The data in table 1.4 shows different values for the MIC depending on the *in vitro* method used. The other *Trichophyton* species studied were *T.equinum, T.concentricum, T.gallinae, T.krajdenii, T.schoenleinii, T.soudanense, T.terrestre, T.violaceum*. *M.audouini, M.cookei, M.nanum, M.langeroni, M.ferrugineum and M.persicolo* were the other *Microsporum* species tested. The other *Candida* species tested for determination of MIC of CPO were C.*brumptii, C.guillermondii, C.lipolytica, C.utilis and C.viswanthi.* The abbreviation CLSI in the table stands for Clinical and Laboratory Standards Institute (formerly the National Committee on Clinical Laboratory Standards). It has been shown that the Minimum Fungicidal Concentration (MFC) value (>256 µg/mL) obtained in the conventional *in vitro* microdilution method is much higher than the nail minimum fungicidal concentration (Nail-MFCs) of 16 - 32 µg/mL obtained using the *in vitro* model of dermatophyte nail infection against seven strains of T. rubrum (Schaller, M. *et al* 2009).This indicates that 16 - 32 µg/mL of CPO was required to kill the seven strains of T. rubrum growing on nail powder. Unlike the other agents (amorolfine and bifonazole) tested, the nail-MFC for CPO was much lower than the MFC obtained from the microdilution technique. This was attributed to possibly different pharmacokinetic properties of CPO in presence of nail and the multiple modes of action of CPO against the microbial cell (Schaller, M. *et al* 2009).

- Antibacterial Activity

Dermatomycoses (including onychomychosis) are further complicated by secondary bacterial infections, especially by gram negative bacteria (Bohn, M. and Kraemer, K. 2000). CPO has shown antibacterial activity with a broader spectrum of action, relative to other antifungal agents. Table 1.5 shows a compilation of minimum inhibitory concentration (MIC) values obtained using standard microdilution method (Gupta, A. and Plott, T. 2004 and Subissi, A. *et al* 2010).

Table 1.5: The Minimum Inhibitory Concentration (MIC) of CPO against bacteria

Microorganism	**MIC (µg/mL)**
Gram positive bacteria	
Staphylococcus aureus	0.5
β-hemolytic Streptococcus Group A	0.06-0.125
Micrococcus luteus	0.25
Micrococcus sedentarius	0.25
Brevibacterium species	<0.06-0.025
Corynebacterium species	0.25
Corynebacterium minutissimum	0.25
Gram negative bacteria	
Pseudomonas aeruginosa	0.5-2
Proteus mirabilis	0.5
Escherichia coli	0.5
Klebsiella pneumoniae	0.5
Gardnerella vaginalis	16-32
Lactobacillus spp.	>128

- Anti-inflammatory Activity

The dermatophytosis of the skin and the nail is worsened by the associated inflammation and pain. CPO is known to demonstrate its anti-inflammatory action by inhibition of the cyclo-oxygenase mediated synthesis of prostaglandins, prevention of release of prostaglandin E_2 (cyclooxygenase inflammatory mediator) and inhibition of formation of 5-HETE and leukotriene B_4 (5-lipoxygenase inflammatory mediators) (Bohn, M. and Kraemer, K. 2000 and Abrams, B. *et al* 1991). CPO has shown significant reduction in the arachidonic acid-induced ear edema in mice, compared to the other antifungal agents like ketoconazole, fluconazole, miconazole, naftifine and amorolfine (Abrams, B. *et al* 1991). The anti-inflammatory activity of CPO was comparable with two reference anti-inflammatory agents – indomethacin and desoximetasone (Abrams, B. *et al* 1991). An *in vivo* human model used to measure the delayed erythema upon exposure to ultraviolet B irradiation, showed that ciclopirox 0.77% was a better anti-inflammatory agent compared econazole nitrate 1%, oxiconazole 1%, ketoconazole 2% and hydrocortisone 2.5% (Rosen, T. *et al* 1997). Seborrhoeic dermatitis and actopic dermatitis are predominantly caused by the release of reactive oxygen species (ROS) from the inflammatory cells (Subissi, A. *et al* 2010). CPO is shown to scavenge the ROS released from the mouse inflammatory cells. This is attributed to the chelation of the intermediary multivalent ions of iron and copper by CPO (Subissi, A. *et al* 2010 and Nakashima, T. *et al* 2007).

1.6.4 Pharmacokinetics

Orally administered ciclopirox is rapidly absorbed and completely eliminated via urine and feces. After oral administration of ciclopirox in dogs and humans, the drug was excreted via the renal route primarily in its glucuronide form (Kellner H.M. *et al* 1981). This indicated that glucuronidation is the major metabolic pathway for CPO. The urinary excretion of CP at predetermined time points was monitored during the daily application of Penlac nail lacquer for 6 months, It was observed that <5% of the applied dose was absorbed into the systemic circulation. Thus the pharmacokinetics of CP is unique in that it does not get metabolized by CYP450 enzymes (like the azoles and allylamines) and therefore is devoid of contradindications and drug-drug interactions.

1.6.5 Chromatographic analysis of CPO

CPO could not be directly quantified using reverse phase or normal phase chromatography. Owing to its chelating properties, CPO would form a complex with the silica based stationary phases leading to tailing. A modification of a reported pre column derivatization method was used for the quantification of CPO in all the samples (Escarrone, A.L. *et al* 2008, Myoung, Y. and Choi, H. 2003 and Lehr, K. and Damm, P.1985). The derivatization process converts the N-hydroxypyridone group to a methyl derivative and thereby blocks its interaction with the silanol groups of the stationary phase. The general procedure of derivatization is used in the current project is addition of 0.1 M sodium hydroxide solution and dimethyl sulfate to the analyte solution, followed by vortexing for 5 min and placing at 37 ºC for 15 - 20 min. To

remove the additional dimethyl sulphate from the sample, an equal volume of triethylamine was added and vortexed for 5 min. The resulting solution was filtered and analyzed using HPLC. Acetonitrile:Water (1:1) was used as the mobile phase with a flow rate of 1 mL/min in a C18 reverse phase column. The wavelength of 298 nm was used for detection of CPO. Any specific alteration in this method will be described in the corresponding chapters. Figure 1.10 shows a representative chromatogram for CPO.

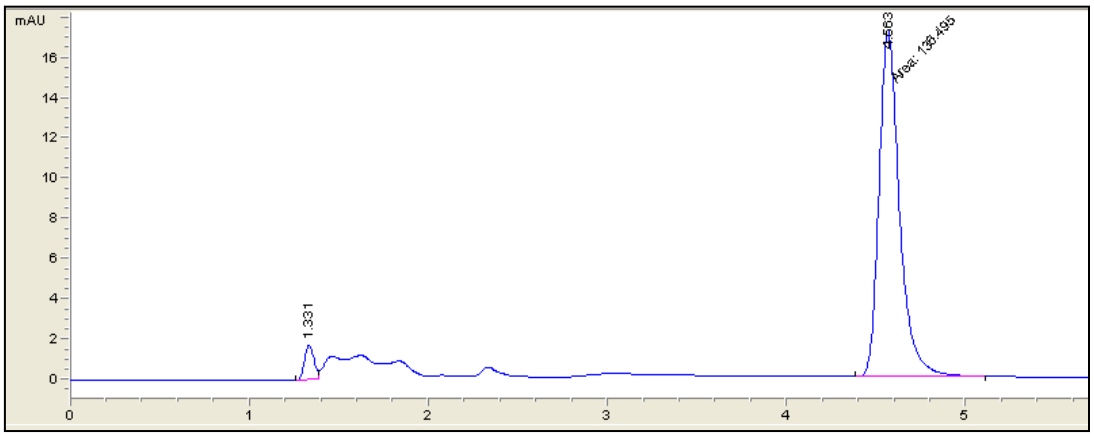


Figure 1.10: HPLC Chromatogram of CPO

1.6.6 Rationale for selection of CPO

Ciclopirox olamine was selected as the antifungal drug because it has a unique structure and mechanism of action which differentiates it from other antifungal drugs. Unlike most of the antifungal agents which act on sterol synthesis in the microbial cell wall, the multi-modal action of CPO on active transport, cell respiratory processes and membrane integrity of the microbial cell makes drug resistance difficult (Abrams, B. *et al* 1992). It is fungistatic and fungicidal with a broad spectrum of action. Due to its antibacterial and anti-inflammatory effects CPO will be effective against secondary bacterial infection and the inflammation associated with OM. CPO is predominantly metabolized by glucuronidation. Therefore, it does not alter the CYP-induced metabolism of co-administered drugs. This is a major advantage over the azole like antifungals which are inhibitors of CYP enzymes.

It is the first FDA approved topical formulation in the US for OM is Penlac® nail lacquer (8% topical solution of ciclopirox). The final formulation for ciclopirox olamine is compared for its *in vitro* effectiveness with the commercial formulation Penlac®. CPO has a low molecular weight and higher aqueous solubility compared to the azoles (Itraconazole, Ketoconazole, Miconazole, Econazole nitrate) and allylamine (Terbinafine hydrochloride) antifungal agents. Hence, it is expected to penetrate the hydrophilic nail better than the other antifungal molecules.

1.7 Strategies to enhance topical delivery of drugs into the nail:

The barrier properties of the nail can be overcome using physical or chemical strategies.

1.7.1 Physical Methods

1.7.1.1 Filing the nail plate

Filing helps to remove the dorsal layer of the nail plate and thereby reduces the barrier and thickness of the nail. Filing is also reported to be used in clinical studies prior to application of Loceryl® nail lacquer. (Pittrof,F.*et al* 1992).

1.7.1.2 Use of acid for surface etching of the nail

20% Tartaric acid or 10% phosphoric acid gel can be applied for 90 sec to increase the roughness of the nail plate surface by 1.3 and 1.7 folds respectively. The increase in roughness increases the surface area for adhering with the polymeric films. This increased contact increases the drug permeation through the nail (Repka M.A. *et al* 2002; Repka, M.A. *et al* 2004)

1.7.1.3 Microporation of nail plate

It is a process of drilling holes with controlled depth in the nail plate using a microcutting device called PathFormer (Path Scientific, USA). This process of controlled hole formation is reported to be well tolerated by participants. However,

extent of enhancement in drug permeation due to this procedure is not known (Murdan, S. 2008)

1.7.1.4 Application of electric current

Iontophoresis is an systematically researched physical method for enhancing the transungual drug permeation. The major advantage of this method is the large increase in drug flux and drug permeation into intact nail plate (Murdan, S. 2008). This method has been reported to enhance the transungual permeation of ciclopirox and terbinafine hydrochloride (Hao, J. *et al*, 2009; Amichai, B. *et al* 2010 and Nair, A.B. 2011)

1.7.2 Chemical Enhancers:

Use of chemical enhancers has a number of advantages over physical methods:

- Ease of drug administration because the enhancer can be incorporated in the formulation.
- On the contrary to some physical methods like drilling and microporation, does not required skilled personal for application.
- Greater activity as entire nail plate can be exposed to the action of chemical enhancers as opposed to physical methods like laser.
- Cheaper than physical methods.

1.8 Penetration enhancers

The low penetration of drugs into the skin and the nail is the primary reason for poor efficacy of topically applied drugs. Use of penetration enhancers is an effective strategy to improve the passive diffusion of the drug into the nail and the skin. Penetration enhancers (PEs) are chemical compounds which interact with the tissue and increase the diffusion of drug molecules through the biological membranes. The properties of an ideal penetration enhancer are summarized below (Williams, A.C. and Barry, B.W., 2004).

- Non-toxic, non-irritant, non-allergenic
- Upon application should work promptly with a predictable and reproducible duration of activity
- Pharmacologically inert
- Should prevent loss of endogenous substances from the body
- Should show reversible change in the barrier properties of the membrane
- Compatible with the active ingredient and other excipients in the formulation
- Comfortable for the patient

During *in vitro* permeation experiments, the membrane is placed between a donor and receiver compartments, where the receiver compartment is maintained under sink conditions for the duration of the study. If the cross sectional area of the membrane is A and its thickness is l, Fick's first law of diffusion can be written as follows (Sinko, P.J. 2006).

$$J = \frac{dM}{A\,dt} = D\left(\frac{C1-C2}{l}\right) \quad \text{.....(1.1)}$$

Where, J= steady state flux, D= diffusion coefficient of the drug (cm^2/sec), M = amount of drug, t = unit time, C_1= Concentration of the drug in the membrane in contact with the donor solution, C_2 = Concentration of the drug in the membrane in contact with the receiver solution.

The concentrations C_1 and C_2 are generally unknown and can be represented as the product of partition coefficient and the concentration of the drug in the respective cells. Therefore $C_1 = K.C_d$ and $C_2 = K.C_r$.

C_d and C_r are the concentrations of the drug in the donor and receiver compartments respectively. The equation (1.1) can be rewritten as

$$J = \frac{dM}{A\,dt} = \frac{DK(Cd-Cr)}{l} \quad \text{.....(1.2)}$$

Under sink conditions C_r is considered as zero and the equation (1.2) is simplified to

$$\frac{dM}{dt} = \frac{DAKCd}{l} = PACd \quad \text{.....(1.3)}$$

Where P is the permeability coefficient or permeability and P = (DK/l).

When a penetration enhancer is applied to the biological membrane (nail or skin), it can alter the following parameters in equation 1.3 (Williams, A. and Barry, B. 2004).

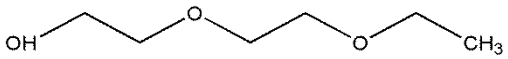
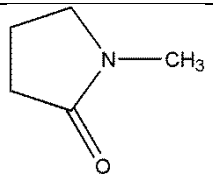
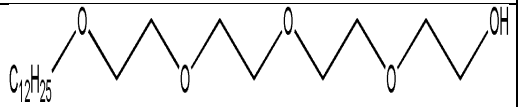
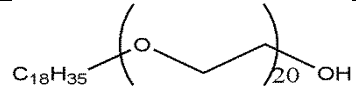
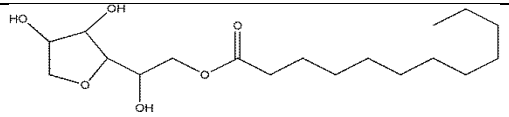
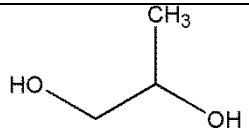
- Increase diffusion coefficient D in the membrane by directly altering the barrier properties of the membrane
- Increase effective concentration of drug, C_d, by increasing the solubility of the drug and increasing its thermodynamic activity

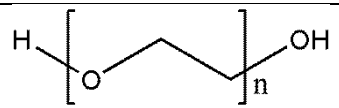
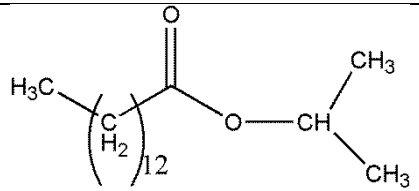
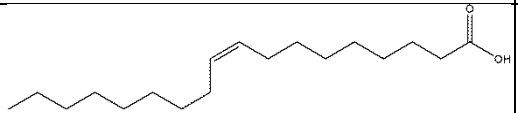
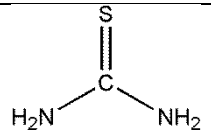
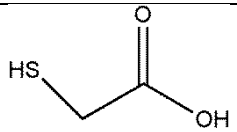
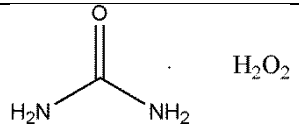
- Increasing the partitioning of the drug from formulation to the membrane by altering solvent nature of the membrane
- Decrease the thickness or path length by providing a less tortuous permeation pathway (less likely)

.

The table 1.6 shows the different penetration enhancers screened in the current project; their chemical structures, concentrations, abbreviations and the mechanism of action (Marstein, S. *et al* 1973; Khengar, R. *et al* 2007; Williams, A and Barry, B. 2004). All the penetration enhancers in table 6 (except for TU, TA and UH) are reported as penetration enhancers for topical or transdermal application on the skin. Thiourea, thioglycolic acid and urea-hydrogen peroxide are good nail penetration enhancers (Khengar, R. *et al* 2007). The mechanism of action mentioned for the skin penetration enhancers are based on their action on the skin alone. It is probable that the effect of these enhancers in increasing transungual drug levels is drug specific (Murdan, S. 2008). Utility of a PE for permeants can be predictable only when the permeants belong to the same series of compounds with comparable physicochemical properties (Williams, A. and Barry, B. 2004). The penetration enhancers tend to show a concentration dependent action.

Table 1.6: Penetration enhancers screened in the current project

No.	Penetration Enhancers (PEs)	Abbreviation	Chemical Structure	Concentration of PEs (%w/w)	Mechanism of Action
1	Diethylene glycol monoethyl ether or (Transcutol®)	TCL		100%	Increase drug solubility and formation of drug depot in skin
2	N-methyl pyrrolidone	NMP		100%	Altering membrane solvent nature, increase drug partition into skin
3	Poly(oxyethylene)(4) lauryl ether or BRIJ 30	B30		2.5%	Potential to solubilize lipids in stratum corneum of skin, increase solubility of drug, emulsify sebum
4	Polyoxyethylene (20) Oleyl Ether or BRIJ 98	B98		2.5%	
5	Sorbitan laurate or SPAN 20	S20		2.5%	
6	Propylene glycol	PG		100%	PG and PEG 400 shown the 'solvent drag' effect within the

7	Polyethylene glycol 400	PEG		100%	skin, solubilizes α keratin in SC
8	Isopropyl myristate	IPM		100%	Penetrates into lipid bilayers and increases lipid fluidity
9	Oleic acid	OA		100%	Modify intercellular lipid domains and fluidizes the membrane
10	Thiourea	TU		5%	Reduces disulphide linkages in keratin of nail
11	Thioglycolic acid	TA		5%	
12	Urea-hydrogen peroxide	UH		8.75%	Oxidizes disulphide linkages in nail.

1.9 Objectives of the project

The moderate to severe cases of OM are treated by oral antifungals or a combination of oral and topical antifungal formulations. The prolonged duration of therapy with the systemic drugs mandates periodic liver function tests for the patients (Elewski, B. *et al* 2005). With the lingering risk of drug-drug interactions, administration of systemic agents to patients with co-morbidities should be dealt with caution. The limitation with systemic antifungals is limited penetration of the drug from the nail bed to the nail plate (Gupta, A.K. and Simpson, F.C. 2012). Currently the topical formulations are limited to mild forms of the infection or in combination with oral antifungals. The formulation development of an effective topical therapy of OM is challenging due the barrier properties of the nail, compact structure of the nail plate, location of the infection within the layers of the nail plate and thickness of the nail (Hui, X. *et al* 2004). The first US FDA approved topical formulation for treatment of OM is Penlac® nail lacquer (8% ciclopirox topical solution). The nail lacquer consists of ethyl acetate and isopropanol as the solvents which evaporate upon application and leave a thin film of the drug on the nail plate (Murdan, S. 2008). As the solvents evaporate the concentration of the drug in the film increases from 8 % to 34.8 % (Gupta A.K. *et al* 2005). Thus the lacquer formulation improves the drug contact with the nail plate and the concentration gradient across the nail. However, the mycological cure rate for the nail lacquer is reported to be only 29 %-36 % (Gupta. A.K. *et al* 2000a and Gupta. A.K. *et al* 2000b).

This indicates that there is a need to improve the efficacy of a topical formulation. For successful topical therapy of OM, the topical formulation must have the following properties (Murdan, S. 2008):

- Ease of use
- Maintain adequate contact with the nail plate
- Contain drug in the form which is easily released from the formulation and easily diffused into the nail.
- High thermodynamic activity
- Ability to hydrate the nail to increase drug permeation
- Contain penetration enhancers

Some of the drawbacks of Penlac® nail lacquer are difficulty in use which causes discontinuation of therapy by patients, inability to hydrate the nail and very low mycological cure rate.

The current project explores the strategy to develop a novel transungual formulation (polymeric patch) which would meet the above criteria. Such a formulation would be more effective than the current topical formulations in treating moderate to severe cases of the disease. The patch formulation would be more convenient for application and removal, unlike the nail lacquer formulation, where the formulation has to be applied for a week and then all the layers of the nail lacquer have to be removed prior to application on the second week. The selection of appropriate patch matrix will help to maintain adhesion on the nail plate. The penetration enhancers which improve drug penetration into the skin (nail folds) and the nail, will be incorporated in the patch to

maintain high levels of solubilized drug for permeation. The occlusive backing of the novel patch will increase the hydration of the nail. In presence of this hydration, the fungal spores will germinate to form hyphae which are more susceptible to the action of antifungal drugs (Flagothier, C. 2005).

It is well reported in literature that penetration of a drug into the nail is affected by number of factors, namely physicochemical properties of a drug molecule (size, shape, charge, and hydrophobicity), the nature of the formulation (nature of the vehicle and drug concentration), presence of enhancers, nail properties (thickness and hydration), and interactions between the drug and the membrane (Elkeeb, R. *et al* 2010). Understanding the roles of different interactions between the drug and the target tissues (nail and nail folds) and the impact of selected enhancers on these interactions will help in development of an effective transungual formulation for treatment of the disease. The figure 1.11 gives the schematic of the experimental work.

1.9.1 Hypothesis

"An antifungal drug can successfully penetrate the human skin and nail from a patch formulation, when formulated with the appropriately selected penetration enhancer(s); a polymer matrix which maintains reasonable solubility of the drug and the enhancer(s)."

1.9.2 Specific aims

1. To determine the factors critical to the *in vitro* transungual delivery of ciclopirox olamine.
2. To design and develop a transungual formulation containing ciclopirox olamine and the tissue specific penetration enhancers.
3. To characterize the novel transungual formulation for physical characteristics and functional properties.

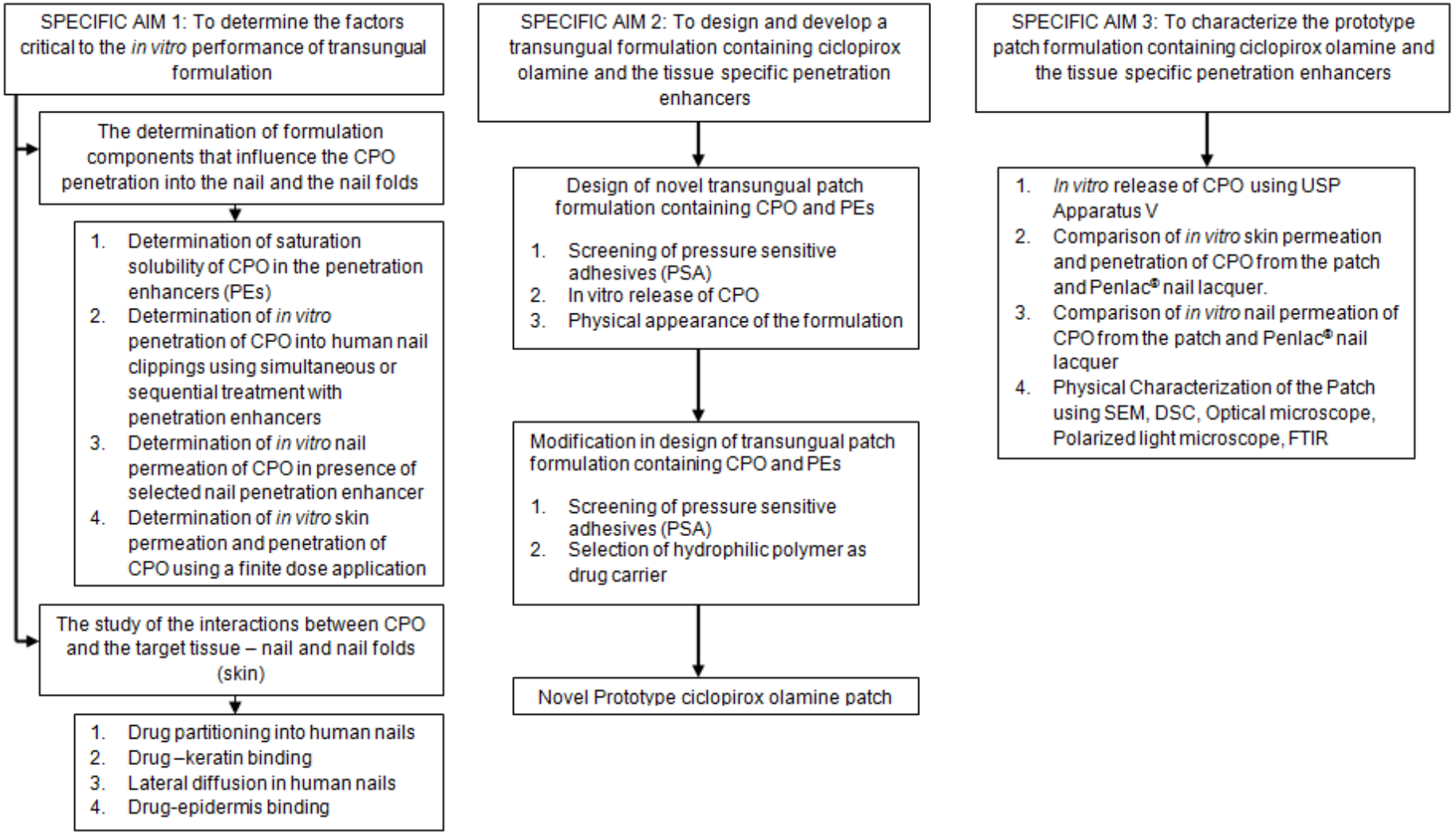


Figure 1.11: Schematic of the Experimental work

CHAPTER 2

THE SELECTION OF THE PENETRATION ENHANCERS: A PREFORMULATION STRATEGY

2.1. Abbreviation

B 30: Brij 30

B 98: Brij 98

CPO: Ciclopirox olamine

IPM: Isopropyl myristate

NMP: N-methyl pyrrolidone

OA: Oleic acid

PEs: Penetration enhancers

PEG 400: Polyethylene glycol 400

PG: Propylene glycol

pH 7.4 PBS : 10 mM Phosphate Buffered Saline pH 7.4

S 20: Span 20

TA: Thioglycolic acid

TCL: Transcutol

TU: Thiourea

UH: Urea-hydrogenperoxide

2.2. Introduction

One of the approaches used to increase passive permeation of topically applied antifungal drugs into and through the nail is the use of chemical penetration enhancers (PEs). It was hypothesized that drug delivery into the two target tissues – human hail plate and the nail folds (skin) with the use of specific PEs will increase drug levels in these target tissues and subsequently increase the antifungal activity of the resulting topical formulation. The main objective of the study was to screen the PEs (Table 1.6) and select suitable enhancers for delivery of the antifungal drug CPO into the nail and nail folds. Figure 2.1 shows the proposed preformulation screening strategy for selection of PEs for transungual and epidermal drug delivery.

2.3. Materials

CPO was purchased from Haorui Pharma-chem Inc., Edison, New Jersey, USA. NMP and PEG (MW 400) were obtained from JT Baker. TCL and B98 were purchased from Acros Organics. B30, S20, sodium hydroxide, gentamicin sulfate, dimethyl sulfate and triethyl amine were purchased from Sigma Aldrich. PG was obtained from MP Biochemicals. OA was bought from EMD. IPM was purchased from Alfa Aesar. Thiourea and sodium phosphate dibasic were obtained from Acros Organics. Franz diffusion cells and neoprene nail adapters were purchased from PermeGear. Cadaver skin and cadaver toenails were purchased from Allosource, Cincinnati and Anatomy Gifts Registry, Maryland respectively. Nanopure water was used for preparation of 10 mM pH 7.4 phosphate buffered saline. HPLC grade acetonitrile from Fischer scientific was used

for preparation of mobile phase. All the chemicals were used as received without any further purification.

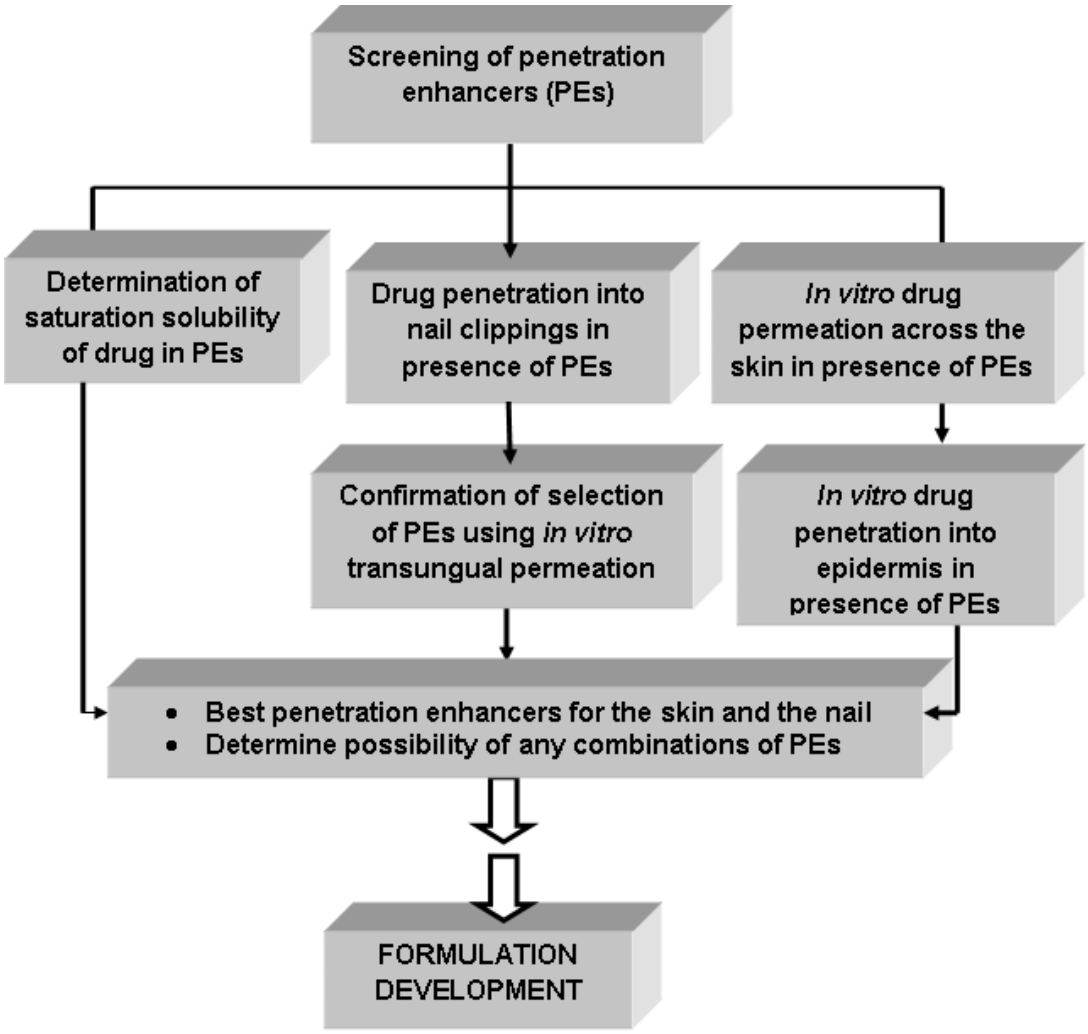


Figure 2.1: Preformulation Strategy for selection of penetration enhancers

2.4. Experimental Methods

2.4.1 Sample Preparation for analysis

The assay of CPO in the nail was performed by first dissolving the nails in 0.5 mL of 1 M sodium hydroxide solution. The nail solutions were filtered through Restek® 0.45 µ syringe filters (cellulose acetate membrane). These solutions were diluted sufficiently to reduce the strength of 1 M NaOH to 0.1 M before derivatization. For the derivatization of CPO, 25 µL of dimethyl sulfate was added to 0.2 mL of the dilute nail solution, and vortexed for 5 min. These solutions were maintained at 37 °C for 20 min. 25 µL of triethylamine was added and vortexed for 1 min to terminate the reaction. The solutions were then filtered through Restek® 0.22 µ syringe filters (cellulose acetate membrane) and analyzed using the HPLC method. In order to quantify the CPO in the buffer, a similar pre-column derivatization process was used. To 0.2 mL of the samples, 50 µL of 0.1 M sodium hydroxide and 25 µL of dimethyl sulfate were added and vortexed for 5 min. The solutions were maintained at 37 °C for 20 min prior to the addition of 25 µL of triethyl amine. The solutions were vortexed, filtered through Restek® 0.22 µ syringe filters (cellulose acetate membrane) and analyzed using HPLC.

2.4.2. High Pressure Liquid Chromatography

CPO content in the samples was assayed using an Agilent1100 series HPLC with dual wavelength detector, using a modified, pre-assay derivatization method (Escarrone, A.L. *et al* 2008, Lehr, K-H. and Damm, P. 1985 and Myoung, Y. and Choi, H-K., 2003).

Acetonitrile: water (50:50 v/v) was used as the mobile phase with a 150 mm by 4.6 mm Eclipse Plus C18 column (Agilent). The flow rate was 1 mL/min and the injection volume was 50 µL. The wavelength of detection for derivatized CPO was 298 nm.

2.4.3. Recovery of ciclopirox olamine from human nails

Solutions of CPO in pH 7.4 PBS with concentrations 0.0253 mg/mL, 0.0505 mg/mL, 0.202 mg/mL, 0.809 mg/mL, 3.24 mg/mL and 6.47 mg/mL were prepared. Approximately 20 mg of human nail clippings were added to each concentration of CPO solution. Each concentration was studied in triplicate. The nail clippings were treated with 200 µL of each of these solutions for 3 hr at 32 ± 1 °C. To dissolve the nails, 200 µL of 1 M NaOH was added to each sample. The nail solutions were diluted to reduce the concentration of 1 M NaOH to 0.1 M and the sample preparation method was followed to quantify the concentration of CPO.

2.4.4. The Recovery of ciclopirox olamine from epidermis and dermis

A stock solution containing 1.6 mg/mL of CPO in pH 7.4 PBS was prepared. Solutions with the concentrations 0.1 mg/mL, 0.2 mg/mL, 0.4 mg/mL, 0.8 mg/mL, 1.2 mg/mL were prepared using the stock solution. 50 µL of each of these solutions, including the stock contains 5.2 µg, 10.4 µg, 20 µg, 40 µg, 60 µg and 80 µg of CPO respectively. Each of the above concentrations was studied in triplicate. 9 mm diameter skin sections were punched from a piece of human cadaver skin, using a cork screw borer. The epidermis and the dermis were separated for each section, using the modified heat separation

method (Frank, J.D. *et al* 1995 and Kassis,V. and SØndergaard, J.1982). Both the epidermis and the dermis, for each skin section were separately treated with 50 µL of the drug solutions for 4 hr in microcentrifuge tubes. After the drug treatment, 1 mL of the mobile phase was added to each of the microcentrifuge tubes and equilibrated for 24 hr for drug extraction at 37 ºC. The extracting medium was filtered through 0.22 µ syringe filters, derivatized and analyzed using HPLC.

2.4.5. Determination of the saturation solubility of ciclopirox olamine in PEs

An excess amount of CPO was added to 0.5 mL of each PE at the concentration levels shown in table 6. The drug was allowed to equilibrate with the enhancers for a period of 72 hr at 32 ± 1 ºC. A saturated solution of CPO in pH 7.4 PBS served as the control solution. At the end of 72 hr, the solutions were filtered through a 0.22 µ syringe filter. The samples were derivatized and analyzed using the HPLC method mentioned previously. The enhancement in solubility, EF_{sol}, of ciclopirox olamine in presence of the enhancers was calculated using the equation below.

$$EF_{sol} = \frac{[CPO]_{PE}}{[CPO]_{pH\ 7.4\ PBS}} \quad \ldots\ldots(2.1)$$

Where $[CPO]_{PE}$ is equilibrium concentration of CPO in presence of PEs and $[CPO]_{pH\ 7.4\ PBS}$ is the equilibrium concentration of CPO in pH 7.4 PBS (Control).

2.4.6. The in vitro nail penetration of ciclopirox olamine

The PEs were screened for their ability to increase the concentration of the drug within the nail, using human nail clippings. Human nail clippings were obtained from healthy volunteers (20-50 years of age) in the Department of Pharmaceutical Sciences, Temple University. The nail clippings were washed thrice with ethanol:water (70:30 v/v) and then rinsed with nanopure® water. The nail clippings were air dried at room temperature and stored at 2-8 ºC until use. Two sets of experiments for the preliminary *in vitro* nail penetration studies were performed based on the method described in the literature (Murthy, S.N. *et al* 2009).

- Simultaneous exposure study – The nail clippings were exposed to the drug and the PEs simultaneously.
- Sequential exposure study – The nail clippings were treated with the PEs and then with the drug solution. The drug and penetration enhancer do not come in contact with each other.

The study was approved for use of human nail clippings by the Temple University Institutional Review Board (TIRB) (Protocol number 13671).

The formulation design differs significantly for the addition of the PEs selected in each of the above studies. It is relatively simple to design a formulation with the drug and the enhancer in it. However, for a pre-treatment (sequential study), two separate formulations were required - one containing just the enhancer and the other containing only the drug.

2.4.6.1 Sequential exposure study

The sequential study identifies the enhancers which act predominantly by altering the barrier properties of the nail. Approximately 14 mg of the nail clippings were weighed for each sample. These clippings were treated for 24 hr with the PEs at the concentration mentioned in table 1.7 at 32 ± 1 ºC. In the control samples, the nail clippings were pre-treated with pH 7.4 PBS (without PEs). The pretreated nail clippings were washed thrice with 3 mL of nanopure water to remove the PEs from the nail surface. The nail clippings were wiped with tissue paper and dried in the oven at 45 ºC for 3 hr to remove moisture. The dry nail clippings were treated with saturated solution of ciclopirox olamine in pH 7.4 PBS for 24 hr at 32 ± 1 ºC. The drug treated nail clippings were washed thrice with 3 mL of nanopure water to remove surface adherent drug, and wiped with tissue paper. The nail clippings were dissolved in 1 M NaOH solution and the drug derivatized and analysed as discussed in previous sections. The enhancement factor (EF_{seq}) drug penetration into the nail clippings were calculated using equation:

$$EF_{seq} = \frac{[CPO]^{seq}_{PE}\ (\mu g/mg \text{ of nail}) \text{ within the nail}}{[CPO]^{seq}_{pH\ 7.4\ PBS}\ (\mu g/mg \text{ of nail}) \text{ within the nail}} \quad \text{.....(2.2)}$$

Where $[CPO]^{seq}_{PE}$ and $[CPO]^{seq}_{pH\ 7.4\ PBS}$ are the concentrations of CPO within the nail clippings after pretreatment with the PEs and pH 7.4 PBS (control) respectively.

2.4.6.2 Simultaneous exposure study

The saturated solutions of CPO in the PEs and 10 mM pH7.4 PBS were prepared. Fourteen milligram of previously washed and dried, human nail clippings were weighed into micro centrifuge tubes. The nail clippings were treated for 24 hr with 0.5 mL of saturated solution of the drug in the enhancers at 32 ± 1 ºC. As a control, nail clippings were treated with a saturated solution of CPO in pH 7.4 PBS. After 24 hr of drug treatment, the nail clippings were washed three times with 5 mL of nanopure water to remove any surface adherent drug. The nail clippings were dried using tissue paper and were dissolved in 0.5 mL of 1 M sodium hydroxide solution at 37 °C. The nail solutions were filtered using 0.45 µ syringe filters, derivatized and analyzed using HPLC as described in the previous sections. This method is a modification of the screening method described in literature (Khengar, R. et al 2007, Murthy, S.N. et al 2009 and Manda, P. *et al* 2012). The enhancement factor, EF_{sim}, gives the improvement in CPO penetration into nail clippings relative to that in the control.

$$EF_{sim} = \frac{[CPO]^{sim}_{PE}\ (\mu g/mg \text{ of nail}) \text{ within the nail}}{[CPO]^{sim}_{pH\ 7.4\ PBS}\ (\mu g/mg \text{ of nail}) \text{ within the nail}} \quad \ldots\ldots\ldots\ldots\ldots (2.3)$$

Where $[CPO]^{sim}_{PE}$ and $[CPO]^{sim}_{pH\ 7.4\ PBS}$ are the concentrations of CPO within the nail clippings in presence of PEs and pH 7.4 PBS (control) respectively.

2.4.7. The in vitro transungual permeation of ciclopirox olamine

The nail penetration study helps to screen different PEs; however, the results needed to be confirmed with an *in vitro* transungual permeation study. The *in vitro* transungual permeation of CPO was performed using only the PEs which showed promise in the nail penetration screen. The human cadaver toenails (Anatomy Gifts Registry) were washed with nanopure water and blotted with tissue paper. The weights and thicknesses of the toenails were measured. The human cadaver toenails were hydrated at 100 % RH for 24 hr (Figure A.3, Appendix A), prior to mounting on Franz diffusion cell with nail adapters (Figure 2.2). The nail adapters (3 mm orifice diameter) with toenails were placed between the donor and receiver compartments of the Franz cells (Figure 2.3). The receiver compartment was filled with 3 mL of pH 7.4 PBS containing 0.1 % w/v gentamicin sulfate. Gentamicin sulfate was added to prevent microbial growth in the receiver compartment. The donor compartment was dosed daily with 21.4 µL of saturated solution of CPO in the potential enhancer from the nail penetration experiment. The temperature of the receiver compartment solution was maintained at 32 ± 1 °C for the duration of 27 days. The study was performed under occlusion by covering the donor compartment with parafilm. On days 5, 10, 15, 18, 21, 24 and 27, 0.5 mL of the receiver compartment solution was sampled and analyzed for drug content. Additionally, on day 27, the nails were dismounted from the nail adapters and the drug content was quantified by separating the nail just below the orifice and the nail surrounding the orifice (Figure 2.4). The drug quantification was performed using the process described in the sample preparation.

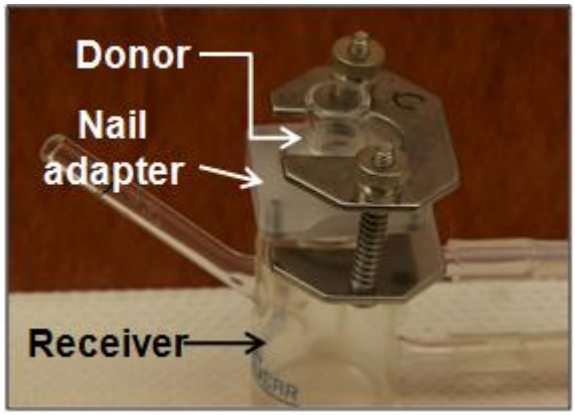


Figure 2.2: Franz diffusion cell with the nail adapter between the donor and receiver compartments

Figure 2.3: Neoprene nail adapter with 3 mm orifice diameter

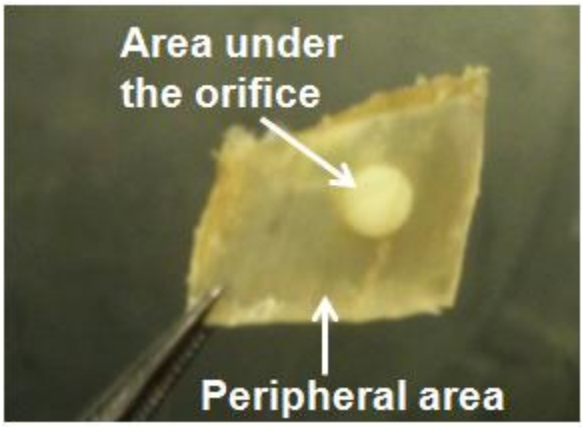


Figure 2.4: Toenail after in vitro permeation study showing the hydrated area under the orifice and the dry peripheral area

2.4.8. The in vitro skin permeation of ciclopirox olamine

The *in vitro* skin permeation of CPO was performed using the PermeGear Franz diffusion apparatus with the internal volume of 3 mL and orifice diameter of 9 mm. The dermatomed human cadaver skin (Allosource) was thawed at room temperature for 15 min. Skin pieces (2.25 cm^2) were cut and placed in pH 7.4 PBS for 20 min to remove the glycerine used for cryopreservation of the cadaver skin. The skin pieces were blotted with tissue paper and placed between the donor and the receiver compartments. The receiver compartment was filled with 3 mL of pH 7.4 PBS and the skin was allowed to equilibrate with the receiving medium for 60 min, prior to drug dosing. Twenty microlitre of the saturated solution of the drug in the PEs was added to the donor compartment to form a thin film on the surface of the skin. The three commercially available formulations of ciclopirox were also studied to compare its permeation in presence of the PEs to that of the commercial formulations. The three formulations studied were 0.77% w/w

Ciclopirox olamine topical suspension, USP; Loprox® gel containing 0.77% ciclopirox and Penlac® nail lacquer (8% topical solution of ciclopirox). The receiver compartment solution was maintained at 32 ± 1 ºC, for a period of 24 hr under occlusion by covering the donor compartment with parafilm. Each of the PEs and the commercial formulations were tested in triplicate and the data was compared with the control. The control cells were dosed with 20 µL of saturated solution of CPO in pH 7.4 PBS. At 2, 4, 6, 8, 22 and 24 hr, 0.5 mL of the receiver compartment solution was withdrawn and replaced with 0.5 mL of fresh buffer to maintain sink conditions. The drug content in the samples was analyzed using the HPLC method. The steady state flux (J) for permeation (µg/cm^2/hr) was obtained from the slope of the linear potion of the plot of cumulative amount of CPO permeating per unit surface area of skin (µg/cm^2) against time (hr). The permeability coefficient Kp was calculated from the ratio of the steady state flux (J) and the concentration in the donor compartment (C_d). Since saturated solutions were used as donor solutions, C_d was the saturation solubility of CPO for all practical purposes of the calculation. The enhancement in flux for skin permeation (EF_{flux}) was calculated using the equation:

$$EF_{flux} = \frac{\text{Flux}_{PE} \text{ of CPO permeation } (\mu g/cm^2/hr)}{\text{Flux}_{\text{pH 7.4 PBS}} \text{ of CPO permeation } (\mu g/cm^2/hr)} \quad \ldots\ldots\ldots\ldots (2.4)$$

Where $Flux_{PE}$ and $Flux_{pH\ 7.4\ PBS}$ are the stead state flux values for permeation of CPO across the skin in presence of the PEs and pH 7.4 PBS (control) respectively.

2.4.9. Finite dose skin penetration of ciclopirox olamine

The skin used in the finite dose permeation study was further analyzed for drug content by separating the epidermis and the dermis using a heat separation method (Kassiv,V and Sondergaard,J., 1982 and Frank,J.D. et al 1995). A modification of the reported heat separation method was used for separation of epidermis and dermis. Post the finite dose skin permeation experiment, the skin was dismounted from the Franz cell and washed with nanopure water thrice on both the epidermal and the dermal sides to remove any surface drug. The skin was then blotted with tissue paper; the area exposed to the drug was separated from the peripheral skin and wrapped in aluminum foil. Each of the samples was heated in the oven at 45 ºC for 10 min. The epidermis was then peeled from the dermis using sharp forceps. The thickness of the epidermis and the dermis were measured using electronic digital micrometer (Marathon Management). The two layers were then placed in separate glass vials containing 1 mL of the mobile phase each at 37 ºC for 24 hr to extract CPO. The concentration of the drug in the extracting medium was quantified using HPLC. The drug levels were reported in µg/mL to determine if the minimum inhibitory concentration (MIC) was achieved in the epidermis and the dermis. The enhancement factors, $EF_{epidermis}$ and EF_{dermis} give the increase in accumulation of CPO in the epidermis and dermis relative to the control.

$$EF_{epidermis}/EF_{dermis} = \frac{[CPO]_{PE}\ (\mu g/mL) \text{ in epidermis or dermis}}{[CPO]_{pH\ 7.4\ PBS}\ (\mu g/mL) \text{ in epidermis or dermis}} \quad \text{……..(2.5)}$$

Where $[CPO]_{PE}$ and $[CPO]_{pH7.4\ PBS}$ are concentrations of CPO within the epidermis/dermis in presence of PEs and pH 7.4 PBS (control) respectively.

2.4.10. Analysis of Results

Relationship plots with EF_{sim}, EF_{flux}, $EF_{epidermis}$ or EF_{dermis} on the y-axis and EF_{sol} on the x-axis were prepared to understand how the different enhancement factors were affected by change in solubility. These plots also helped to appreciate the mechanism of action of the PEs in the two different biological membranes- skin and the nail. Each of the relationship plots were divided into four quadrants (I to IV) by drawing lines parallel to the x and y axes, passing though enhancement factor 1 on each axes. The EF of 1 stands for the control sample used in the determination of the respective enhancement factor. This method of analysis is a modification of the reported method (Murthy, S.N. et al 2009).

2.4.11. Statistical Analysis of Experimental Data

All the experiments were performed in triplicate and the data was reported as mean value (± standard deviation). One way analysis of variance, ANOVA (Minitab 16 software) was used to determine statistical significance. The post hoc test (Dunnett's test for multiple comparisions) was used to compare the results for the PEs against the control, where *p-value* <0.05 was considered as statistically significant.

2.5. Results and Discussion

2.5.1. High Pressure Liquid Chromatography

A linear plot was attained in the range of 0.09 to 46.73 µg/mL with a correlation coefficient of 0.9996. The retention time, the limit of quantification (LOQ) and limit of detection (LOD) for the derivatized CPO were 4.6 min, 90 ng/mL and 25 ng/mL respectively.

2.5.2. Recovery of ciclopirox olamine from human nail

A recovery of 86-97 % was obtained for CPO in the concentration range of 0.25 µg/mg nail to 64.7 µg/mg nail after dissolution in 1 M NaOH and the pre-column derivatization process.

2.5.3. Recovery of ciclopirox olamine from epidermis and dermis

74.5 to 95.6 % of ciclopirox olamine was recovered when epidermis with a diameter of 9 mm was treated with 5.2 µg to 80 µg of CPO after the extraction of the epidermis. The recovery for the dermis was greater than 95 %.

2.5.4. Saturation solubility of Ciclopirox olamine in PEs

Figure 2.5 shows that the solubility of CPO was significantly higher in PG, oleic acid OA, TCL, NMP and TU compared to the control (pH 7.4 PBS) (p-value < 0.05). The nail PEs TA and UH did not significantly increase the solubility of CPO. TA caused precipitation of the free acid form of CPO. An increase in solubility of CPO in penetration enhancer increases concentration gradient across the skin. CPO solubility in

the enhancers was determined to elucidate any correlation between improvement in permeation and/ or penetration into the target tissues.

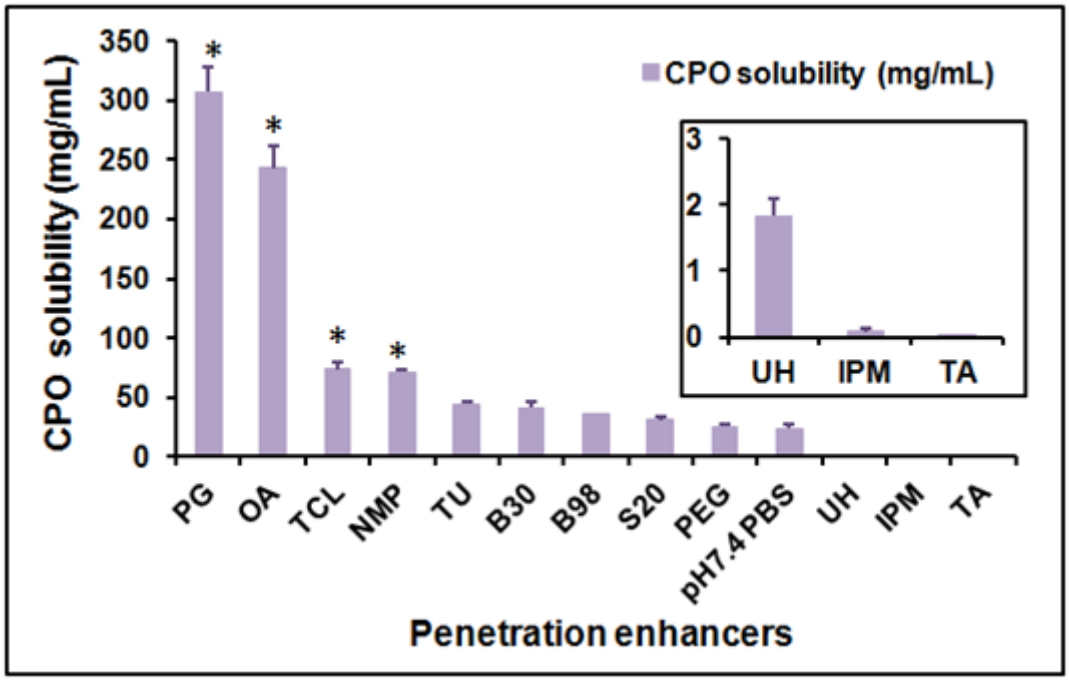


Figure 2.5: Saturation solubility (mg/mL) of CPO in presence of PEs

2.5.5. In vitro nail penetration of ciclopirox olamine

2.5.5.1. Preliminary in vitro nail penetration studies

The preliminary *in vitro* nail penetration studies were performed at two concentration levels of the reported nail PEs – UH, TA and TU. UH (at 8.75% and 17.5%), TA (at 5% and 10%) and TU (at 5% and 10%) were studied in both simultaneous and sequential exposure studies. Figure 2.6 shows the preliminary data for the three nail PEs. The

statistical significance (p-value <0.05) over the control is shown in the figure with an asterisk. UH at both concentration levels showed better enhancement factors for the simultaneous exposure compared to pretreatment of the nail clippings with the enhancer. On the contrary, the enhancement factors for TA were significantly higher for the sequential exposure. It was seen in the solubility studies that the drug precipitated into its free acid form in the presence of TA and a significantly lower saturation solubility of CPO in presence of TA. This was the reason for the reduced penetration of CPO into the nail clippings upon simultaneous exposure in the presence of TA. Ten percent TU showed 2.5 fold and 2.1 fold increase in the drug concentration within the nail in the simultaneous and sequential studies respectively. TU at both 5 % and 10 % worked better in the simultaneous study. The solubility of CPO in presence of TU was 1.86 fold higher than the control (figure 2.5). The level of the solubilized drug was higher in the presence of TU (at 5% and 10%). This explains the increase in the levels of the drug in the simultaneous study relative to the levels in the sequential study. It was noted that there was a concentration dependent effect for all the three PEs in both the simultaneous and sequential studies (Figure 2.6). It is advantageous to have the PEs along with the drug in a formulation, rather than as a pretreatment. For agents like TA and TU, the stability of the drug in these agents and their ability to overcome the barrier properties of the nail are important criteria for selection. It was observed that the nail clippings were very soft and extremely hydrated at the higher concentrations of all three PEs. It was concluded from the preliminary studies that a lower concentration of the nail PEs would be more compatible with the drug and therefore the further studies with nail clippings were performed at lower concentrations of UH, TA and TU.

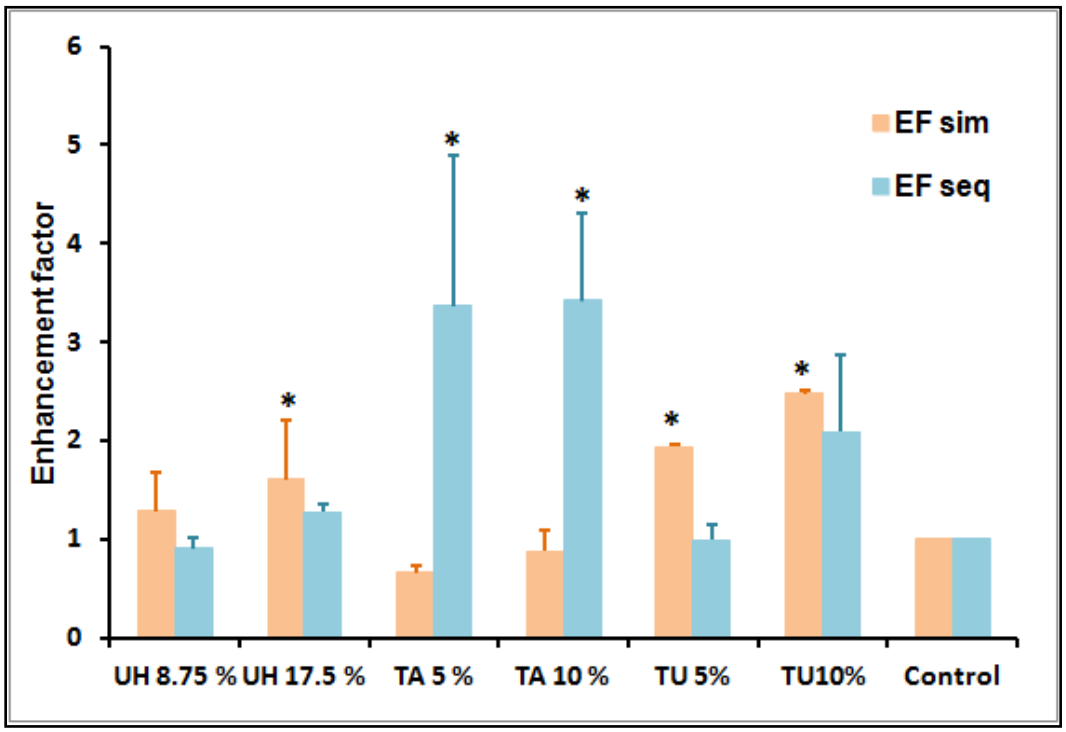


Figure 2.6: Preliminary data for the enhancement factors in simultaneous (EF_{sim}) and sequential (EF_{seq}) exposure studies at two concentration levels of nail penetration enhancers

2.5.5.2. Sequential exposure study

A sequential exposure study was done by treating the nails with the PEs for 24 hr followed by drug treatment for 24 hr. Figure 2.7 gives the enhancement in concentration of CPO within the nail clippings (in decreasing order) after the pretreatment. TA at 5 %w/w showed the greatest accumulation of the drug at 3.4 fold, followed by PG with an enhancement factor of 1.8. All the other skin PEs including TCL, NMP, IPM, OA, B30, B98, S20 did not show a significant improvement in the CPO concentrations within the nail clippings.

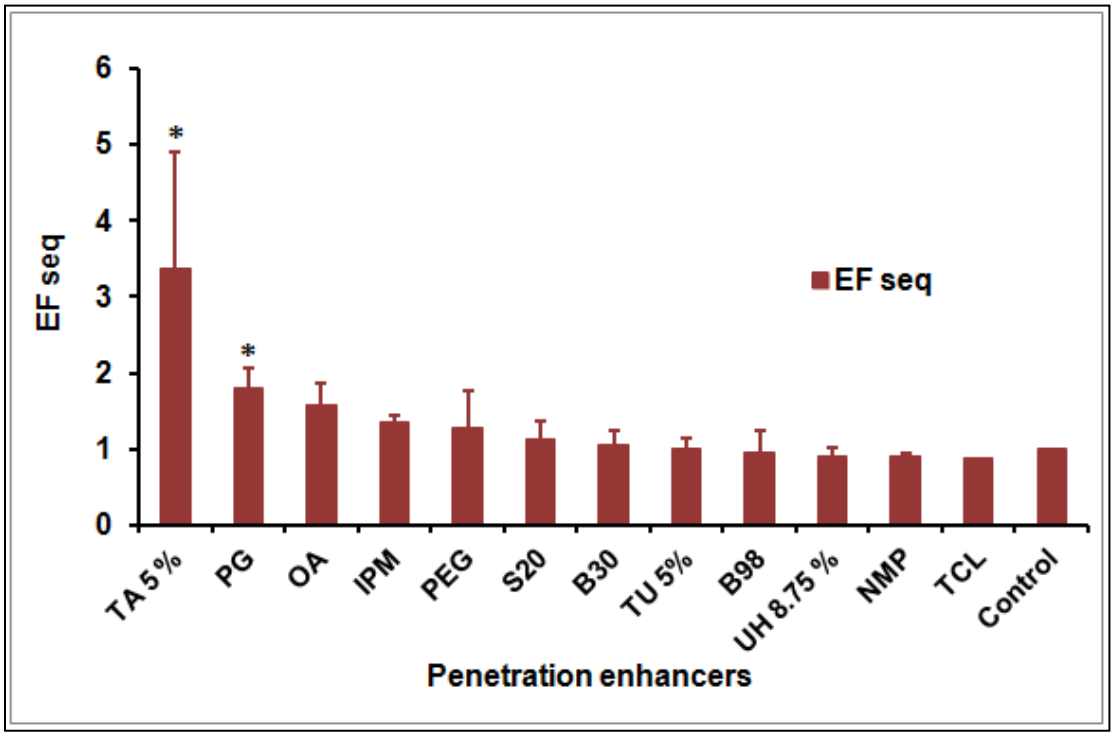


Figure 2.7: Accumulation of CPO within the nail clippings pretreated with the PEs.

The data from the sequential exposure study shows that the pretreatment with 5 % TA resulted in a 3.8 fold higher accumulation of CPO within the nail clippings and it was the best enhancer for CPO if used as a pretreatment. This observation is in alignment with the report showing 3.8 fold higher flux of permeation of caffeine through human nail, pretreated with 5 % TA (Khengar, R. *et al* 2007). One of the methods of action of the skin PEs is increasing the drug solubility. This was an attempt to identify if any of the skin PEs can enhance nail penetration by improving the solubility of CPO in the donor vehicle. One of the long term goals of the current project was to select enhancer(s) which could be incorporated within the transungual formulation containing CPO. The literature reports (Khengar, R. *et al* 2007 and Traynor, M.J. *et al* 2010) and figure 2.7 show a

significant improvement in drug penetration when TA was used for pretreatment of nails. However, inclusion of TA with CPO in a formulation could lead to incompatibility and precipitation of CPO. Therefore, TA was not studied further in the transungual permeation studies.

2.5.5.3. Simultaneous exposure of nail to CPO and PEs

A 5 % w/w TU solution was the only agent to significantly increase the penetration of ciclopirox olamine into the nail clippings compared to the control (Figure 2.8). The other nail PEs, TA and UH did not improve the penetration of CPO into the nail clippings. The skin PEs had no effect on the penetration of CPO into the nail. One of screening methodologies in literature for transungual delivery of antifungal agents, shows that 5 % w/v of TA significantly improves the *in vitro* penetration of CPO into human nail clippings (Chouhan, P. and Saini, T.R. 2012). In the report, CPO was prepared as a 1 mg/mL solution using 0.5 % SLS in pH 7.4 PBS containing TA. However, it was found that CPO had low solubility of 2.5 µg/mL in 5 % TA prepared in pH 7.4 PBS (figure 2.5). The reduced solubility significantly reduced the penetration of CPO into the nail clippings in presence of TA. Thus 5% TA is in quadrant II of the analysis plot in figure 2.9. The plot of enhancement factors for nail penetration and solubility (Figure 2.9) shows that 5 %w/w TU is the only agent in the third quadrant of the analysis plot which indicates improvements in solubility and nail penetration. The concentration gradient is the driving force for the passive diffusion of drug molecules across a membrane. As shown previously in table 1.7, PEs act by various mechanisms; one mechanism is by increasing the concentration gradient of the drug across the membrane by increasing the

solubility of the drug. Higher levels of solubilized form of CPO will ensure maintenance of high concentration gradient across the nail plate and the nail folds (skin). This plays an additive effect to the reducing action of TU on the disulfide bonds in nail keratin.

The *in vitro* simultaneous study identifies PEs that improve the drug concentration in the nail. However, the results of this study have to be confirmed with the *in vitro* transungual study because; both the dorsal and the ventral surfaces of the nail clippings are exposed to the drug and the PE in the nail penetration study. Nevertheless, the nail penetration study is a high-throughput experiment to select potential nail PEs. The 5 %w/w TU showed 1.86 fold improvement in solubility (Figure 2.9) and significant increase in the accumulation of CPO in the nail clippings. This solubility enhancement in presence of TU could be a drug specific phenomenon.

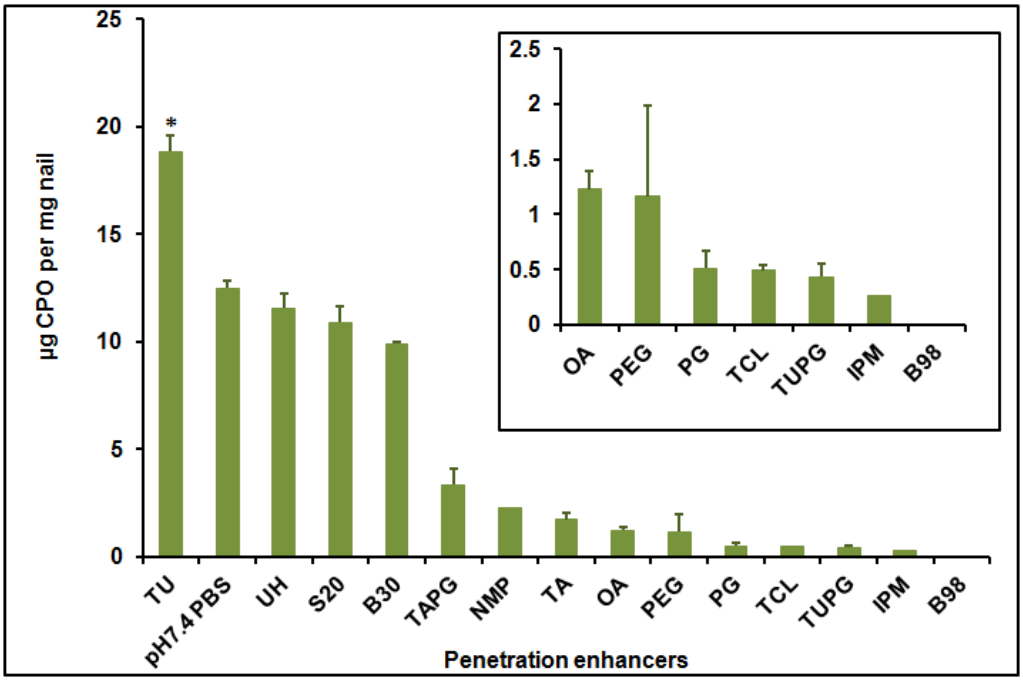


Figure 2.8: Concentration (µg CPO/mg nail) of CPO in the nail clippings in presence of PEs (simultaneous exposure study)

Thiourea is a strong reducing agent, which reduces the disulfide linkage of nail keratin and thereby improves drug penetration into the nail (Khengar,R. *et al* 2007). It can therefore be concluded from figure 2.9 that 5 % TU works by two mechanisms on the nail plate – improving levels of solubilized CPO in the vehicle and reducing the disulfide bonds of nail keratin.

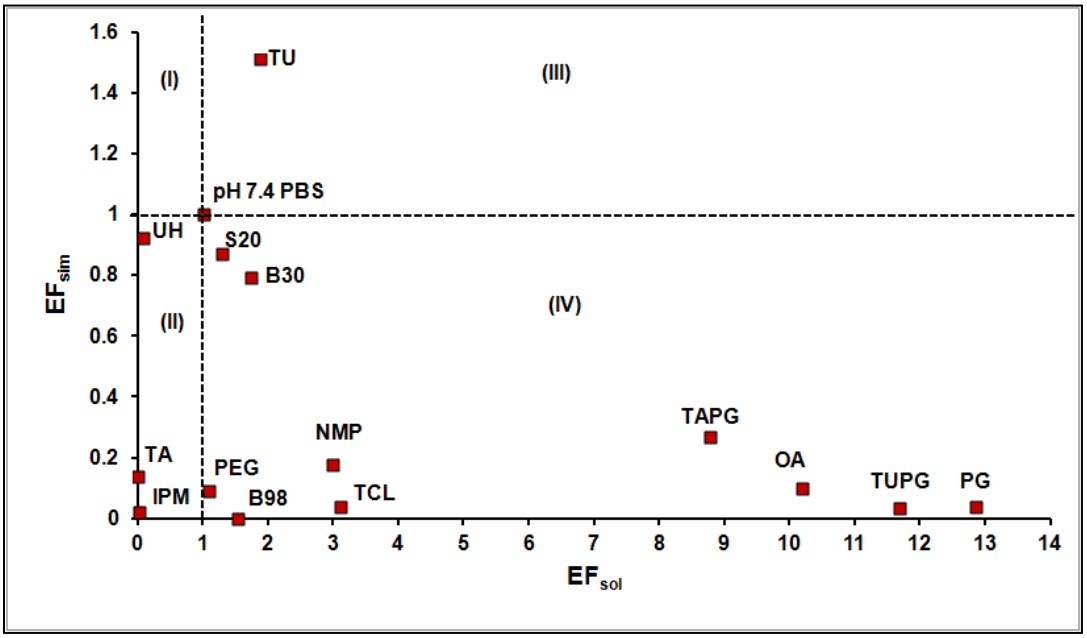


Figure 2.9: Analysis plot showing the relationship between the enhancements in solubility and nail penetration of CPO in presence of PEs.

2.5.6. The in vitro transungual permeation

The ability of TU in enhancing drug penetration was confirmed in the *in vitro* transungual permeation of CPO through human cadaver toe nail. The toenails, obtained from a female donor (55 yrs of age) were smooth on the surface, normal and devoid of any symptoms (excessive thickness, dystrophy and discoloration) of onychomychosis. The average

thickness of the toenails was 0.96 ± 0.014 mm. Figure 2.10 shows the permeation profile of ciclopirox olamine through human cadaver nail. A permeation flux of 1.56 µg/cm^2/day and a lag time of 14.5 days was observed. 188.44 ± 39.83 µg and 6.81 ± 1.24 µg of CPO were attained in the orifice and the periphery of the toenails respectively.

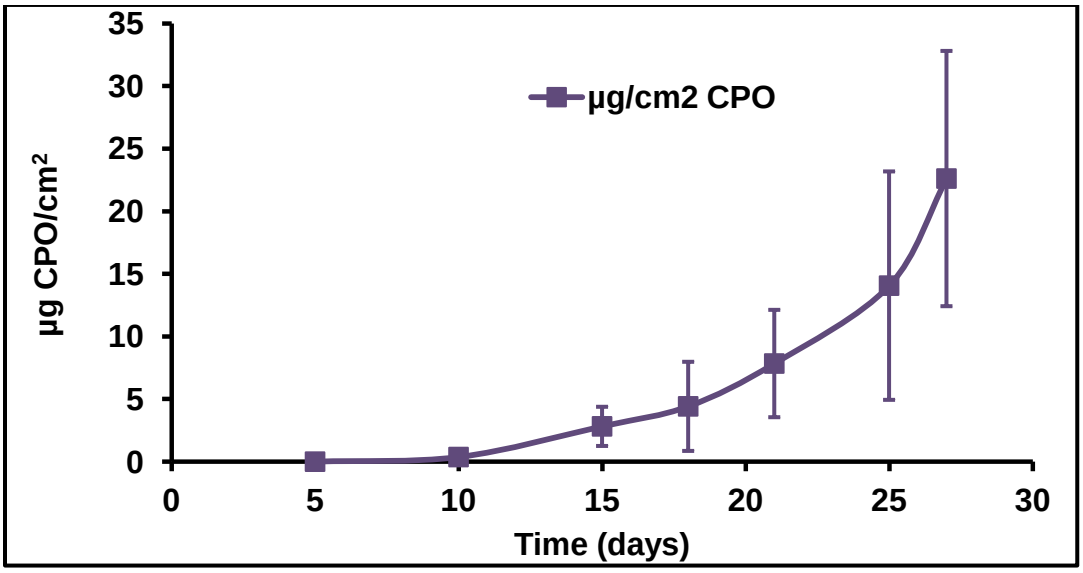


Figure 2.10: Transungual permeation of CPO in presence of 5 %w/w TU

The use of 5 % w/w TU results in good transungual permeation of CPO within 27 days. The reported minimum inhibitory concentration (MIC) of CPO against the dermatophytes is in the range of 0.004 to 1.0 µg/mL (Subissi, A. *et al* 2010, Kokjohn, K. *et al* 2003, Gupta, A.K. and Kohli, Y. 2003, Shehata, A.S. *et al* 2008 and Singh, J. *et al* 2007). The concentration of CPO attained in the receiver medium at the end of 27 days (1.79 ± 1.0 µg/mL) is greater than the reported MIC values for CPO. The concentration of CPO within the nail was calculated as mg/mL using the volume of the nail (product of nail

thickness and area exposed to CPO treatment). Thus the concentration of CPO within the nail is 27.71 ± 5.98 mg/mL and is four orders of magnitude higher than the reported MIC values for CPO (Subissi, A. *et al* 2010, Kokjohn, K. *et al* 2003, Gupta, A.K. and Kohli, Y. , 2003, Shehata, A.S. *et al* 2008 and Singh, J. *et al* 2007). The Nail-MFC (minimum fungicidal concentration in presence of nail powder) of CPO was determined against seven strains of *T.rubrum* growing on keratin (Schaller, M. *et al* 2009). The Nail-MFC for CPO was reported to be in the range of 16 – 32 µg/mL (Schaller,M. *et al* 2009). The concentration attained in the nail is significantly greater than the Nail-MFC of CPO. This high accumulation of CPO within the nail will also serve as a reservoir for drug release after termination of the therapy and prevent relapse or reinfection. Five percent TU was therefore selected as the PE for the nail.

2.5.7. In vitro skin permeation

The permeation parameters are shown in Table 2.1. The permeation flux of ciclopirox olamine through the skin increased 2.5 and 2.4 fold in presence of S20 and B30, respectively. Therefore, based on the preformulation strategy discussed in the chapter, S20 and B30 were found to be potential PEs for transdermal delivery of CPO. The permeation of CPO was lower than the control in presence of TCL and PG (Table 2.1)**.** The analysis plot in figure 2.11 shows the relationship between the enhancements in permeation flux and solubility of CPO in the presence of the PEs. The PEs S20, B30, B98 and NMP in quadrant III indicate their ability to increase the solubility of CPO and permeation through the skin. TCL, PG and OA in quadrant IV significantly improve the solubility multiple folds but do not show a corresponding increase in the flux of

permeation. IPM in quadrant II does not improve solubility or permeation. In topical drug delivery, the enhancers in the quadrants II and IV will be beneficial in minimizing the higher drug concentration reaching the systemic circulation.

The main goal of topical delivery is to maximize skin exposure and minimize the systemic exposure of the drug, thereby reducing the chances of adverse side effects and drug-drug interactions. The enhancers which increase the flux through the skin are beneficial for transdermal applications. The analysis plot in figure 2.11 shows that S20, B30, B98 and NMP belong to quadrant III because they improve both solubility and permeation of CPO. TU, B98, S20 and B30 showed <2 fold increase in solubility, while the enhancement in permeation flux for B98 to S20 was 1.3 to 2.5 fold.

The data suggests than improved solubility is not the only mode for increased flux. These non-ionic surfactants are speculated to alter the barrier properties by solubilizing the lipids in the stratum corneum and emulsifying the sebum (Williams,A. and Barry B., 2004). NMP increases both the drug solubility and permeation across the skin. Thus S20, B30, B98 and NMP are potential PEs for the transdermal delivery of CPO. Hence, these agents cannot be incorporated in the topical formulation containing CPO. For topical formulations, it is important to select PEs which increase the levels of the drug in the target organ, skin (Kumar, S. *et al* 1992). With the objective to identify one single enhancer for the skin and the nail plate, 5 % TU was also tested for its action on transdermal permeation and skin penetration of CPO. In favor of topical delivery, TU did not remarkably increase the skin permeation. However no significant drug accumulation was achieved in the epidermis in presence of TU. IPM in quadrant II and TCL, OA and PG in quadrant IV (figure 2.11) did not increase the permeation of CPO. Relative to the

other skin PEs, the lag time for CPO permeation (Table 2.1) is long in presence of PG at 8.3 ± 0.5 hr.

Table 2.1: The in vitro skin permeation parameters in presence of the PEs

PE	**Flux ($\mu g/cm^2/hr$)**	**Lag-time (hr)**	**$Q_{24\ hr}$ ($\mu g/cm^2$)**
S20	4.84 ± 1.49	6.2 ± 0.2	97.71 ± 8.16
B30	4.77 ± 0.15	5.9 ± 0.2	92.03 ± 2.67
NMP	2.58 ± 1.11	4.0 ± 2.4	50.39 ± 3.86
B98	2.52 ± 0.28	7.8 ± 0.1	42.78 ± 4.87
TU	2.06 ± 0.63	4.2 ± 1.9	42.67 ± 10.19
pH 7.4 PBS	1.95 ± 0.56	5.5 ± 0.4	38.89 ± 16.64
IPM	1.32 ± 0.74	8.1 ± 0.3	23.39 ± 13.27
OA	1.24 ± 0.15	4.6 ± 0.1	24.19 ± 2.91
TCL	0.95 ± 0.25	5.4 ± 1.2	15.71 ± 11.26
TAPG	0.61 ± 0.14	5.1 ± 0.8	10.78 ± 3.75
Loprox	0.19 ± 0.04	0.7 ± 0.5	4.73 ± 0.66
TA	0.16 ± 0.04	11.2 ± 4.1	5.07 ± 1.50
CPO suspn	0.16 ± 0.005	1.08 ± 1.05	4.58 ± 0.11
Penlac	**	**	3.28 ± 0.77
TUPG	0.07 ± 0.03	7.3 ± 0.4	1.16 ± 0.5
PG	0.046 ± 0.01	8.3 ± 0.5	0.85 ± 0.27

***Could not be calculated because of non-steady state permeation profile*

This effect is attributed to the increased tortuosity of the stratum corneum in presence of 100 % PG, caused by dehydration of the epidermis (Thomas, N.S. and Panchagnula, R. 2003). Though PG shows the greatest solubility for CPO, it shows lowest flux for its transdermal permeation. This phenomenon is a concentration dependent effect of PG. PG at higher concentrations is reported to reduce the transdermal flux of the permeant (Touitou, E. and Abed, L.1985 and Bonina, F.P. and Montenegro, L. 1992) due to increase in viscosity and dehydration of the stratum corneum (Thomas, N.S. and Panchagnula, R., 2003). Moreover at higher concentrations of PG, there is extensive water loss from the polar head regions in the epidermal layer, increase in viscosity and decrease in partition coefficient of the drug (Thomas, N.S. and Panchagnula, R. 2003). This indicates that in the presence of 100 % PG, the contribution of EF_{sol} of CPO towards its $EF_{finite\ flux}$ is minimal.

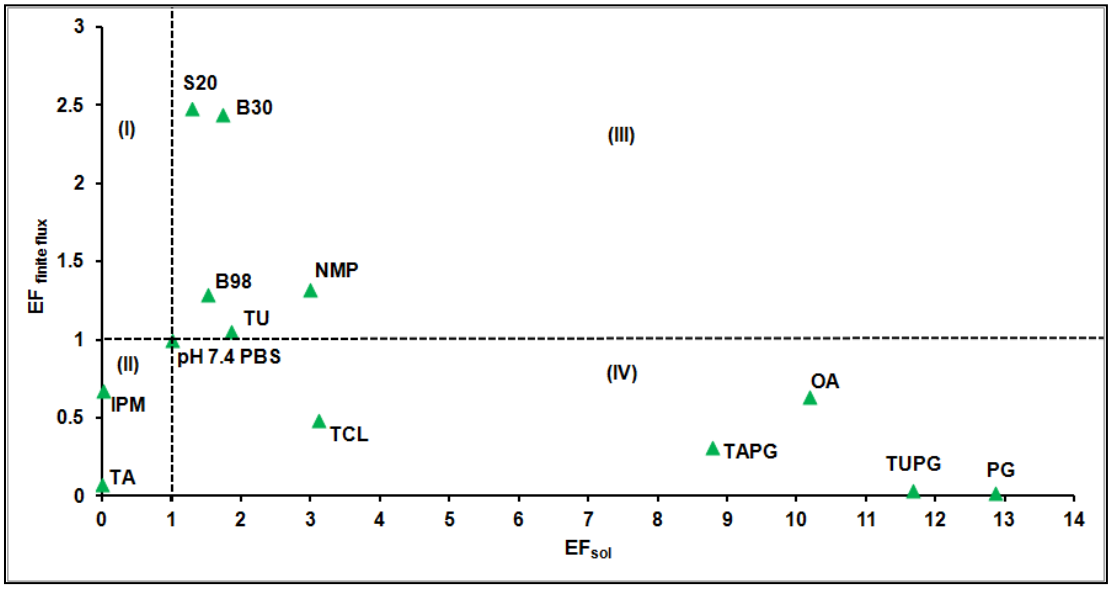


Figure 2.11: Analysis plot showing the relationship between the enhancements in solubility and skin permeation flux for CPO in presence of PEs

2.5.8. In vitro skin penetration

The skin penetration study quantifies the levels of the drug in the epidermal and dermal layers of the skin. One of the target sites for the topical antifungal therapy is the skin, specifically the epidermis. The final selection criteria for the optimum enhancers, is their ability to enhance the drug accumulation in the epidermis. The drug levels in the epidermis and dermis of the skin along with the flux values in the finite dose skin studies will serve as a better criterion for selection of PEs for the skin.

The data in figure 2.12 is arranged in the decreasing order of the enhancement factor in the epidermis. Any value greater than 1 for enhancement factor is desirable in the epidermis because the superficial cutaneous infections reside in the epidermis. Figure 2.12 shows the enhancement in accumulation of CPO in epidermis and dermis of the skin in the presence of the enhancers. PG, TCL, IPM and OA showed 3.2, 2.2, 1.6 and 1.03 fold enhancement in drug levels in the epidermis. The same agents showed 0.5, 2.3, 5.6 and 0.6 fold improvement in drug levels within the dermis. The enhancement levels for the commercially available formulations of CPO are on the lower end. The skin permeation studies (Table 2.1) showed that the flux or drug permeation is high for S20 and B30. In figure 2.12, the quadrant III has NMP, B30, S20 and TU which showed 5.9, 2.1, 1.6 and 0.6 fold accumulation in the epidermis. Though NMP and B30 showed high drug accumulation in the epidermis, the increase in transdermal permeation would increase the amount of drug in the systemic circulation, which could lead to the adverse effects. Drug permeation in the presence of propylene glycol and transcutol is the least, while the enhancement in drug levels in the epidermis is high. PG shows a 3.2 fold enhancement in the epidermis while transcutol shows 2 fold increase. The enhancement levels corresponding to propylene glycol is higher than the commercial formulations. PG

was the only agent which showed accumulation of CPO specifically in the epidermis and a low permeation flux of 0.046 µg/cm^2/hr.TCL did not show any specificity in the accumulation of CPO in any particular layer of the skin. The EF for the drug in dermis in presence of TCL is also 2.2 folds. The blood capillaries are located in the lower layers of the dermis. If the levels of the drug increases in the dermis, it is probable that this drug will eventually partition into the blood. Owing to all these, propylene glycol was chosen over transcutol. Figure 2.13 also shows the relationship between $EF_{epidermis}$ and EF_{sol}. PG has now moved to the third quadrant, showing a 12-fold improvement in solubility and 3.2 fold increase in CPO levels in the epidermis.

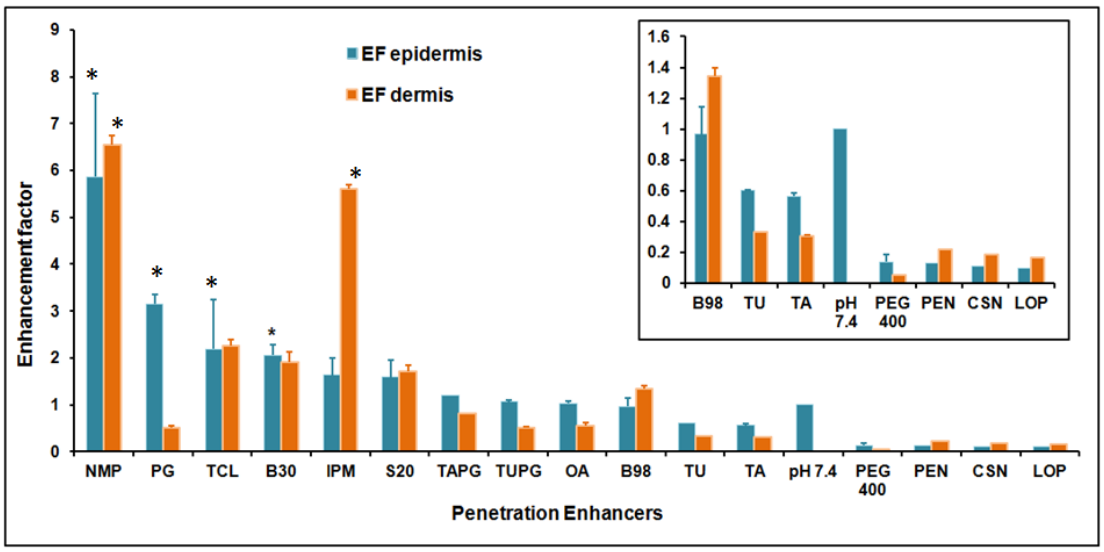


Figure 2.12: Enhancement in accumulation of CPO within epidermis and dermis in presence of PEs and the commercial formulations

IPM showed 1.6 and 5.3 fold accumulation in the epidermis and the dermis respectively (Figure 2.12). Since IPM was not selective in CPO penetration into the epidermis, it was not selected as the skin PE of CPO. Similarly OA was also eliminated as the skin PE because of minimal accumulation within the epidermis. Inspite of higher drug accumulation in the epidermis and dermis in presence of NMP, B30 and S20, these PEs are not preferred for further formulation development, because they also increase the permeation of CPO through the skin which could lead to other toxicities. Therefore, it is important to choose PEs which show greater accumulation in the layers of the skin with minimum flux for the drug permeation. PG and TCL both showed good accumulation of CPO within the epidermis of the skin. PG and TCL are reported to improve drug penetration into the skin while reducing the transdermal permeation (Lorenzetti, O.J., 1979, Ritschel, W. *et al* 1991, Puglia, C. *et al* 2001 and Williams, A. and Barry B., 2004). Additionally, in presence of PG, 5.29 ± 0.19 mg/mL of CPO was quantified within the epidermis. This value is significantly higher than the reported MIC of 0.004 - 1.0 µg/mL. PG was chosen as the best enhancer for the skin penetration of CPO, because it showed much lower permeation flux than the control and enhanced the levels of CPO , selectively within the epidermis.

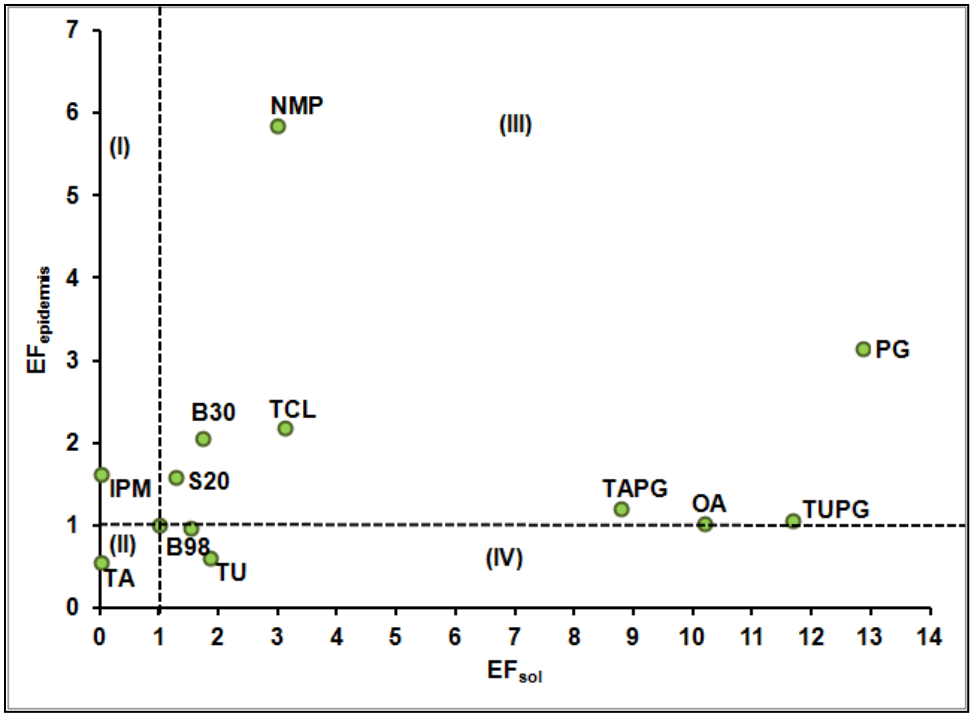


Figure 2.13: Analysis plot showing the relationship between the enhancement factors in solubility and epidermal accumulation of CPO in presence of PEs

2.5.9. Combination of PEs

The simulatneous and sequential nail studies each identified 5 % TU and 5 % TA respectively as the nail PE for CPO. The skin studies showed the potential of PG as the skin PE for topical delivery of CPO. Since two different PEs were selected for the nail and the skin, a combination of the two agents 5 % TU and PG (TUPG) and 5 % TA in PG (TAPG) were studied for saturation solubility, nail penetration, skin permeation and skin penetration. The solubility of CPO in the combinations of PEs was significanlty increased compared to the individual nail PE. In the presence of TUPG, the saturation solubility of CPO was 278.99 ± 8.41 mg/mL and that for TAPG was 209.65 ± 28.70 mg/mL. These

values are much higher than the solubility of CPO in 5 % TU and 5 % TA. These combinations were studied in the simultaneous nail exposure study to determine if any of the combination would give an additive effect. Figure 2.9 shows that like PG, TAPG and TUPG were in quadrant IV of the analysis plot. It was expected that one of these combinations will retain the individual effects of TA or TU and result in improved CPO penetration into the nail clippings. In the presence of TAPG, the skin permeation flux value for CPO was 0.61 ± 0.14 μg/cm^2/hr and a 3 fold lower concentration of CPO was observed in the receiver compartment at 24 hr compared to the control (Table 2.1). TUPG showed results comparable to that of PG with a very low flux value of 0.07 ± 0.03 μg/cm^2/hr and a drug concentration of 1.16 ± 05 μg/cm^2 in the receiver compartment. These results again confirmed that the combinations behaved more like PG in the nail and the skin. This could be attributed to the larger proportion of PG in the combinations. However, the data from the CPO penetration into the epidermis did not show effects similar to PG alone. The inset in figure 2.12 shows that TA and TU did not show any favorable accumulation of CPO within the epidermis relative to the control. Compared to TA and TU, the CPO accumulation in the epidermis was improved in presence of TAPG and TUPG . This enhancement was not comparable to the 3.2 fold enhancement in presence of PG. Thus, the skin penetration data shows that the combination of PEs did not behave like PG within the skin. Though the combinations improved solubility and minimized skin permeation, it was not beneficial in improving drug levels within the nail and the epidermis of the nail folds. The individual affects of 5 % TU and PG were absent when 5 %TU was prepared using PG (TUPG). The combinations TUPG and TAPG did not yield an additive effect with respect to nail penetration and skin penetration. The

cause of loss of the individual effects of 5 % TU, 5 % TA and PG in the combination was not investigated further. Since 5 % TU and PG were selected using the preformulation screening strategy, it was concluded that the formulation has to be designed to incorporate both of the enhancers but without losing the individual positive effect of thiourea and propylene glycol. This poses additional challenge in designing and developing a formulation with these attributes.

2.6. Conclusion

In passive diffusion of the drug, the concentration gradient is the driving force for drug diffusion. It is therefore important to maintain a high concentration gradient between the formulation and the target tissues. This is achieved with the use of specific PEs, which also have other underlying mechanisms towards the target tissues. A preformulation strategy has been proposed for the selection of appropriate PEs for drug delivery into the nail plate and the nail folds. The saturation solubility experiments identified PEs which increase the solubility of ciclopirox olamine and can be potential enhancers. The aim of the study was to identify a single enhancer or enhancer combination which can be effective for skin penetration and nail penetration and permeation of ciclopirox olamine. Skin and nail being very different tissues, no single enhancer or a combination worked well in both tissues. The nail penetration and permeation studies showed that 5 % TU could be a potential enhancer for the nail permeation if the drug and the enhancer are to be incorporated in the same formulation. The skin permeation and penetration studies identified the effect of propylene glycol in increasing the levels of the drug in the

epidermis while minimizing transdermal permeation. Incorporating the two different enhancers into a single topical formulation is challenging.

CHAPTER 3

THE INTERACTIONS BETWEEN CICLOPIROX OLAMINE AND THE TARGET TISSUES – NAIL AND NAIL FOLDS

3.1. Abbreviation

CPO: Ciclopirox olamine

MR: Methyl red sodium salt

pH 7.4 PBS: 10 mM Phosphate buffered saline, pH 7.4

TU: Thiourea

3.2. Introduction

The first specific aim involved study of the critical interactions between CPO and the target tissues and effects on drug delivery into and through the nail.

It was hypothesized that the following interactions between the drug (ciclopirox olamine) and the biological membranes (nail and skin) can have an impact on the transungual delivery of CPO.

1. Drug partitioning into human nail
2. Drug-keratin binding
3. Lateral diffusion
4. Drug-epidermis binding

It is important to understand the interaction between CPO and the two target site membranes (nail and skin), to identify the critical interactions which can impact the levels of drug penetrating the skin and nail and the levels of drug permeating through the nail to reach the nail bed. This also has an implication on the effects of the enhancers incorporated in the formulation. Exploring these interactions will help to understand the process of drug permeation through the nail plate. All of the following studies utilize 5 % TU as the penetration enhancer for the nail. TU was selected as the transungual penetration enhancer for CPO based on the preformulation strategy elaborated in chapter 2.

3.2.1. Drug partitioning into human nails (healthy and infected)

The ability of the drug to partition into the nail is critical in topical drug delivery. For optimal permeation across the nail, the topically applied drug should partition favorably into the nail plate from the formulation and diffuse out of the nail, from the ventral surface to reach the nail bed. The drug partitioning experiments were performed using healthy and infected cadaver nails. The healthy cadaver nails and nail clippings were used throughout the preformulation screening in Chapter 2. Any difference in the partitioning behavior of CPO in the healthy and diseased nails can be detected by a change in lag times of permeation across infected cadaver nails.

3.2.2. Drug – keratin binding

Ciclopirox olamine is reported to show 94-97% protein binding (Kellner, H.M. *et al* 1981). It was therefore hypothesized that the CPO will bind to keratin. Keratin is present in both the nail plate (90 % of total nail plate mass) and the stratum corneum (80 % of total protein content) of the skin (Rice, R.H. *et al* 2010 and Bragulla HH and Homberger DG. 2009). CPO-keratin binding, if significant will imply that CPO can bind to the two target tissues and have an effect on the transungual permeation and skin penetration of CPO. The bound drug is unavailable to diffuse out of the tissue and is rendered inactive against the dermatophytes (Tatsumi, Y. et al 2002). This strong binding coupled with the partitioning effect can reduce the overall concentration of the drug available at the ventral side of the nail plate in the *in vitro* transungual permeation studies. The opposite perspective is that, this drug-keratin binding can also be viewed as an advantage, because the bound drug can serve as a reservoir, once drug therapy is stopped.

3.2.3. Lateral diffusion in human nails

In passive diffusion, the concentration gradient across the nail plate acts as the only driving force for drug permeation. It was hypothesized that the lateral movement of drug molecules around the site of application on the nail or *lateral diffusion*, during an *in vitro* experiment could lead to a reduction in the overall permeation of the drug molecule across the nail plate (Figure 3.1). The lateral diffusion beyond the area of the donor compartment or dosing area can lengthen the lag-time and initial

permeation rate until the drug reaches an equilibrium in the adjacent tissue. The greater the excess area of the tissue surrounding the receptor dosing area, the larger the negative impact on transungual permeation.

When there is simultaneous lateral and transungual permeation during the *in vitro* permeation experiment, the generated data underestimates the potential of the penetration enhancer or a prototype formulation compared to the values that would be obtained *in vivo*. This data can therefore lead to early elimination of a penetration enhancer or formulation because it does not meet the target *in vitro* permeation rate. Therefore, the main objective was to demonstrate the process of passive lateral diffusion in the human nail plate and its effect on passive transungual permeation of the antifungal drug ciclopirox olamine. A secondary goal was to minimize lateral diffusion in the *in vitro* transungual permeation studies in order to generate more reliable data that would be reflective of in vivo permeation. It is important to note that the impact of lateral diffusion on transungual permeation is profound in passive drug delivery relative to iontophoresis.

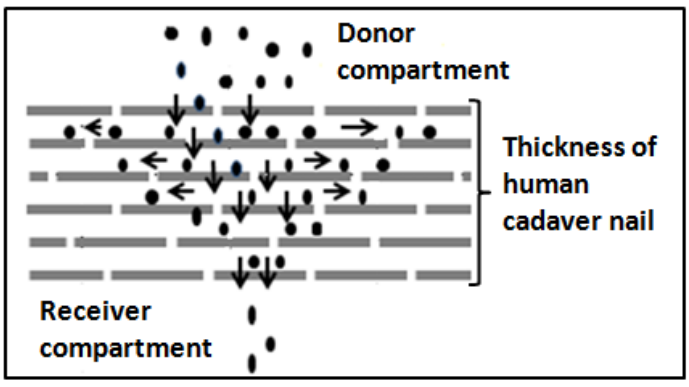


Figure 3.1: Lateral diffusion in human nail plate

3.2.4. Drug-epidermis binding

The epidermis of the nail folds, hyponychium and the nail bed is one of the target sites identified for topical therapy of onychomychosis. The uppermost layer of the epidermis is the stratum corneum, which is rich in keratin. The epidermal binding when compared with keratin binding can give insight to the other possible binding sites for CPO other than keratin in the stratum corneum (Banning,T.P. and Heard, C.M. 2002). Extensive of binding CPO to epidermis would minimize the amount of CPO permeating across the skin into the systemic circulation. In epidermal delivery of CPO, the main goal is to minimize the levels of CPO reaching the systemic circulation.

3.3. Materials

Ciclopirox olamine (CPO) was purchased from Haorui Pharma-chem Inc., Edison, New Jersey, USA. Potassium phosphate monobasic, sodium hydroxide, gentamicin sulfate, methyl red sodium salt (MR) (95% dye content), dimethyl sulfate and triethyl amine were purchased from Sigma Aldrich. Thiourea and sodium phosphate dibasic were obtained from Acros Organics. Soft stainless steel wire with 0.02 gauge size was bought from Malin Co. (Brookpark, Ohio). Keratin powder was obtained from Spectrum Chemicals. Franz diffusion cells and neoprene nail adapters were purchased from PermeGear. The human nail clippings were donated by 20 healthy volunteers in the Department of Pharmaceutical Sciences, Temple University. Human cadaver toe nails were purchased from Anatomy Gifts Registry (Hanover, Maryland). Nanopure

water was used for preparation of 10mM pH 7.4 phosphate buffered saline. HPLC grade Acetonitrile from Fischer scientific was used in preparation of mobile phase. All the chemicals were used as received without any additional purification.

3.4. Experimental Methods

3.4.1. Sample Preparation for analysis

To quantify the amounts of ciclopirox olamine within the nail, a modification of the reported derivatization method (Lehr, K-H and Damm, P. 1985 and Escarrone, A.L. *et al* 2008) for CPO was used. The human nails were firstly dissolved in 1 M sodium hydroxide solution at 37 °C. The nail solutions were filtered through Restek® 0.45 µ syringe filters (cellulose acetate membrane). These solutions were diluted with pH 7.4 PBS to reduce the strength of 1 M NaOH to 0.1 M before derivatization. For the derivatization of CPO, 25 µL of dimethyl sulfate was added to 0.2 mL of the dilute nail solution, and vortexed for 5 min. These solutions were maintained at 37 °C for 20 min. 25 µL of triethylamine was added to terminate the reaction and vortexed for 1 min. The solutions were then filtered through Restek® 0.22 µ syringe filters (cellulose acetate membrane) and analyzed using the HPLC method. For assay of CPO in the buffer, a similar pre-injection derivatization process was used. To 0.2 mL of the samples, 50 µL of 0.1 M NaOH and 25 µL of dimethyl sulfate were added and vortexed for 5 min. The solutions were maintained at 37 °C for 20 min prior to addition of 25 µL of triethyl amine. The solutions were vortexed, filtered through 0.22 µ syringe filters and analyzed using HPLC.

3.4.2. Method of Analysis

3.4.2.1. High Pressure Liquid Chromatography

Ciclopirox olamine (CPO) in the derivatized samples were quantified using the modified HPLC method discussed in Chapter 2 (Escarrone,A.L. *et al.* 2008 and Lehr K-H and Damm P. 1985). An Agilent1100 series HPLC with autosampler and dual wavelength detector was used. The mobile phase was acetonitrile:water (50:50 v/v) with a flow rate of 1 mL/min. The column was a 150 by 4.6 mm Eclipse Plus C18 column (Agilent). The sample injection volume was 50 µL. The detection wavelength was 298 nm.

3.4.2.2. UV/Vis Spectroscopy

The sodium salt of methyl red sodium (MR) level in the nail and the buffer was quantified using visible spectroscopy at 480nm. The absorbance of solution of nails dissolved in 1 M sodium hydroxide, without MR was used to correct the absorbance of the samples. A standard curve for methyl red in the nail solution was obtained using a concentration range of 6.77 µg/mL to 108.33 µg/mL.

3.4.3. Recovery of ciclopirox olamine from human nails

Solutions of ciclopirox olamine in pH 7.4 PBS with concentrations 0.0253 mg/mL, 0.0505 mg/mL, 0.202 mg/mL, 0.809 mg/mL, 3.24 mg/mL and 6.47 mg/mL were prepared. Approximately 20 mg of human nail clippings were weighed for each sample. Each concentration was studied in triplicate. The nail clippings were treated

with 200 µL of each of these solutions for 3 hr at 32 ± 1°C. Two hundred µL of 1M NaOH was added to dissolve the nail clippings. The nail solutions were diluted to reduce the concentration of 1 M NaOH to 0.1 M and the sample preparation method from section 2.2 was followed using the dilute nail solution to quantify the concentration of CPO. The use of human nail clippings in the study was approved by Temple University Institutional Review Board (Protocol number 13671).

3.4.4. Drug partition into human nails (healthy and infected)

Whole human cadaver toenails (healthy and infected) obtained from Anatomy Gifts Registry were placed at 100 % RH for 24 hr prior to start of the experiment to ensure equilibrium hydration of the nails (Figure A.3, Appendix A). The drug partitioning into the nail was studied in saturated solution of CPO in pH 7.4 PBS (CPO solubility in pH 7.4 PBS is 23.92 ± 3.71 mg/mL) and in saturated solution of CPO in 5 % w/w TU in pH 7.4 PBS (CPO solubility in 5 % TU is 44.40 ± 2.0 mg/mL). The following methodology remained the same for healthy and infected human nails. Each of the samples was studied in triplicate. Upon hydration, the nails were cut into two along their lengths. The weight of each of the nail halves were measured before the experiment. The schematic in figure 3.2 shows the treatment of the nail pieces with the drug solution. The pieces A and C were dissolved in 1 M NaOH solution to determine the drug content (µg) per mg of the nail. The nail pieces B and D were placed in 0.7 mL of pH 7.4 PBS to determine the levels of drug partitioning from the nail into the buffer in 72 hr. At the end of 72 hr, the nail sections B and D were wiped with tissue paper and dissolved in 0.5 mL of 1 M NaOH. The nail solutions were evaluated for the content of CPO using HPLC. The levels of drug remaining within

the nail and amount that partitioned into pH 7.4 PBS was determined. The concentration of CPO (µg/mg of nail) was compared between A and B in set 1 and C and D in set 2 using one way analysis of variance ANOVA (Microsoft Excel 2007). The study was performed in triplicate, using three whole cadaver toe nails. Three separate toe nails from the same donor were used to reduce inter-subject variability. The same process was followed for the infected nails. The variability increased significantly for infected nails because the stage of the infection, the thicknesses of the nails and dystrophy of the nails from the same donor are highly variable.

3.4.5. Drug-keratin Binding

Drug-keratin binding was performed using keratin powder. Three milligram of CPO was weighed and then dissolved in sufficient volume of 5 % TU in pH 7.4 PBS solution to obtain a 10 mL, 300 µg/mL CPO stock solution. Serial dilution of the stock solution was performed to obtain ten concentrations in the range of 0.59 µg/mL to 300 µg/mL CPO. Approximately 20 mg (19 – 23 mg) of keratin powder was used for each sample. Each of the concentrations was studied in triplicate. The weighed keratin powder (spectrum chemicals) was added to 2 mL micro centrifuge tubes. To each tube 0.2 mL of the drug solutions were added and the tubes were placed in the water bath at 32 ± 1 ºC for 48 hr. Each of the samples was emptied into micro-centrifuge tubes containing 0.22 µm filter cartridges (Ambion). The samples were centrifuged at 10000 rpm for 10 min. The unbound concentration of CPO in the filtrate was quantified using HPLC. This method is a modification of a previously reported method (Banning, T.P. and Heard C.M. 2002). The bound concentration of

CPO was calculated by subtracting the unbound concentration from the incubation concentration. The incubation concentration and the bound concentration of CPO were normalized with the weight of keratin powder in the sample and plotted.

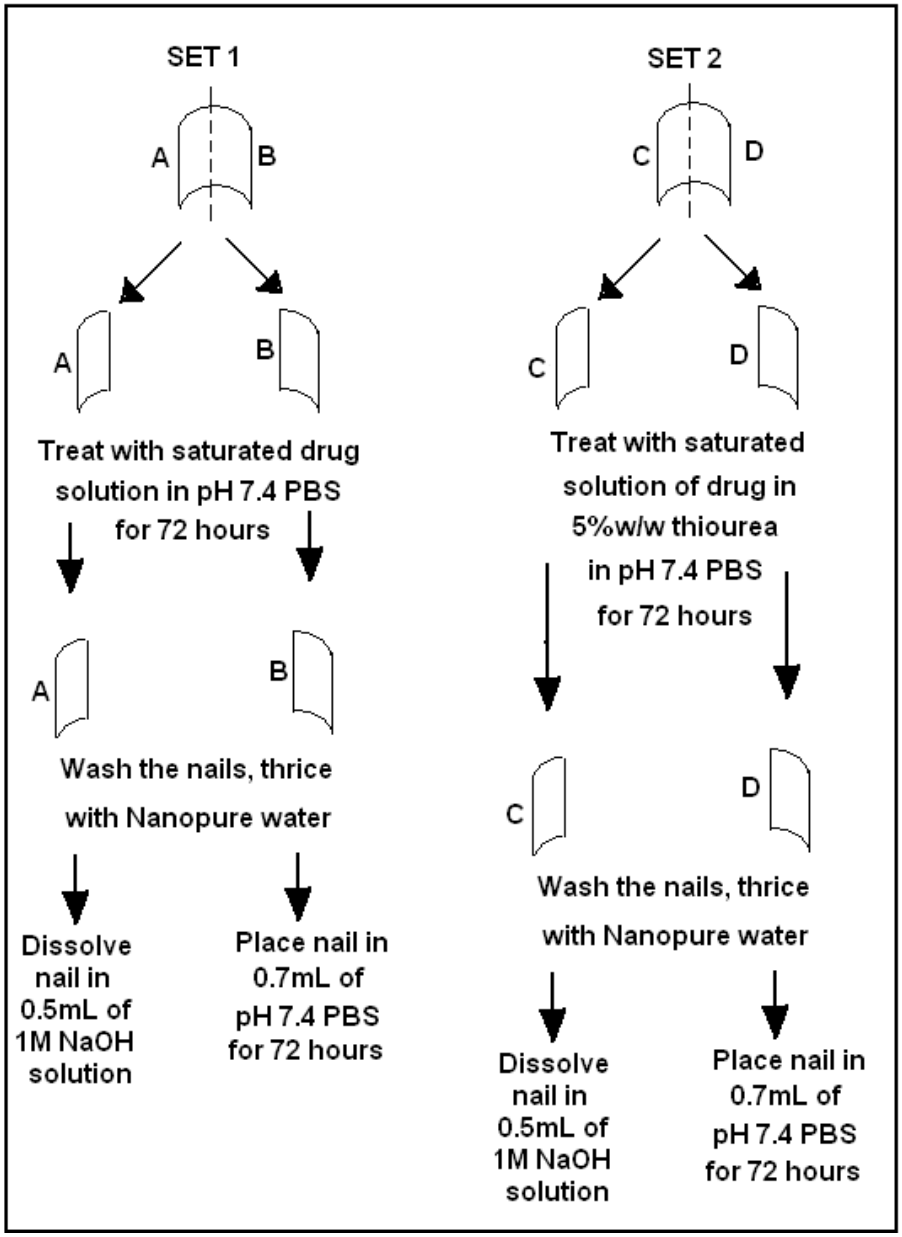


Figure 3.2: Schematic for the CPO-Nail partition study

3.4.6. Methyl red-keratin Binding

Three mg of MR was weighed and then dissolved in sufficient volume of 5 %TU in pH 7.4 PBS solution to obtain 10 mL, 300 µg/mL MR stock solution. Serial dilutions of the stock solution produced ten standard concentrations in the range of 0.59 µg/mL to 300 µg/mL MR. Serial dilution of the stock solution was performed to obtain ten concentrations in the range of 0.59 µg/mL to 300 µg/mL. Approximately 20 mg (19 – 23 mg) of keratin powder was used for each sample. Each of the concentrations was studied in triplicate. The weighed keratin powder (spectrum chemicals) was added to 2 mL micro centrifuge tubes. 0.2 mL of the drug solutions were added to each tube and the tubes were placed in the water bath at 32 ± 1ºC for 48 hr. Each of the samples was emptied into micro centrifuge tubes containing 0.22 µm filter inserts (company name of filter insert). The samples were centrifuged at 10000 rpm for 10 min. The unbound concentration of MR in the filtrate was quantified using Visible Light Spectroscopy at detection wavelength of 490 nm. The bound concentration of MR was calculated by subtracting the unbound concentration from the incubation concentration. The incubation concentration and the bound concentration of MR were normalized with the weight of the keratin powder in the sample and plotted.

3.4.7. Lateral Diffusion in human nails

3.4.7.1. Lateral Diffusion of Ciclopirox olamine and Methyl red in the nail

Sodium methyl red was used to visualize the lateral diffusion of CPO in human cadaver toe nails. A 5 % TU solution was prepared using pH 7.4 PBS, containing

0.01% w/v gentamicin sulfate as an antimicrobial agent (PBS-GS). This buffer was used to prepare a 3 mg/ml MR solution. An excess amount of CPO was added to the above solution and equilibrated for 72 hr to form a saturated solution of CPO in presence of MR and TU (C-MR-TU). Two cadaver toe nails were hydrated at 100% RH for 24 hr prior to the start of the experiment. Each of the toe nails was cut longitudinally to produce two equal pieces. Each nail piece was suspended vertically with the proximal end suspended 2 mm into the C-MR-TU solution. Figure 3.3 shows the schematic of the experimental method. The study was performed in duplicate. The duration of the experiment was determined by the time required for the methyl red to diffuse to the distal end of the nail, when observed visually. At the end of experiment, the nails were dismounted and cut into different sections (based on the color gradient of the dye). The weights, thicknesses and the lengths (x_1 to x_6) (figure 3.3) of each section was measured. Each of the sections was dissolved in 0.2 mL of 1M NaOH solution. The nail solution was derivatized and analysed using HPLC. The absorbance of each of the diluted nail solutions, prior to derivatization was quantified using the process described in the previous sections.

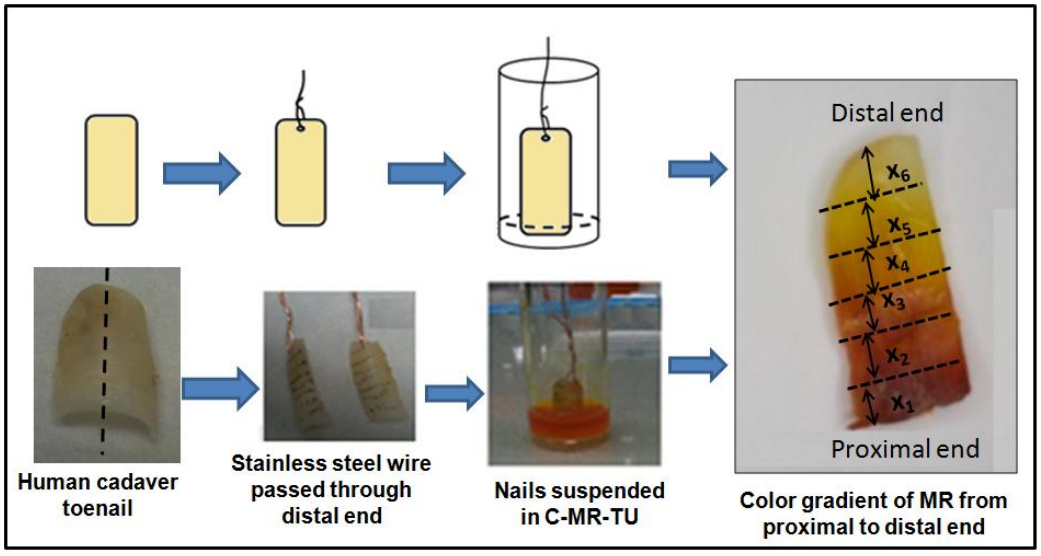
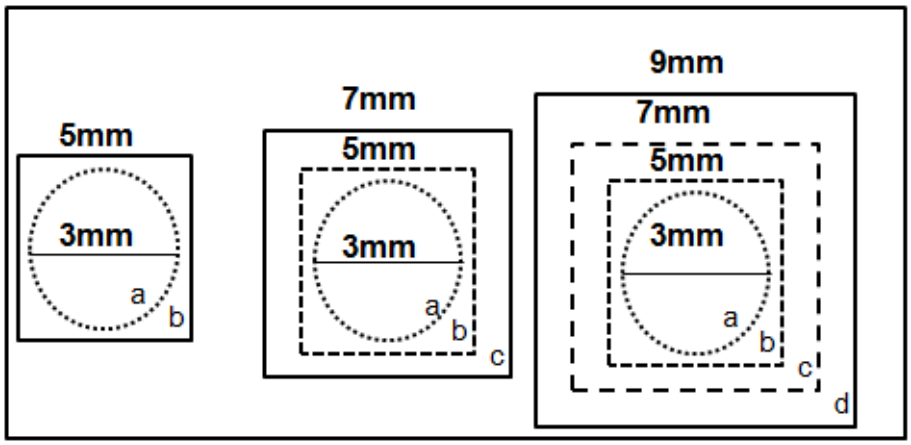


Figure 3.3: Schematic for lateral diffusion of CPO and MR in the nail

3.4.7.2. *In vitro* transungual permeation of CPO

The C-MR-TU solution was prepared as described in the previous section. The cadaver toe nails were pre-hydrated at 100 % RH for 24 hr. The thickness of the nails was measured using digital calipers (Marathon), prior to mounting on 3 mm diameter nail adapters (PermeGear) (Figure 2.3). Three toenails each of the 5 X 5 mm (25 mm^2), 7 X 7 mm (49 mm^2) and 9 X 9 mm (81 mm^2) were trimmed (Figure 3.4). The different dimensions were used to study the extent of lateral diffusion of CPO and MR during the *in vitro* transungual permeation. The receiver compartment of the Franz cell (Figure 2.2) was filled with 3 mL of PBS-GS (pH 7.4) and the temperature was maintained at 32 ± 1ºC. To mimic daily dosing, the nails were dosed with 21.4 µL of C-MR-TU and kept occluded for 40 days. The solution from the previous day was removed from the donor compartment, prior to application of subsequent doses of C-

MR-TU. On days 5, 10, 15, 20, 25, 30, 35 and 40, a sample of 0.5 mL of receiver medium was removed and an equal volume of fresh pH 7.4 PBS-GS was added. On day 40, the nails were dismounted and the area underneath the orifice and surrounding sections of the periphery were cut (as shown in figure 3.4) for separate analysis. These sections were separately dissolved in 0.1mL 1 M NaOH. The levels of CPO and MR in the nail were quantified using HPLC (298 nm) and visible light spectroscopy (480 nm), respectively. Similarly the samples withdrawn at the different time points were quantified for ciclopirox olamine. The experiment was performed in triplicate for each nail size.



[a]The circular section under the orifice; [b,c,d] The peripheral areas around the orifice.

Figure 3.4: The sectioning of the toenails after in vitro transungual permeation for CPO quantification in the nail orifice and the periphery.

3.4.8. Drug-Epidermis binding

Human cadaver skin was thawed at room temperature and placed in pH 7.4 PBS for 20 min prior to start of the experiment. Skin pieces each with a diameter of 9 mm were cut using a cork screw borer. The epidermis was separated from the dermis in each of the pieces using a modification of the heat separation method (Kassis,V. and Sondergaard,J. 1982). Each of the skin pieces were wrapped in aluminum foil and placed in the oven at 45 ºC for 10 min. Using sharp forceps, the epidermis was pulled away from the dermis and placed in a 2 mL micro centrifuge tube. 21.3 mg of CPO was weighed and dissolved in a sufficient volume of 5 %TU in pH 7.4 PBS solution to obtain a 10 mL of stock solution with a concentration of 2.13 mg/mL. A serial dilution of the stock solution was performed to obtain ten concentrations in the range of 4.2 µg/mL to 2.130 mg/mL. Each concentration was studied in four replicates. The epidermis was dosed with 0.2 mL of the standard solutions and maintained at 32 ± 1 ºC for 24 hr. The samples were transferred to micro centrifuge tubes with 0.22 µ filter cartridges (Ambion) and centrifuged at 10,000 rpm for 10 min. The unbound drug concentration was quantified in the filtrate using HPLC. The bound concentration of CPO was calculated by subtracting the unbound concentration from the incubation concentration. A plot of the molar concentrations of the bound drug against incubation concentration was constructed.

3.5. Results and Discussion

3.5.1. Method of Analysis

3.5.1.1. High Pressure Liquid Chromatography

A linear response was attained for CPO in the range of 0.09 µg/mL to 46.73 µg/mL with a correlation coefficient of 0.9996 (figure A.1, Appendix A). The retention time for the derivatised CPO was 4.6 min. The limit of detection (LOD) was 25 ng/mL and the limit of quantification (LOQ) was 90 ng/mL.

3.5.1.2. Visible Spectroscopy

A linear response with a correlation coefficient of 0.9981 was obtained for MR in the concentration range of 6.77 µg/mL to 108.33 µg/mL (Figure A.2, Appendix A).

3.5.2. Recovery of ciclopirox olamine from the nail

A recovery of 86-97 % was obtained for CPO in the concentration range of 0.25 µg/mg nail to 64.7 µg/mg nail.

3.5.3. Drug partitioning into human nails (healthy and infected)

For treatment of OM, it is important that the drug applied on the dorsal surface partitions and diffuses into the nail plate. However, the drug should also partition out of the nail from the ventral side to reach the nail bed. Table 3.1 shows the concentration (µg of CPO/mg of nail) of CPO in each section of the nail for healthy nails. In table 3.1 the total concentration of CPO in sections A and B were not significantly different from each other in healthy nails (*p-value*>0.05). The total concentration in section B is the sum of concentration of CPO retained in the nail (X)

and concentration of CPO diffusing out (Y) of the nail into pH 7.4 PBS at end of 72 hr (table 3.1). The CPO concentrations retained in the nail and the partitioning into the buffer were not significantly different in the absence of TU (*p-value >0.05*) as shown in figure 3.5. In the presence of 5 %TU, the total concentration of CPO in the nail sections C and D were not significantly different (*p-value* >0.05). The total concentration of CPO in the absence of TU (section B) and in presence of TU (section D) were not significantly different (*p-value* >0.05).

It was expected that in the presence of the enhancer TU, the CPO concentration would be higher (section D). This result can be explained on the basis of the relationship plot (figure 2.9) between EF_{sim} and EF_{sol}, where TU was the only penetration enhancer in quadrant III, with a 1.5 fold higher penetration into the nail clippings. The most interesting finding was that CPO partitioned into the nail and remained preferably in the nail in the presence of 5 %TU, with much lower CPO levels diffusing out in the buffer at end of 72 hr (*p-value* <0.05) (figure 3.5). This indicates that less drug partitions out of the nail in presence of 5% TU. In absence of TU (Section B), percent of drug partitioning out of the nail is not significantly different from the percent of drug remaining in the nail at end of experiment (*p-value* >0.05).

A similar trend was observed in infected nails. The data for the CPO partitioning into the infected human toenails is shown in Table 3.2. Infected nails are thicker and contain loose corneocytes compared to healthy nails (Rashid,A. *et al* 1995). In the absence of TU, levels of CPO in the control nail (A) and sample nail (B) were not significantly different (*p-value* >0.05). In presence of 5 %TU, the concentrations of CPO in the control (Nail C) and test nail (Nail D) were not significantly different

indicated by the *p-value* >0.05. However, significantly lower levels of CPO diffused out of the infected nail in presence of TU (Figure 3.6). This phenomenon was also observed in healthy nails in presence of TU. The looser arrangement of corneocytes in the infected nail provides additional binding sites for CPO even in absence of TU. This can be seen from the 13.36 percent increase in CPO remaining in infected nails (64.52 ± 12.99 %) relative to that in healthy nails (51.16 ± 7.27 %) in absence of TU. In the presence of 5 % TU, the percent CPO retained in the healthy and infected nails were increased compared to the corresponding nails in absence of TU. The infected nails retain 76.91± 2.76% of CPO in presence of TU, which is greater than the 67.87± 9.31 % of CPO retained in the healthy nails. This could also be due to the interaction CPO has with the fungal elements within the infected nails.

This means that once the drug enters the nail (in the presence of TU), it does not effectively partition out of the nail. CPO binds extensively to nail keratin. TU acts as a penetration enhancer by reducing the disulfide bonds in keratin. The keratins in the nail are in the form of an α–helix (Garson, J. *et al* 2000). As the disulfide bonds break, the keratin structure opens up, revealing greater number of binding sites (Ciaccio, N.A. and Laurence, J.S. 2009). It is hypothesized that this greater binding also contributes towards lower CPO partitioning out of the nail in presence of TU. This can pose a potential problem during the *in vitro* nail permeation experiment for ciclopirox olamine, by increasing the lag time for transungual permeation. The opposite hypothesis is that this increased CPO level within the nail can serve as a reservoir after termination of therapy.

Table 3.1 Partitioning of ciclopirox olamine into the healthy human toenails in the presence and absence of 5 %w/w thiourea

CPO in control section A (in absence of TU)	CPO in nail section B (in absence of TU)					CPO in control section C (in presence of TU)	CPO in nail section D (in presence of TU)				
	CPO retained in nail at end of partitioning (X)	CPO partitioned into pH 7.4 PBS at end of partitioning (Y)	Total CPO in section B (X+Y)	% CPO retained in the nail at end of partitioning	%CPO partitioned into pH 7.4 PBS at end of partitioning		CPO retained in nail at end of partitioning (M)	CPO partitioned into pH 7.4 PBS at end of partitioning (N)	Total CPO in section D (M+N)	% CPO retained in the nail at end of partitioning	%CPO partitioned into pH 7.4 PBS at end of partitioning
30.0335	13.4697	9.1507	22.6204	59.55	40.45	38.1967	20.6131	5.7959	26.4090	78.05	21.95
23.1392	12.5884	14.1581	26.7466	47.07	52.93	42.3420	23.3080	12.1603	35.4683	65.72	34.29
39.0383	19.6388	23.2833	42.9221	45.75	54.24	26.1536	13.0512	8.7611	21.8123	59.83	40.17
30.74 ± 7.97	**15.23 ± 3.84**	**15.53 ± 7.17**	**30.76 ± 10.73**	**50.79 ± 7.62**	**49.21 ± 7.61**	**35.56 ± 8.41**	**19.00 ± 5.32**	**8.91 ± 3.18**	**27.90 ± 6.95**	**67.87 ± 9.30**	**32.14 ± 9.30**

Table 3.2: Partitioning ciclopirox olamine into the infected human toenails in the presence and absence of 5 % w/w thiourea

CPO in control section A (in absence of TU)	CPO in nail section B (in absence of TU)					CPO in control section C (in presence of TU)	CPO in nail section D (in presence of TU)				
	CPO retained in nail at end of partitioning (X)	CPO partitioned into pH 7.4 PBS at end of partitioning (Y)	Total CPO in section B (X+Y)	% CPO retained in the nail at end of partitioning	%CPO partitioned into pH 7.4 PBS at end of partitioning		CPO retained in nail at end of partitioning (M)	CPO partitioned into pH 7.4 PBS at end of partitioning (N)	Total CPO in section D (M+N)	% CPO retained in the nail at end of partitioning	%CPO partitioned into pH 7.4 PBS at end of partitioning
14.2331	9.3278	4.222	13.5498	68.84	31.16	6.2482	5.446	1.3592	6.8051	80.03	19.97
10.5298	7.8377	2.6396	10.4773	74.81	25.19	15.9877	9.5663	3.03	12.5960	75.95	24.05
27.7177	13.4508	13.4941	26.9449	49.92	50.08	20.3629	8.9332	3.02	11.9497	74.76	25.24
17.49 ± 9.05	**10.21 ± 2.91**	**6.79 ± 5.86**	**17.00 ± 8.76**	**64.52 ± 13.00**	**35.48 ± 13.00**	**14.20 ± 7.23**	**7.98 ± 2.22**	**2.47 ± 0.96**	**3.17 ± 10.45**	**76.91 ± 2.76**	**23.09 ± 2.76**

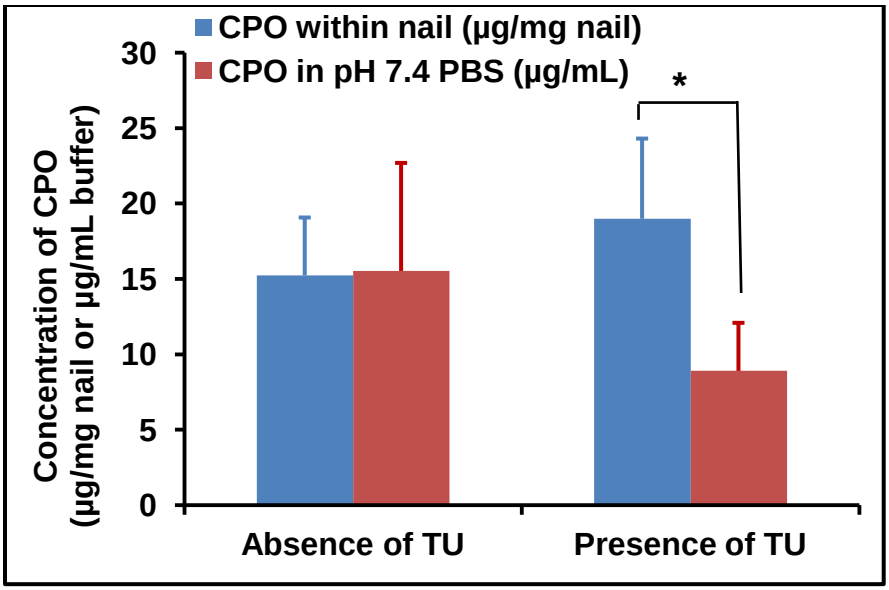


Figure 3.5: Concentration of CPO within healthy human toenail and the buffer after the partitioning study (n=3)

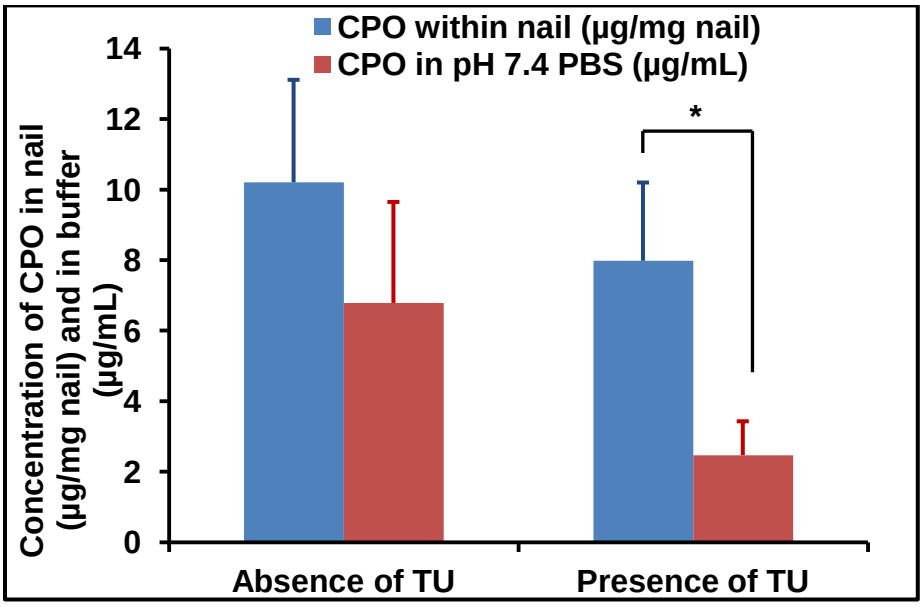


Figure 3.6: Concentration of CPO within the infected human toenails and the buffer after the partitioning study (n=3)

3.5.4. CPO-keratin Binding

Figure 3.7 shows the binding of CPO to keratin powder in the presence of 5 % TU. CPO shows linear binding over the concentration range studied. Saturation was not attained at the concentrations studied for keratin binding. Five percent TU is a reducing agent which acts by reducing the disulfide bonds in keratin (Khengar, R. *et al* 2007). These disulfide bonds contribute to the secondary and tertiary structure of keratin (Freedman, M. H. *et al* 1962). In the presence of TU, a greater number of binding sites may become available for binding with CPO. This is because the secondary and tertiary configurations of keratin are modified in the presence of disulfide bond breaking agents (Freedman, M.H. *et al* 1962). It is reported that pretreatment with a reducing agent (thioglycolic acid) increases the available binding sites for the antifungal drug griseofulvin (Freedman, M.H. *et al* 1962). The groups on CPO which can interact with the available sites on keratin are shown in figure 3.8. The methyl ($–CH_3$) group can show weak van der waals interactions with the proteins The hydroxyl group (-OH) and the carbonyl oxygen are available for hydrogen bonding with keratin. The cyclohexane and pyridine groups (shown with blue arrows in figure 3.8) potentially undergo hydrophobic interactions. It is reported that these non-covalent interactions between the drug and keratin are reversible (Freedman, M.H. *et al* 1962). The literature suggests that antifungal agents which show extensive keratin binding in the nail show reduced activity (Tatsumi, Y. *et al* 2002).

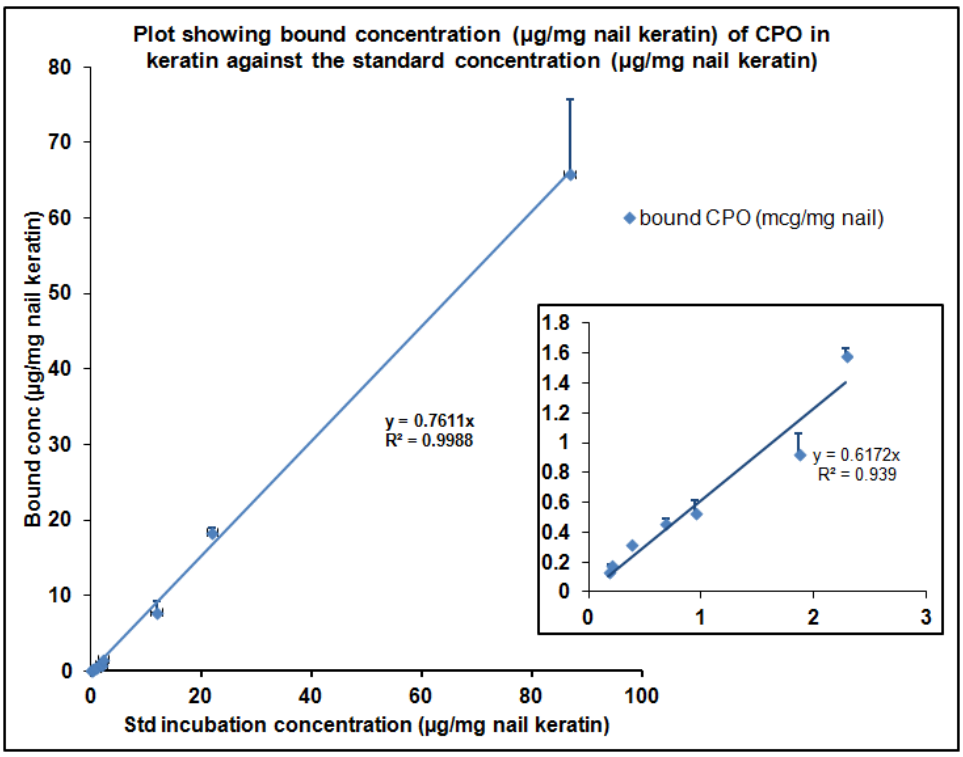


Figure 3.7: Concentration of keratin bound CPO against the standard incubation concentration

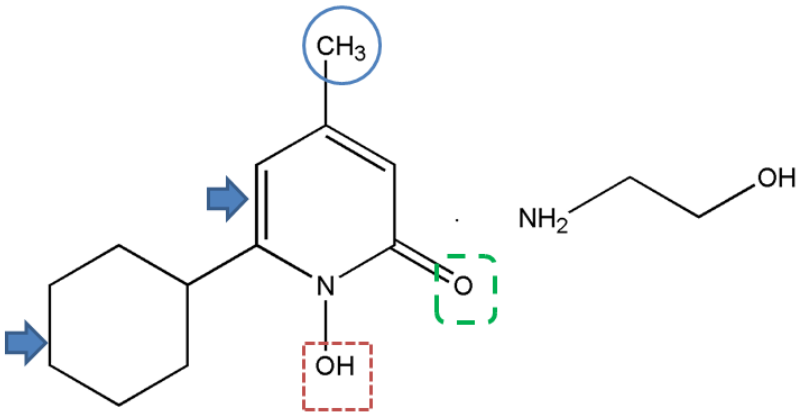


Figure 3.8: Chemical structure of CPO showing potential groups for keratin binding

Terbinafine is reported to have 96 % of keratin binding which leads to its inactivation in presence of keratin (Tatsumi, Y. *et al* 2002 and Uchida, K. and Yamaguchi, H. 1993). Additionally, it is the unbound fraction of the drug which is available for permeation across the nail plate. Upon saturation of the binding sites, there is an increase in concentration of the free drug to permeate the nail plate. The concentration of CPO within the nail after *in vitro* transungual permeation was found to be 27.71 ± 5.98 mg/mL (Chapter 2) which is significantly higher than the highest incubation concentration of 300 µg/mL and was concluded to be also greater than the saturation concentration for the nail. The time required to saturate all the available binding sites may be a factor contributing towards the lag-time for transungual permeation (Banning, T.P. and Heard, C.M. 2002). A high keratin binding for a topically applied drug like CPO may potentially reduce its efficacy due to slow release of the unbound concentration of the drug (Murdan, S. 2012).

In contrast to these observations is the hypothesis that drug binding can be beneficial if a drug reservoir is formed within the nail plate. This would release therapeutic drug after the termination of therapy (Murdan, S. 2012). As the drug partitions into the corneocytes it binds to the keratin present within the corneocytes (Banning, T.P. and Heard, C.M. 2002). It was therefore important to study the CPO keratin binding to substantiate the results obtained in the CPO partitioning study and lateral diffusion study.

3.5.4. Methyl red – Keratin Binding

The keratin binding of MR was also studied in presence of 5 % TU in order to better understand why MR showed similar lateral diffusion as CPO in human toenails. The results of the lateral diffusion experiment are discussed in detail in the following section. Similar to the drug keratin binding, MR showed linear binding to keratin at all the incubation concentrations (figure 3.9). The bound and incubation concentrations were normalized with the amount of keratin powder used for each sample.

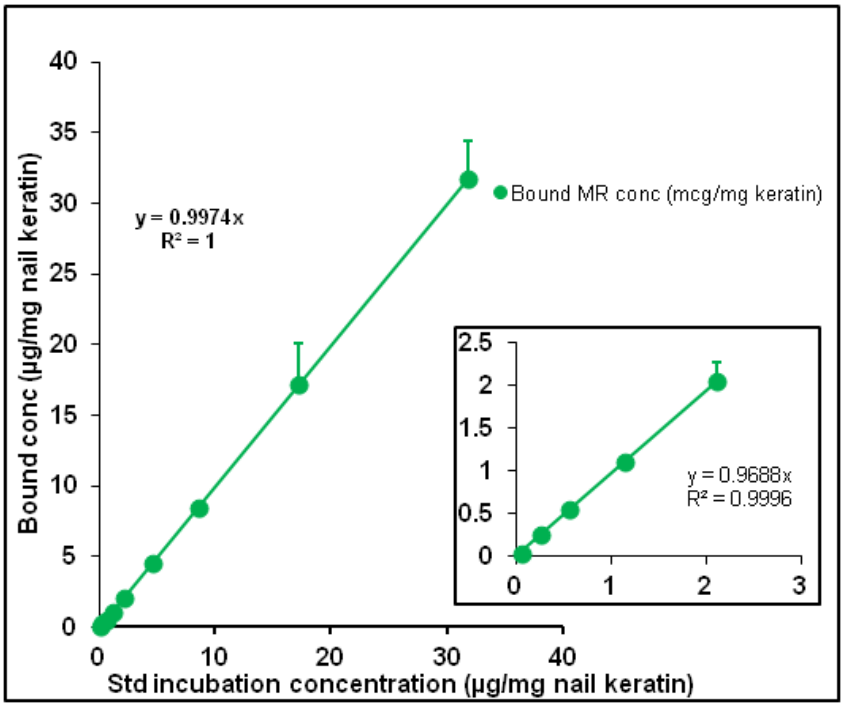


Figure 3.9: Concentration of MR bound to keratin against the incubation concentration

Figure 3.10 shows the chemical structure of MR (methyl red sodium salt). The groups in the structure are also capable of interacting with keratin in a similar manner as CPO. This could be one of the factors for similar binding behavior of MR and CPO with keratin.

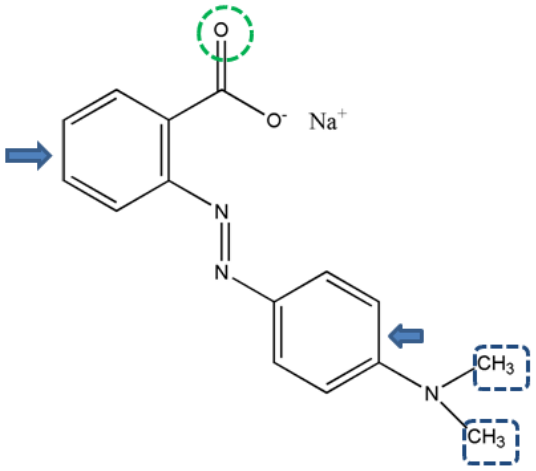


Figure 3.10: Chemical structure of MR showing potential groups for keratin binding

3.5.4. Lateral diffusion in human nails

3.5.4.1. The Lateral Diffusion of Ciclopirox olamine and Methyl red in nail

Figure 3.11 shows the lateral diffusion of methyl red in the suspended toe nail and the sectioning of the toe nails for analysis. The levels of CPO from the proximal to the distal end revealed its migration along the lengths of the two toe nails A and B (figure 3.12). The lines did not show any standard deviation because the nails A and B had different lengths. This would result in a different concentration gradient for each nail. Therefore, the concentration at each distance would be different as expected. The original purpose for the use of the methyl red was to estimate the length of time needed for the

experiment. Methyl red sodium salt was selected to visualize the process of lateral diffusion of CPO in the human toe nail because it is water soluble, has a molecular weight (291.62 g/mol) similar to that of CPO (268.36 g/mol) and is readily taken up by the nail. It did not affect the stability of the drug and it itself remained stable in presence of 5% w/w thiourea (nail penetration enhancer). In the preformulation screening studies, 5% w/w thiourea had shown the greatest enhancement in penetration of ciclopirox olamine into the nail (Chapter 2).

Interestingly, a strong correlation was observed between the concentrations of CPO and MR in each of the toe nails (Figure 3.13). This correlation was not expected. This correlation indicates that MR can be used to visualize the lateral diffusion of CPO in the human toenails. This was also validated in the *in vitro* transungual permeation of CPO and MR. The process of lateral diffusion was studied using a novel approach. In order to mimic the lateral movement of molecules in the nail plate, the nails were suspended in the solution containing CPO, MR and 5 % TU.

There was no significant increase in the lateral movement of CPO and MR in presence of 5 % TU in the suspended nail. This observation was different from the literature report that in the presence of 5 % urea as the skin penetration enhancer for ibuprofen, there was increase in both penetration and lateral distribution of ibuprofen in the skin (Schicksnus, G. and Muller-Goymann, C. 2004). This difference could be attributed to the difference of the physical and chemical composition of the skin and the nail.

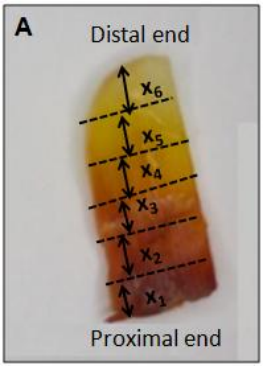


Figure 3.11: Color gradient of MR in the suspended toenail and the dotted lines representing the sectioning of the toenails

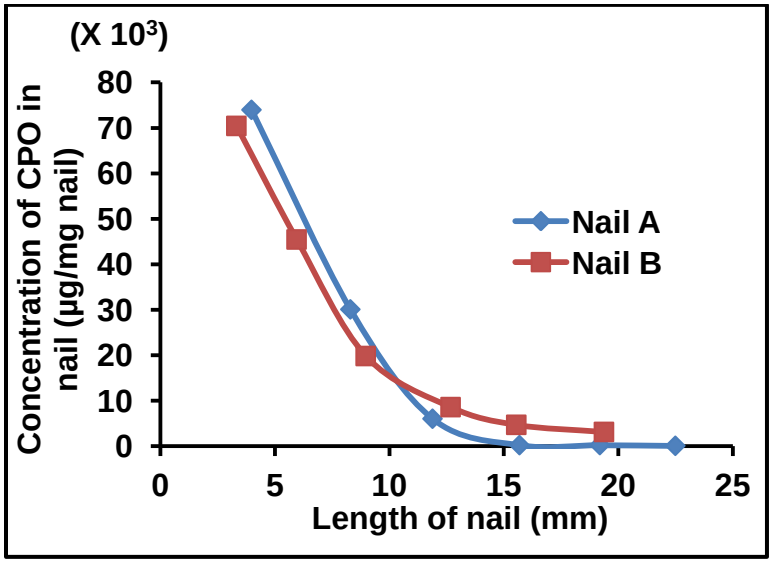


Figure 3.12: Decline in the concentration of CPO from the proximal to the distal end along the length of the suspended toenails

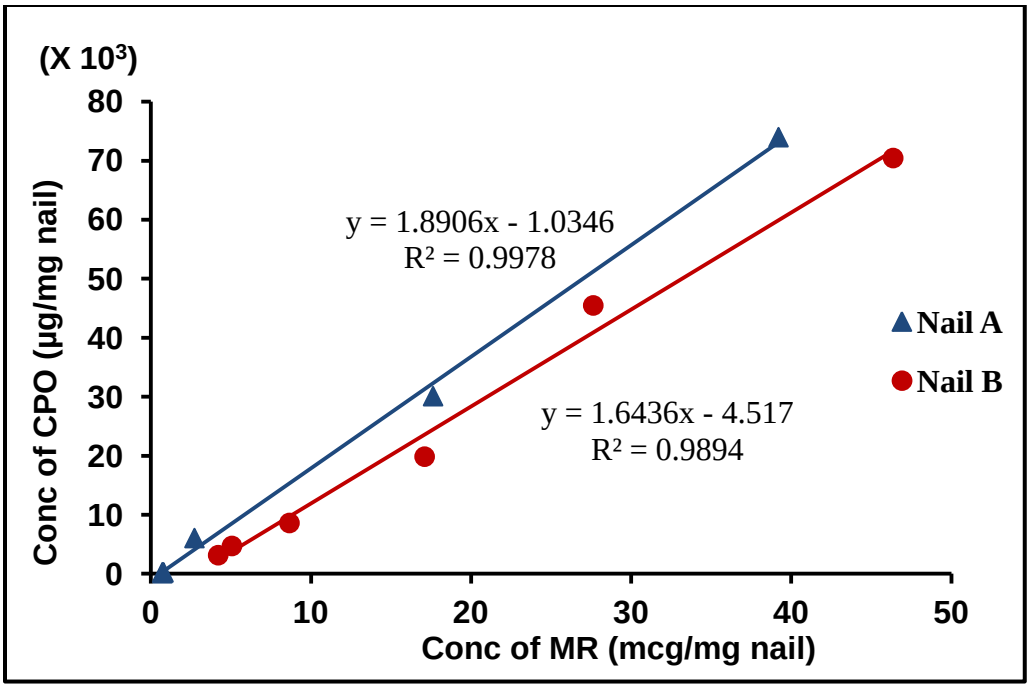


Figure 3.13: Correlation between the concentrations of CPO and MR in the nail sections

The diffusion of molecules along length of the suspended nail could be a favored direction for diffusion because of the orientation of the keratin fibers in the nail. The intermediate layer is the thickest and softest layer of nail plate (Kobayashi, Y. *et al* 1999 and Murdan, S. 2002). The keratin fibers are arranged parallel to the free end of the nail in the intermediate layer (Garson, J. *et al* 2000). The keratin fibers orient in both parallel and perpendicular direction in the dorsal and ventral layers of the nail (Garson, J. *et al* 2000).

The similarity in the trends of decline in concentrations of CPO and MR laterally in the toe nails indicates similar lateral movement of CPO and MR. This is also evident from the good correlation between the concentrations of CPO and MR in each of the two toe

nails suspended in the drug solution. One of the contributors to this similarity in lateral movement of MR and CPO is similarity in their respective binding abilities with keratin. Methyl red sodium salt can therefore be used to visualize the lateral diffusion of CPO. Previously Lucifer yellow (fluorescent dye) was used to show the presence of a lateral diffusion pathway in the skin, which is suggested to have a physiological role in the transport of lymphokines and inflammatory mediators (Mansbridge, J. and Knapp, M. 1993).

The physiological role and the effect of lateral diffusion on antifungal therapy in human nail need to be explored further. Lateral diffusion of drugs in the nails has been reported as an observation by researchers during iontophoretic transungual drug delivery of terbinafine hydrochloride (Nair, A.B. *et al* 2009a, 2009b, 2009c, 2009d). In the case of iontophoresis, a high flux of drug is attained across the thickness of the nail, with the aid of electric current and is much greater than passive diffusion. Therefore, the effect of passive lateral diffusion does not markedly reduce iontophoretic transungual drug permeation. However in passive topical delivery, lateral diffusion through the nail can have a significant impact on *in vitro* transungual permeation resulting in underestimation of in vivo permeation. It is therefore important to understand the effect of lateral diffusion during passive *in vitro* transungual permeation. To date this is the only study on lateral diffusion which provides an insight on its effect on passive transungual permeation during *in vitro* studies.

3.5.4.2. In vitro transungual permeation

Human cadaver toe nails from a single donor were trimmed to get three different dimensions (5 X 5 mm (25 mm^2), 7 X 7 mm (49 mm^2) and 9 X 9 mm (81 mm^2)) in order to study the effect of the size of the peripheral areas (around a constant area of drug application) on lateral diffusion. Transungual permeation fluxes of 4.94 ± 2.61 µg/cm^2/day, 1.16 ± 0.57 µg/cm^2/day and 0.81 ± 0.41 µg/cm^2/day were attained for the toe nails of the dimensions 5 X 5 mm (25 mm^2), 7 X 7 mm (49 mm^2) and 9 X 9 mm (81 mm^2) respectively (figure 3.14). The lag times for the 5 X 5 mm (25 mm^2) and the 7 X 7 mm (49 mm^2) nail pieces were 6.4 ± 1.9 days and 6.3 ± 0.2 days respectively. These values are not significantly different. A longer lag-time of 8.8 ± 2.6 days was obtained for the nail size of 9 X 9 mm. This indicates that a longer lag-time is obtained for a larger piece of toenail using the same donor solution. Therefore the flux and the lag-times are a function of the extent of lateral diffusion which in turn depends on the size of the toenail used. This indicates that lateral diffusion in the nail has to be minimized to obtain reliable permeation flux and lag-time values in an *in vitro* transungual permeation study that would be representative of in vivo results.

The concentration of ciclopirox olamine in the orifice and each of the subsequent section of the periphery is shown in figure 3.12. In each of the toenails used, 7.07 mm^2 of toenail area was exposed to the donor solution. The drug concentrations within the orifice (7.07 mm^2) for each of the three dimensions of the toe nails are not significantly different (p-value >0.05). Similarly, the CPO concentrations in the sections B and C of the 5 X 5 mm, 7 X 7 mm and 9 X 9 mm are not significantly different (p-value >0.05).

Figure 3.13 shows the correlation between the concentrations (µg/mg of nail) of CPO and MR in the area of drug exposure (7.07 mm^2) and the sequential peripheral sections for each nail dimension studied. This confirms the initial observation that MR concentrations correlate with the CPO concentrations in each section of the nail and that it acts as an indicator for CPO lateral diffusion. The area of drug exposure for the nail adapters is only 7.07 mm^2. The nail specimen with a measurement of 5 X 5 mm (25 mm^2) has the smallest peripheral surface area of 17.93 mm^2 while maintaining a leak proof seal within the nail adapter. The other extreme, 9 X 9 mm (81 mm^2) is the largest dimension of toe nails which can be used to maintain the duration of the experiments within practical limits of 30 – 40 days.

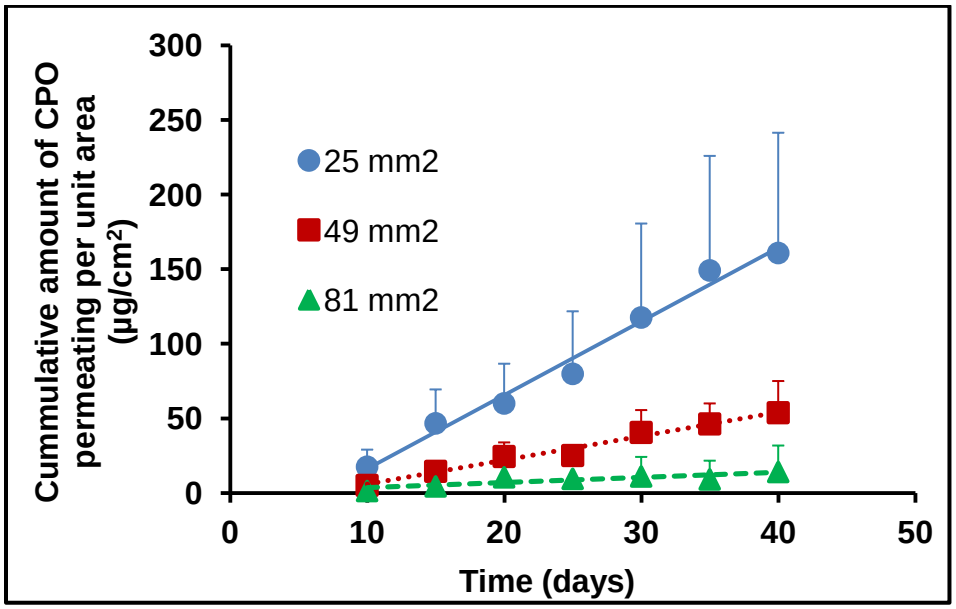


Figure 3.14: Reduction in the permeation of CPO with increase in the surface area of toenail used in the in vitro experiment

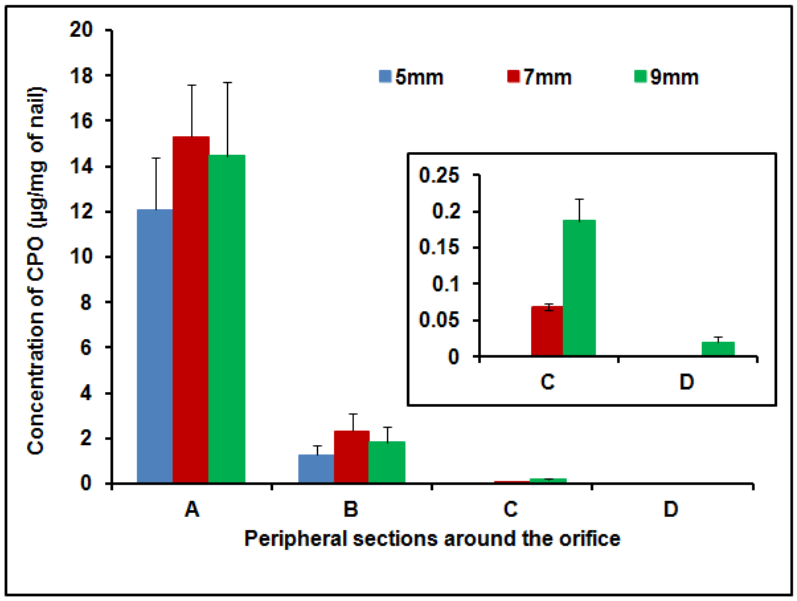


Figure 3.15: Concentration of CPO in the nail sections in the area of drug exposure and in the periphery for the three dimensions of toenails

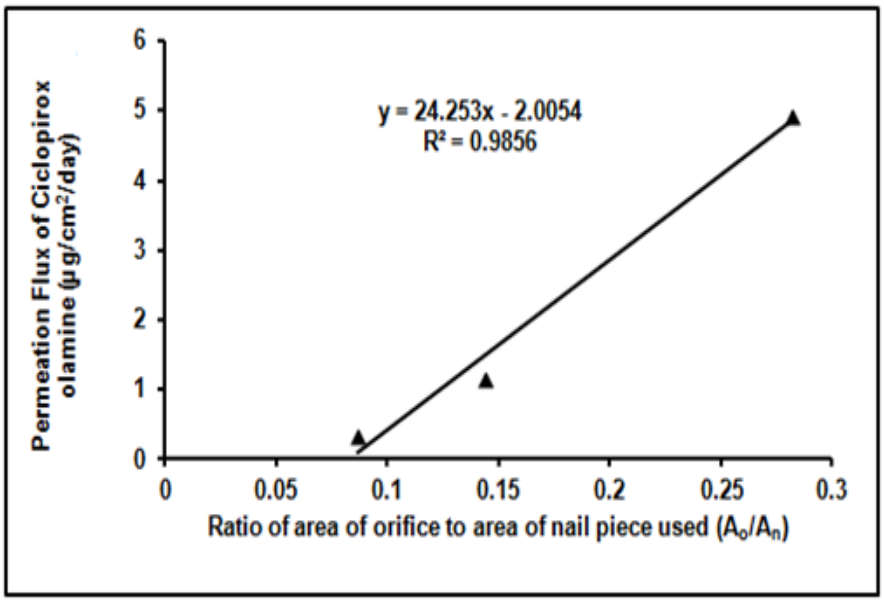


Figure 3.16: Correlation between the CPO flux and the ratio A_0/A_n

A strong correlation exists between the flux of ciclopirox olamine and ratio of orifice area to total nail area (A_o/A_n) (figure 3.16). If a toe nail with dimension of 11 X 11 mm (121 mm^2) is used with a nail adapter of 3 mm orifice diameter, this ratio falls to 0.0584. The increase in nail area will lead to a significant drop in permeation of ciclopirox olamine through the nail. To obtain a measurable quantity of the drug, the experiment may have to be continued for an extended duration of time. In a preliminary experiment, when untrimmed nails were used, no permeation was observed after 27 days (The duration of the experiment). Hence the largest dimension of toe nail in the study was limited to 9 X 9 mm (81 mm^2).

The CPO concentration at the site of application on the nail was much greater than the reported minimum inhibitory concentration (MIC) of CPO against the dermatophytes (0.004 to 1.0 µg/mL) (Subissi,A. et al 2010, Kokjohn,K. et al 2003, Gupta,A.K. and Kohli, Y. , 2003, Shehata, A.S. et al 2008 and Singh, J et al 2007). The 3 mm orifice diameter provides 7.065 mm^2 of surface area for permeation of the drug and the dye. This area is 28.26 %, 14.42 % and 8.72 % of the total area of the nail specimens with dimensions 5 X 5 mm (25 mm^2), 7 X 7 mm (49 mm^2) and 9 X 9 mm (81 mm^2) respectively.

When cadaver skin is placed between the donor and the receiver compartments of the Franz diffusion cell, there is a tight seal and the pressure exerted by the clamp on the skin greatly limits the lateral diffusion of the molecules beyond the area of application.

However the human nail plate is a hard, rigid structure which does not get compressed within the nail adapter. Hence, lateral diffusion cannot be prevented in the nail *in vitro*.

CPO and MR showed lateral diffusion around the area of application in the *in vitro* transungual permeation studies. Previously, a photothermal imaging technique is reported to show the lateral distribution of the drug into the proximal end of the nail when the formulation was applied on the proximal nail fold (B. Gotter et al 2010).

Figure 3.17: The ideal cylinder and the pyramid model for lateral diffusion

When the nail under the orifice is treated with the drug, some drug molecules diffuse into the upper layers of the nail plate. Ideally if there is no lateral diffusion, the drug molecules should diffuse through the entire thickness of the nail (h) under the orifice (Figure 3.17). As the permeation process continues, this cylinder with the height of h and diameter 3 mm, forms a concentration gradient of the drug and eventually CPO diffuses out from the ventral side of the nail plate. However, it was observed that, as the drug fills up the upper layers of the nail under the orifice, it also moves laterally from the cylinder to the periphery. The drug from the upper saturated layers of the nail then diffuses into the layers underneath. All this time the drug is also moving from the central part of cylinder towards the periphery. Thus, lateral diffusion, which itself is a passive diffusion process, is dependent on the concentration gradient formed between the cylinder and the area surrounding the cylinder. As this process continues, there is formation of a three

dimensional pattern of diffusion which can be best approximated as a pyramid (figure 3.17).

The volume of this pyramid (V_p) is given by:

V_p= 1/3*Area of base*height …….(3.1)

Where area of base = Total area of nail and

height = thickness of nail)

The volume of the ideal cylinder (Vc) is given by:

$V_c = 1/3*\pi r^2*$height …….(3.2)

Where radius of the nail adapter orifice, r = 1.5 mm

height = thickness of nail

Volume of the nail outside the central cylinder, which contains the laterally diffused drug, is given by:

$V_n = V_p - V_c$ ……..(3.3)

The nail underneath the orifice, for each orifice was found to contain the same amounts of drug in all the three nail dimensions. The volume V_n can be used to calculate the concentration of CPO in the nail around the central cylinder. The flux of transungual permeation of CPO strongly correlates with the concentration of CPO in the nail surrounding the cylinder (Figure 3.18). This correlation shows that the highest flux is observed in the nail dimension, where the concentration developing in the peripheral

layer immediately outside the central cylinder is highest. This concentration decreases drastically as the peripheral area and V_n increases. In the presence of lateral diffusion, the required driving force for transungual permeation is the high CPO concentration in the orifice and the periphery. As the size of the nail increases from 5 X 5 mm to 7 X 7mm and finally to 9 X 9mm, the concentration of CPO in the peripheral area of the nail drops significantly, resulting in decline in transungual permeation. The concentration drop may be caused by the gradient that forms between the treated area and the peripheral area of the nail. A large peripheral area would lengthen the gradient and drop the peripheral concentration.

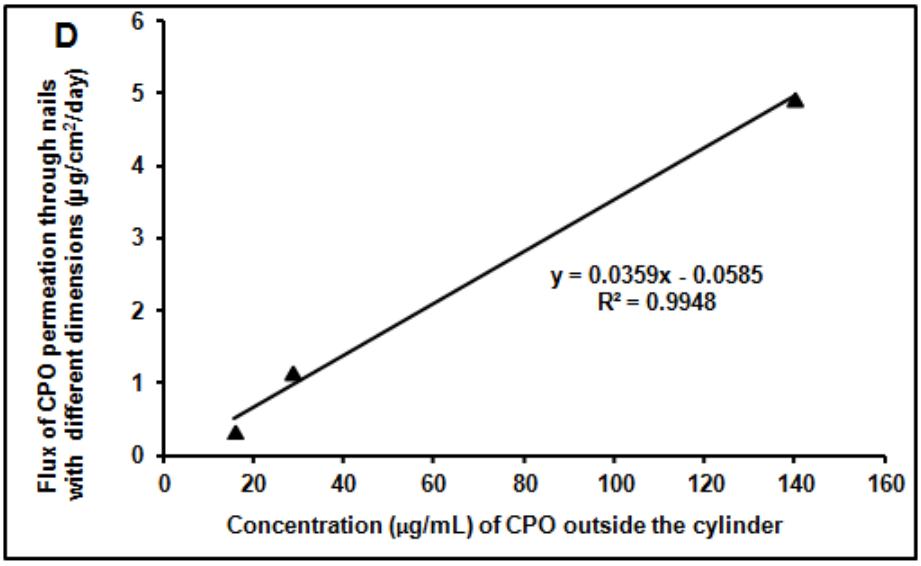


Figure 3.18: Correlation between the CPO permeation flux and CPO concentration outside the cylinder

Lateral diffusion in the human nail plate cannot be completely prevented, only its effect on transungual permeation of drugs can be controlled by minimizing the available peripheral surface area. The lag times for 5 X 5 mm and 7 X 7 mm nail pieces were not significantly different. This indicates that the two dimensions did not affect the lag time as much as it affected the permeation of CPO. However when 9 X 9 mm nail size was used, the lag time increased. Additionally, untrimmed nails used in a similar experimental design showed the lag times greater than the duration of the experiment (27 days). There was no measurable amount of drug permeating the nails during the 27 days. A similar increase in lag-time as a consequence of lateral diffusion was seen with ibuprofen in the skin (Schicksnus, G. and Muller-Goymann, C. 2004). Longer lag-times correspond to a longer time before detectable levels of CPO can reach the receiver compartment.

These results show that lateral diffusion occurs in the nail and this can have a profound impact on the experimental data acquired from *in vitro* drug permeation studies. The decision of selecting a penetration enhancer or elimination of a potential formulation from further studies is dependent on this *in vitro* experimental data. If lateral diffusion overrides the permeation process, an enhancer or an optimum formulation may be falsely eliminated from selection. Hence, it is important to select the appropriate dimension and surface area of nails, considering the area of the orifice on the nail adapters for the *in vitro* experiments. When the patient applies the topical transungual formulation, it covers the entire surface area of the nail and greatly reduces the area available for lateral diffusion to the nail under the nail folds. The lateral diffusion that would occur in nail during therapy would actually be advantageous because this would increase the drug concentration in the unexposed nail underneath the nail folds. It is therefore expected

that passive transungual permeation *in vivo* is closer to the ideal cylinder model. This *in vivo* scenario can be mimicked in the *in vitro* setting by using the appropriate size of the nail which is just sufficient to provide a tight leak proof seal around the orifice in the nail adapter. However, if lateral diffusion is extensive as in the *in vitro* experiments, passive lateral diffusion competes with the passive transungual permeation, causing a shift from the cylinder to the pyramid. This leads to an overestimation of lag time and underestimation of permeation flux. Thus, this understanding of the effect that lateral diffusion has on passive transungual permeation of drugs has helped to define the best experimental in vitro methodology, using nails with minimal excess surface area, to better predict *in vivo*, transungual permeation and aid in selection of penetration enhancers and formulations that met the target permeation profile.

3.5.5 .Drug-Epidermis binding

The drug keratin binding was studied for CPO primarily because epidermis was one of the target tissues for drug delivery. The stratum corneum is reported to show the largest levels of keratin in the human body (Banning, T.P. and Heard, C.M. 2002). The binding of CPO to epidermis is shown in figure 3.19. The epidermal binding in the nail folds surrounding the nail may be saturable. However CPO showed dose-dependent linear binding to epidermis at incubation concentrations up to 2130 µg/mL. This linearity in drug-epidermal binding is reported for doxycycline (Banning, T.P. and Heard, C.M., 2002). The highest incubation concentrations in the epidermal and keratin binding studies were 2130 µg/mL and 300 µg/mL respectively. Since keratin is the major component

available for binding in the epidermis, it can be safely assumed that 2130 µg /mL is still below the saturation concentration of CPO for keratin. Nevertheless, there may be other sites within the epidermis to which CPO can potentially bind. In topically delivery, binding of the drug to the epidermis helps to retain the drug within its target site, Though CPO shows strong affinity for keratin, the binding to keratin is via non covalent bonds and these interactions are reversible. Hence, CPO bound to the epidermis will serve as a drug reservoir.

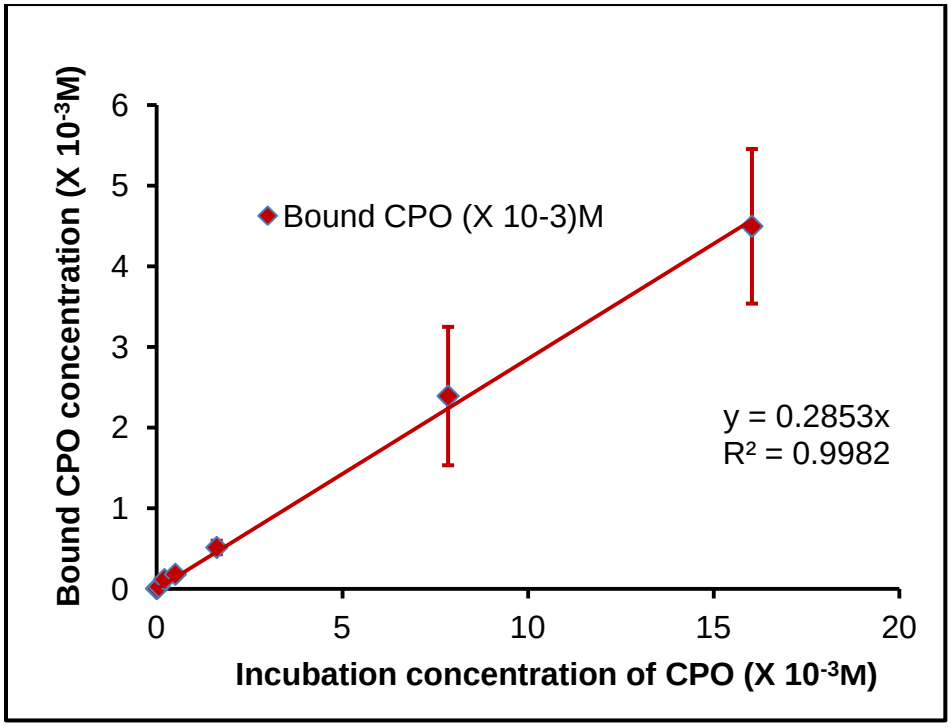


Figure 3.19: Epidermal binding of CPO

3.6. Conclusion

The human nail and the stratum corneum are rich in keratins. It was therefore important to assess the drug-membrane interactions and their affect on the drug delivery to the target tissues. Ciclopirox olamine partitioned favorably into the human nail and showed greater partitioning into infected human nails. This is regarded beneficial in case of topical delivery, as the released drug will readily diffuse into the nail plate. However, lower levels of the drug diffused out from the nails in presence of penetration enhancer (TU).

The results indicated significant drug binding to nail keratin and only the unbound drug will be available for permeation across the nail plate. Only the unbound drug is active against the causative organisms. Therefore sufficiently high amounts of the drug have to be delivered into the nail plate in order to saturate the nail and allow adequate levels of unbound drug to diffuse out to the nail bed.

It is shown that lateral diffusion occurs in the nail and can reduce the *in vitro* passive transungual drug permeation. Methyl Red sodium salt was a useful tool for visualizing the lateral diffusion of a drug such as ciclopirox olamine. The significance of lateral diffusion is greater in the *in vitro* experiments because significant lateral diffusion would mean practically no drug permeation which can lead to false conclusions pertaining to the selected enhancer or design of formulation. It is important to control the dimensions and excess surface area of the toe nails during *in vitro* passive drug delivery experiments to obtain data which is predictive of *in vivo* permeation. It is therefore recommended that the cadaver toe nails used for the permeation experiments be trimmed to cover the orifice

in the nail adapter and provide a leak-proof seal to minimize any excess peripheral surface area.

Ciclopirox olamine also showed significant binding to the epidermal tissue. This binding in association with the mechanism of action of propylene glycol in the epidermis, enabled selective delivery of CPO into the epidermis

CHAPTER 4

DESIGN AND FORMULATION DEVELOPMENT OF THE TRANSUNGUAL PATCH

4.1. Abbreviation

CPO: Ciclopirox olamine

DIA: Drug-in-adhesive

HPMC: Hydroxypropyl methylcellulose

PEs: Penetration enhancers

P301: Polyox WSR-301

P303: Polyox WASR-303

TU: Thiourea

TAC: Triacetin

TEC: Triethyl citrate

4.2. Introduction

There is limited literature available on the methodology and key aspects for formulation development of passive transungual drug delivery formulations. The key properties (Murdan, S. 2008) identified for a successful topical formulation for transungual delivery include:

- Ease of use
- Maintain adequate contact with nail
- Easy release and diffusion of drug from the formulation
- High thermodynamic activity
- Ability to hydrate the nail
- Presence of penetration enhancers

It was hypothesized that a well-designed nail patch formulation can meet the above criteria for a topical formulation. Nail patches can also help to overcome some of the limitations of a nail lacquer formulation such as Penlac. These limitations include: the possibility of drug crystallization in the film after the solvent evaporates from the nail lacquer, filing of the nail prior to lacquer re-application and the absence of penetration enhancer, etc. (Murdan, S. 2008). The reformulation strategy for ciclopirox olamine was to attain better transungual drug delivery compared to the Penlac® nail lacquer formulation (Gupta, A. K. and Simpson, F.C 2012). Ciclopirox olamine is reported to show greater solubility than the free acid Ciclopirox, and therefore was expected to permeate the hydrophilic nail plate better than the free acid form. Therefore CPO was

selected as the active ingredient in the transungual formulation. For effective drug delivery into and across the nail, it is important to obtain a favorable balance between the solubility of the drug dose in the polymer, release of the drug from the dosage form and the partitioning of the drug from the dosage form to the membrane.

The selection criteria for the polymer matrix of a patch include:

- The patch design
- Stability of the drug
- Compatibility with penetration enhancers
- Duration of wear (24 hrs)
- Ease of manufacture
- Ease of use

The design of a transungual patch was based on the designs for transdermal patches. Transdermal patches can be categorized into two main types – reservoir type and drug-in-adhesive (DIA) type. The DIA type of patch has the drug and PEs in a dissolved or highly dispersed state in the pressure sensitive adhesive (Williams, A. 2003). The DIA design was explored for development of the transungual patch using PSAs.

The major components of a DIA patch are a backing membrane (BM), adhesive, drug, plasticizer, permeation enhancer (if required) and release liner (RL). The desirable characteristic of each component are described below.

- The backing membrane should be inert and compatible with the other components of the patch (Güngör,S. *et al* 2012 and Williams, A., 2003). It is important that it is

impermeable to the drug and penetration enhancers if they are present in the formulation. In case of transungual patches, occlusive backing membrane was proposed to maintain the optimal hydration of the nail plate and thereby increase transungual permeation of the antifungal drugs. However, considering the prolonged use of the patch (one patch daily for few months) and its contact with skin, a non-occlusive backing membrane was used to avoid skin irritation.

- Plasticizers are low molecular weight compounds which incorporate themselves between the polymer chains and alter the polymer – polymer interaction and therefore lower the glass transition temperature (Güngör,S. et al 2012, Rajan, R. et al, 2010; Gal, A. and Nussinovitch,A., 2009). The resultant increase in the movement of the polymer chains makes the polymer more pliable (Rajan, R. et al, 2010). Appropriate selection of the plasticizer and its concentration in the patch helps to control drug release (Güngör,S. et al 2012).
- The adhesive should not cause any irritation to the skin or leave any residue upon patch removal. It should easily adhere to the site of application and maintain contact throughout the duration of application. The pressure sensitive adhesives (PSAs) used for transdermal patches are typically selected from the classes of polyisobutylenes, acrylates and polysiloxanes (Tan,H. and Pfister,W. 1999 and Williams,A. 2003).
- The release liner should be impermeable to the components of the matrix, occlusive and easily removed from the adhesive layer prior to application of the patch on the skin (Güngör,S. et al 2012 and Williams, A., 2003)..

The main objective of this chapter was to select the final design for a prototype transungual patch. The developed patch would have the following properties:

- CPO and the penetration enhancers (TU and PG) dispersed or dissolved in the polymer matrix
- Once daily application
- Not less than 80 % of CPO released in 24 hr
- No crystallization of CPO or TU or phase separation in the polymer matrix
- Ability to deliver CPO at levels greater than the Minimum Inhibitory Concentration in the target tissues.

An initial DIA design was explored which was later modified to include a separate hydrophilic polymer matrix layer in the patch design. Based on the drug release data obtained, the type and concentration of plasticizers were altered in the subsequent formula for the patches. To clearly explain the rationale for design modifications, the method of development and the corresponding results will be discussed completely for each formulation. Hence, there are no separate experimental methods and results sections in this chapter. The chapter is divided into two parts describing the Drug-in-Adhesive design and the modified drug in hydrophilic matrix design.

4.3. Materials

CPO was purchased from Haorui Pharma-chem Inc., Edison, New Jersey, USA. PG was obtained from MP Biochemicals. Thiourea was obtained from Acros Organics. The

PSAs with the brand name DURO-TAK® e.g. polyisobutylene (DURO-TAK® 87-6908) and acrylates (DURO-TAK® 87-9301, DURO-TAK® 87-9088, DURO-TAK® 87-4287, DURO-TAK® 87-4098, DURO-TAK® 87-2287) were gift samples from Henkel. The silicone PSAs compatible with amine functional drugs (Dow Corning® BIO-PSA 7-4302, BIO-PSA 7-4502 and BIO-PSA 7-4602) were provided as a gift by Dow Corning Corp. All of these PSAs are listed for transdermal and/or topical use on the respective company websites. The cellulose ethers Methocel® K15M and Methocel® K100M were obtained from the Dow Chemical Company. The water soluble resins Polyox® WSRN-80, WSRN-750, WSR-301 and WSR-303 were purchased from the Dow Chemical Company. The non-occlusive ethylene vinyl acetate (EVA) backing membrane, 3M™ CoTran™ 9707 and the fluoropolymer coated polyester film 3M™ Scotchpak™ 9742 were obtained from 3M (St.Paul, MN).

4.4. Drug-in-adhesive design

The initial design for the transungual patch was based on the simple DIA design. In order to meet the key properties for a successful topical patch a DIA type nail patch was designed as shown in figure 4.1. The drug (CPO) and the PEs (TU and PG) were dissolved or uniformly dispersed in the PSAs and spread onto a release liner. The films were air-dried for 24 hr in the hood, followed by drying in the oven at 45 ºC for 3 hr. The non-occlusive backing membrane was laid over the dry PSA film. It was recognized early on that the main challenge of developing a DIA type patch was uniform distribution of the water soluble PEs into the relatively hydrophobic PSAs.

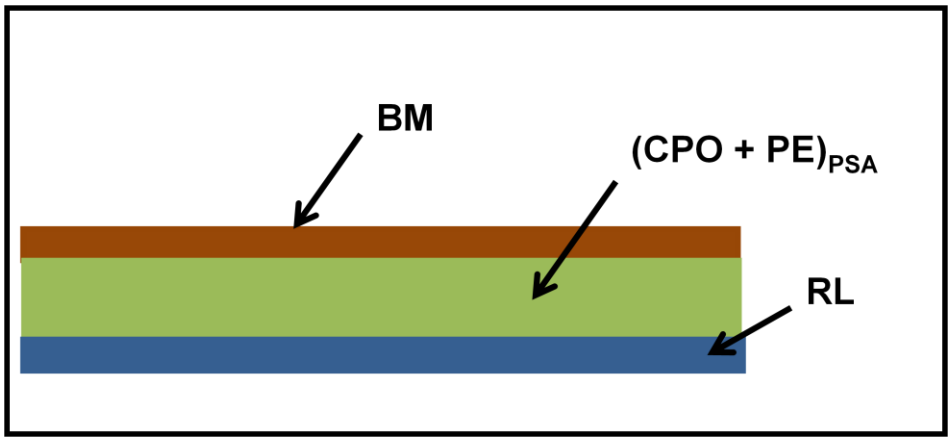


Figure 4.1: *Drug-in-adhesive (DIA) design of the transungual patch consisting of Backing Membrane (BM), CPO and PE dissolved or dispersed in pressure sensitive adhesive (CPO +PE)$_{PSA}$ and Release liner (RL)*

4.4.1. General method of fabrication

The general method of fabrication remained the same for each design of the patch. The drug (CPO) and the nail PE (TU) were weighed out in a glass bottle and dissolved in solvent (methanol). Other solvents (if required) such as isopropyl alcohol, ethyl acetate and n-heptane were added as per the formula and vortexed for 1 min. The skin PE, PG was weighed and vortexed with the methanol solution until a single phase was obtained. If the formula involves the use of hydrophobic plasticizers (Triacetin and Triethyl citrate), these agents were weighed and mixed with the solvents prior to addition of PG. Finally the polymer was weighed out into the glass bottle and vortexed for 5 min to form a homogenous mixture. The bottles were then placed on the bottle roller (Wheaton) (figure 4.2) and mixed overnight at medium speed to complete the mixing and remove the air bubbles generated during vortexing the polymer. The mixture was then poured on

to the release liner and spread into a layer with uniform thickness. The film was air dried in the hood for 24 hr. The backing membrane was laid onto the dry film.

4.4.2. Determination of drug release

The percent drug release was determined from each of the films using Franz diffusion cells. Square patches with 4 cm^2 area were cut out from the films. The thickness of the patch was measured using digital micrometers (Marathon). The average thickness of the release liner and the backing membrane were subtracted and total patch thickness to calculate the thickness of the acrylate film. The release liner was peeled off and the release surface was placed facing the donor compartment of the Franz diffusion cells. Three mL of pH 7.4 PBS was filled in the receiver compartment. Samples (1.0 mL) were withdrawn from the receiver compartment at 1, 2, 4, 8, 12 and 24 hr and replaced with equal volume of pH 7.4 PBS. Since it was a screening experiment, only one patch per sample was studied (n = 1). The cumulative percent release for CPO was calculated.

4.4.3. Determination of Concentration of TU and PG

One of the initial processes was determination of concentration of TU and PG that can be loaded into the PSA to result in patch without crystallization and phase separation. The main goal was to determine an optimum concentration of TU and PG that can be maintained throughout the subsequent screening of all the PSAs. DURO-TAK® 87-4287 is a polyacrylate with 39 % solid content dissolved in ethyl acetate. Three concentrations of TU (1 %w/w, 5 %w/w and 10 %w/w) and four concentrations of PG (1 %w/w, 5 %w/w, 10 %w/w and 20 %w/w) in the acrylate matrix were prepared. In each of the

patches prepared, the CPO load was fixed at 1 % w/w. Table 4.1 gives the composition of the formulations prepared. F1 to F8 were the formulations generated in the preliminary screening.

Table 4.1: Composition of patches for determination of TU and PG load for further screening

Ingredient	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**
CPO (%)	1	1	1	1	1	1	1	1
TU (%)	1	5	10	0	0	0	0	5
PG (%)	0	0	0	1	5	10	20	10
DURO-TAK® 87-4287 (%)	98	94	89	98	94	89	79	84
Methanol (g)	1.0	2.0	3.0	1.0	1.0	1.0	1.0	1.0

Figure 4.2: Bottle roller

4.4.3.1. Results and Discussion

All of the 8 polymer mixtures shown in table 4.1 were clear and devoid of any undissolved particles after 24 hr of mixing on the bottle roller. The film F1 with 1 % TU did not show any crystallization. However the crystallization increased in films F2 and F3 with 5 % TU and 10 % TU, respectively (Figures 4.3 and 4.4). Since there was a concentration dependant increase (visual observation) in the number of crystals throughout the film and no crystallization in the film with 1 % TU, it was concluded that it is TU that crystallizes out of the acrylate matrix at 5 % and 10 % load. A similar crystallization of ciclopirox free acid has been reported in acrylate PSA at a drug load of 10 % w/w (Myoung, Y. and Choi, H., 2003). However CPO is readily soluble in methanol and ethyl acetate. Methanol was used as a cosolvent to dissolve TU and form a homogenous mixture with the polymer. As the solvents evaporate, TU is no longer in solution and it crystallizes out. Crystal formation was also found to significantly reduce the tack of the film. Reduced transdermal permeation of ketoprofen and change in the adhesive property of acrylate PSA has been related to the crystallization of ketoprofen in the adhesive matrix (Myoung, Y. and Choi, H., 2003 and Kim, J.H. and Choi, H.K., 2002). The formulations F4 to F7 resulted in clear films without any crystallization. However crystallization was observed in film formed using formulation F8. Even with the addition of 10 % PG, TU crystallized out upon drying (Figure 4.5). It was therefore concluded that all the future screening studies will be carried out at 1 % CPO and 1 % TU. The proportion of other ingredients in the formula will be altered in order to get the desired physical and functional characteristics of the patch.

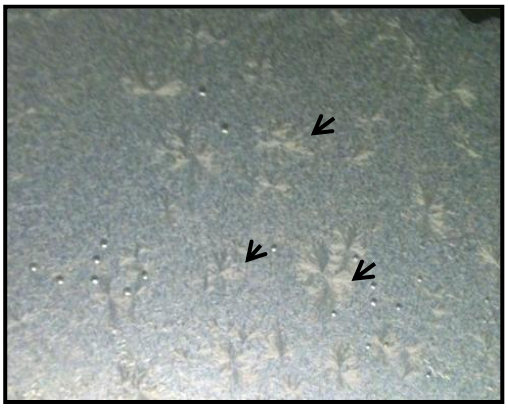


Figure 4.3: Crystallization in DURO-TAK® 87-4287 Film containing 1 % CPO and 5 % TU

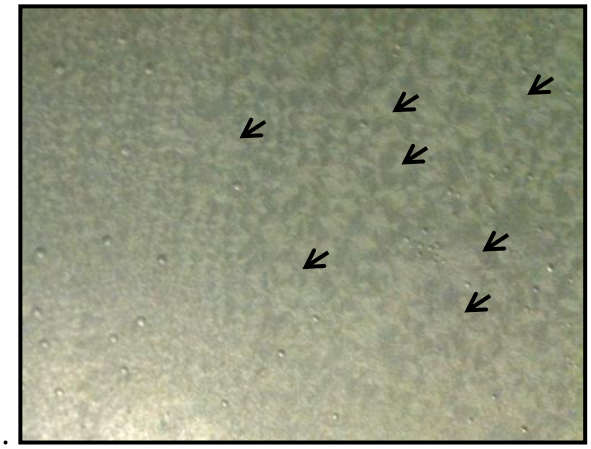


Figure 4.4: Crystallization in DURO-TAK® 87-4287 Film containing 1 % CPO and 10 % TU

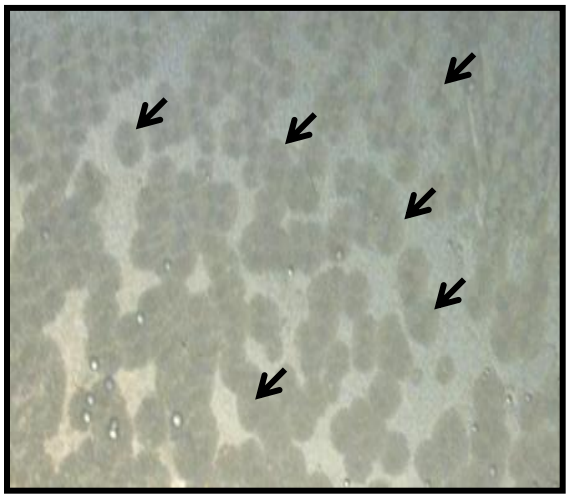


Figure 4.5: Crystallization in DURO-TAK® 87-4287 Film containing 1% CPO, 5 %TU and 10 % PG

4.4.4. Screening of Polyisobutylene

4.4.4.1. Experimental Method

Keeping the percentage of TU and CPO at a constant level of 1 %w/w, polyisobutylene films were fabricated with 5 levels of PG. The polyisobutylene grade DURO-TAK® 87-6908 was used to fabricate the patches (A1 to A6) using the formulae shown in table 4.2. The total weight of the mixture prepared was 5 g. The polymer DURO-TAK® 87-6908 contains n-heptane as the solvent. Therefore Isopropyl alcohol (IPA) was used as a co-solvent to make the methanol solution (containing CPO and TU), miscible in n-heptane. The general method of fabrication discussed earlier was used for preparation of the films.

*Table 4.2: Formulae for Polyisobutylene (*DURO-TAK® *87-6908) films containing CPO, TU and PG*

Ingredients	**A1**	**A2**	**A3**	**A4**	**A5**	**A6**
CPO (%)	1	1	1	1	1	1
TU (%)	1	1	1	1	1	1
PG (%)	1	5	10	20	30	50
DURO-TAK® 87-6908 (%)	97	93	88	78	68	48
Methanol (g)	0.1	0.1	0.1	0.1	0.1	0.1
Isopropyl Alcohol (g)	0.1	0.1	0.1	0.1	0.1	0.1
n-heptane (g)	0.3	0.3	0.3	0.3	0.3	0.3

4.4.4.2. Result and discussion

There was significant phase separation in all films (A1 to A6) after drying. It was observed that the components do not form a homogenous mixture, regardless of the presence of solvents like methanol, isopropyl alcohol and n-heptane. Compared to the acrylates and silicone PSAs selected for screening, polyisobutylene was more hydrophobic and therefore incorporation of TU into the patch caused more prevalent precipitation of TU. It was therefore decided to discontinue the use of polyisobutylene for fabrication of the patch.

4.4.5. Screening of Acrylates

Keeping the percentage of TU and CPO at a constant level of 1 % of the weight of total mixture, acrylate films were fabricated with 4 levels of PG. Four acrylate grades DURO-TAK® 87-4287, DURO-TAK® 87-9088, DURO-TAK® 87-9301 and DURO-TAK® 87-4098 were used to fabricate the patches using the formula shown in table 4.3. The total weight of the mixture was 5 g. The general process of film fabrication, discussed in the previous sections was used. Methanol was the only solvent used for the dissolving the components in the acrylates films.

Table 4.3: Formulae for the films fabricated using DURO-TAK® *87-4287,* DURO-TAK® *87-9088,* DURO-TAK® *87-9301 and* DURO-TAK® *87-4098*

Ingredients	**B1/C1/D1/E1**	**B2/C2/D2/E2**	**B3/C3D2/E2**	**B4/C4D2/E2**
CPO (%)	1	1	1	1
TU (%)	1	1	1	1
PG (%)	1	5	10	20
Acrylate (%)	97	93	88	78
Methanol (g)	2.0	2.0	2.0	2.0

[B] Prepared using DURO-TAK® 87-4287 [C] Prepared using DURO-TAK® 87-9088 [D] Prepared using DURO-TAK® 87-9301 [E] Prepared using DURO-TAK® 87-4098

4.4.5.1. Results and Discussion

All the mixtures appeared clear, devoid of any phase separation. All the films were clear with a faint yellow color without any crystal formation except B4, C3 and C4 where the films were opaque and colorless. Table 4.4 shows the data for thickness of the drug film, % CPO release and amount of drug in the patch. In the case of DURO-TAK® 87-4287 (B series of patches), DURO-TAK® 87-9301 (D series of patches) and DURO-TAK® 87-4098 (E series of patches) the films did not show any increase in drug release with increase in PG content in the films. Although there was a trend in increase of drug release with increase in PG content for DURO-TAK® 87-9088, the increase in PG content did not show a corresponding increase in drug release in 24 hr. The CPO release from the patches did not meet the specification of not less than 80 % in 24 hr. Propylene glycol at 5 % w/w was used as an enhancer in fabrication of physostigmine transdermal patches using acrylate PSA (Kim, J-H. *et al* 2002). It was also noted that the tack for each of the acrylates dropped considerably with increase in PG content. Hence the approach to increase the PG content to increase drug release in acrylates was discontinued. It was recommended that use of hydrophobic plasticizers such as triacetin and triethyl citrate would be beneficial in increasing the drug release from the drug loaded acrylate films. It was hypothesized that addition of the hydrophobic plasticizers into the PSAs would reduce the glass transition temperature (Tg) of the adhesives and increase the polymer chain mobility.

Table 4.4: Characteristics of the [B]DURO-TAK® 87-4287, [C]DURO-TAK® 87-9088, [D]DURO-TAK® 87-9301 and [E]DURO-TAK® 87-4098 patches containing 1 % CPO (n = 1)

Patch	**PG (%)**	**Dry Film Thickness (mm)**	**CPO in patch (µg / cm^2)**	**% CPO Release at 24 hr**
B1	1	0.366	393.6	21.46
B2	5	0.359	405.8	15.12
B3	10	0.336	342.8	20.49
B4	20	0.361	411.7	21.88
C1	1	0.266	310.8	3.4
C2	5	0.378	449.2	4.04
C3	10	0.380	500.6	5.14
C4	20	0.419	539.8	13.65
D1	1	0.374	486.6	8.38
D2	5	0.287	306.4	14.19
D3	10	0.315	364.4	14.27
D4	20	0.331	365.3	9.06
E1	1	0.215	420.6	13.9
E2	5	0.246	446.6	14.09
E3	10	0.129	300.5	21.46
E4	20	0.244	432.2	18.92

4.4.6. Screening of Silicone PSAs

The Dow Corning® BIO-PSA 7-4302 is described as amino functional drug compatible adhesive for transdermal and topical applications. BIO-PSA 7-4502 and BIO-PSA 7-4602 are described as adhesives for application of transdermal systems on the skin. BIO-PSA 7-4302 and BIO-PSA 7-4602 are reported to show high tack whereas BIO-PSA 7-4502 shows medium tack. All of the three grades of silicone adhesives have ethyl acetate as the solvent.

4.4.6.1. Fabrication of drug loaded films

Drug loaded silicone films were prepared using the formulations similar to that of the acrylate films, with 1 % each of CPO and TU and varying the levels of PG at four levels using methanol and ethyl acetate as the solvents. There was considerable phase separation using Dow Corning® BIO-PSA 7-4302. However BIO-PSA 7-4302 showed the best tack of all the three grades. The dispersion of solids was found to be better in the other two grades (BIO-PSA 7-4502 and BIO-PSA 7-4602). Therefore, combination of the silicone adhesive grades was used as an approach to obtain desirable tack property from BIO-PSA 7-4302 and drug distribution within the film using BIO-PSA 7-4502 and BIO-PSA 7-4302. Table 4.5 gives the formulation for the four combinations of silicone adhesives screened. The weights of the ingredients were calculated for total mixture weight of 5 g each. In each of the silicone adhesive combinations with two grades, a 1:1 weight ratio was used. Similarly, for a combination of three grades of the adhesives, 33 %

of each grade was added in the combination. The percent of polymer in table 4.5 corresponds to the total polymer used. In table 4.5, G1 has a combination of BIO-PSA 7-4302 and BIO-PSA 7-4602 (1:1). The formulation G2 and G3 have the combinations BIO-PSA 7-4502: BIO-PSA 7-4602 (1:1) and BIO-PSA 7-4302:BIO-PSA 7-4502 (1:1), respectively. All three grades in equal quantities form the total polymer content in formulation G4. The general method of fabrication described in section 4.4.1 was used for preparation of the patches.

Table 4.5: Formulae for CPO films made using combinations of silicone adhesives

Ingredients	**G1**	**G2**	**G3**	**G4**
CPO (%)	1	1	1	1
TU (%)	1	1	1	1
PG (%)	2.5	2.5	2.5	2.5
Polymer (%)	95.5	95.5	95.5	95.5
Methanol (g)	0.2	0.2	0.2	0.2

4.4.6.2. Determination of Drug Release

The percent CPO release from the films G1, G2, G3 and G4 was determined using the method described earlier for the acrylate films in section 4.4.2. One film per formulation was tested using the Franz diffusion cell (n = 1).

4.4.6.3. Result and Discussion

The increase in PG content reduced the tack of the acrylate films. Therefore, a lower PG content of 2.5 % was used for the screening of combinations of silicone adhesives. There was no phase separation in any of the combinations used for preparation of CPO loaded films. The drug release was similar to that for the acrylate films for the three formulations G1, G2 and G4 with 26.7 %, 19.45 % and 16.14 %, respectively. The highest drug release among the acrylates in Table 4.4 and the silicone combinations in Table 4.5 was attained in G3 with a percent CPO release of 45.49 %. The results of drug release showed that the combination of BIO-PSA 7-4302:BIO-PSA 7-4502 (1:1) showed the best release. The use of plasticizers like Triacetin and Triethyl citrate was proposed to improve the drug release in all the combinations of the silicone adhesive grades.

4.4.7. Addition of Hydrophobic Plasticizers in acrylate and silicone films

Triacetin (TAC) and Triethyl citrate (TEC) were the hydrophobic plasticizers used in the current project. These hydrophobic plasticizers have limited miscibility in water (Snejdrova,E. and Dittrich,M., 2012). Since there was phase separation associated with incorporation of high percentages of PG in the acrylate formulations and the corresponding improvement in drug release was not significant, a lower concentration of PG was incorporated with TEC and TAC into the acrylates and the combinations of silicone adhesives. Tables 4.6 and 4.7 show the composition and drug releases from the formulations containing plasticizers in the acrylate adhesives and silicone adhesives,

respectively. In each of the formulations shown in tables 4.6 and 4.7, the concentrations of CPO and TU are constant at 1 % of the weight of polymer mixture.

In the formulations H1 to H8 in Table 4.6, the CPO release was not significantly affected by the presence of either of the two hydrophobic plasticizers. However, there was an increase in the CPO release in formulations H9 and H10 upon increasing the levels of TAC and TEC in acrylate 87-4287. The films H13 and H14 showed a 3 fold increase in CPO release compared to H5 and H6, respectively. There was a similar increase in drug release when the TAC and TEC levels were increased to 10 % in the acrylate 87-4098. Still this increase was not significantly greater than the CPO release attained for films with 20 % PG in table 4.4. Certainly the release of CPO in presence of TAC and TEC was not close to the desired release of ≥ 80% in 24 hr for an immediate release patch.

The formulation I1 with 77.35 % CPO release in table 4.7 showed the drug release comparable to the acceptance criterion of ≥ 80% CPO release in 24 hr. It was decided to evaluate formulation I1 further for the *in vitro* skin permeation and *in vitro* skin penetration prior to the *in vitro* transungual permeations study.

Table 4.6: Formulae of films fabricated using acrylate PSA and hydrophobic plasticizers

Formulation No.	**TAC (%)**	**TEC (%)**	**PG (%)**	**DURO-TAK® grade and (%)**	**% CPO release at 24 hr (0.64 cm^2 release area)**
H1	5	0	5	87-4287 (88)	15.94
H2	0	5	5	87-4287 (88)	16.88
H3	5	0	5	87-9088 (88)	12.91
H4	0	5	5	87-9088 (88)	12.67
H5	5	0	5	87-9301 (88)	10.61
H6	0	5	5	87-9301 (88)	13.27
H7	5	0	5	87-4098 (88)	16.05
H8	0	5	5	87-4098 (88)	19.02
H9	10	0	2	87-4287 (86)	44.48
H10	0	10	2	87-4287 (86)	29.7
H11	10	0	2	87-9088 (86)	7.51
H12	0	10	2	87-9088 (86)	5.12
H13	10	0	2	87-9301 (86)	31.64
H14	0	10	2	87-9301 (86)	36.33
H15	10	0	2	87-4098 (86)	31.9
H16	0	10	2	87-4098 (86)	35.31

Table 4.7: Formulae of films fabricated using silicone PSAs and hydrophobic plasticizers

Formulation No.	**TAC (%)**	**TEC (%)**	**PG (%)**	**Polymer grade and %**	**% CPO release at 24 hr**
I1	5	0	5	A(88)	**77.35**
I2	0	5	5	A (88)	33.31
I3	5	0	5	B (88)	10.13
I4	0	5	5	B (88)	59.75
I5	5	0	5	C (88)	17.38
I6	0	5	5	C(88)	16.12
I7	5	0	5	D (88)	18.67
I8	0	5	5	D (88)	13.28
I13	10	0	2	C (86)	30.27
I14	0	10	2	C (86)	33.52

[A] Combination of 7-4302 and 7-4502 (1:1) [B] Combination of 7-4302 and 7-4602 (1:1) [C] Combination of 7-4502 and 7-4602 (1:1) [D] Combination of all the three grades in equal proportions

4.4.7.1 *In vitro* skin permeation of CPO

4.4.7.1.i. Experimental Method

The formulation I1 was studied for the transdermal permeation of CPO using human cadaver skin (Allosource, Cincinnati, OH) and Franz diffusion cells. Three films each

with an area of 4 cm^2 square patch were cut from film I1. The process of *in vitro* skin permeation as discussed in Chapter 2 was performed using the films and Penlac nail lacquer. The dermatomed human cadaver skin (Allosource, Ohio) was thawed at room temperature for 15 min. The skin was cut into 1.5 cm by 1.5 cm pieces and placed in 100 mL of pH 7.4 PBS solution for 20 min. The skin pieces were mount on the Franz diffusion cells with orifice diameter of 9 mm and allowed to equilibrate with the receiving medium (pH 7.4 PBS) for 60 min prior to the application of the patch. The release liner was removed from the films and the matrix surface was placed in contact with the skin. The area of 0.64 cm^2 of the skin was in contact with the receiving medium. The receiver compartment was sampled at 1, 2, 4, 8, 12 and 24 hr by withdrawing 0.5 mL of the receiving medium and replacing with equal volume of pH 7.4 PBS. At the end of 24 hr, epidermis and dermis were heat separated and extracted for CPO content. Since the skin was very thin, it was difficult to handle the epidermis and the dermis for thickness measurements. Hence the drug level in each of these layers was reported as amount/unit area. The samples were analyzed for CPO using the HPLC method described in Chapter 2.

4.4.7.1.ii. Results and Discussion

The *in vitro* permeation of CPO across the human skin is shown in figure 4.6. Penlac lacquer shows a non-steady state permeation. The amount of CPO permeating the skin from Penlac nail lacquer was approximately 50 % higher at the initial time points (1 to 4 hr) compared to the formulation I1. The PSA film I1 however shows Fickian diffusion

profile with a permeation flux of 0.85 ± 0.36 µg/cm^2/hr and a lag time of 3.8 ± 0.6 hr. The cumulative amounts CPO permeating the skin in 24 hr ($Q_{24\ hr}$) in presence of I1 film and Penlac were 17.77 ± 7.25 µg/cm^2 and 9.90 ± 5.15 µg/cm^2, respectively. Greater amounts of CPO permeated the skin upon treatment with I1 film. This was the major drawback of the formulation as one of the objectives of topical delivery of CPO to the skin is to minimize transdermal permeation into the systemic circulation and increase the epidermal accumulation of CPO.

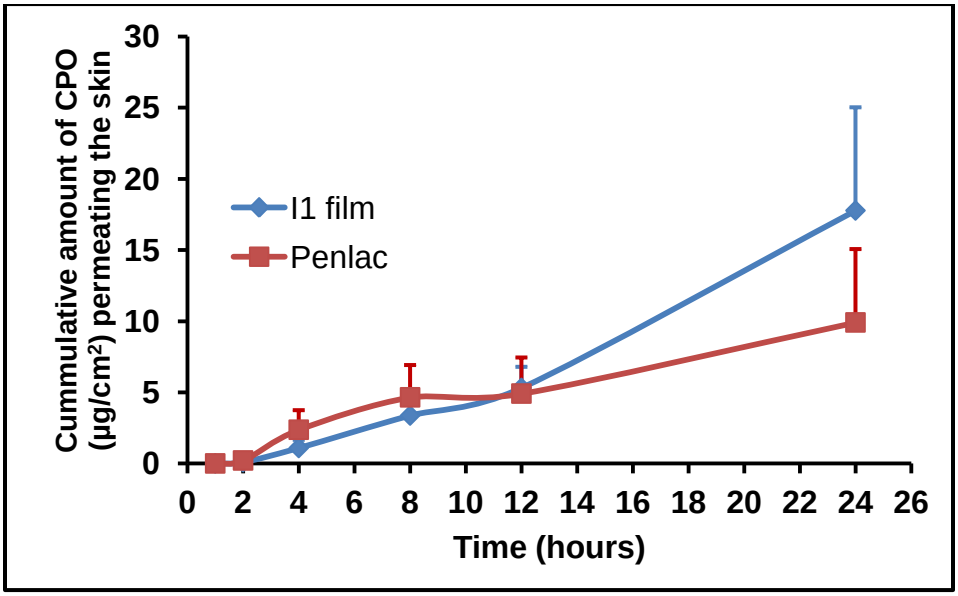


Figure 4.6: The in vitro skin permeation of CPO from I1 film and Penlac® nail lacquer

The ability of the formulation I1 to deliver CPO into the skin layers is shown in figure 4.7. The amounts of CPO in the epidermis and dermis upon treatment with I1 film were

not significantly different from that of Penlac® nail lacquer (p-value >0.05 obtained using ANOVA (Microsoft Excel 2007)).

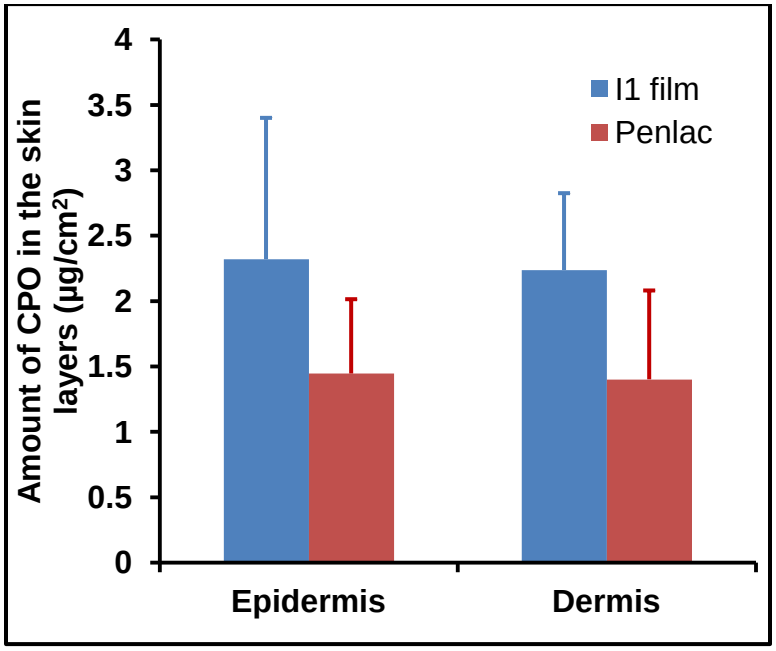


Figure 4.7: The in vitro skin penetration of CPO from I1 film and Penlac® nail lacquer

4.4.7.2. *In vitro* transungual permeation of CPO

4.4.7.2.i. Experimental Method

A method similar to the methods described in Chapter 2 was used. The cadaver toenails (Anatomy Gifts Registry) were thawed to room temperature. The nails were washed with nanopure water three times and dried using tissue papers. The toenails were cut into 7 mm X 7 mm (49 mm^2) pieces in order to minimize the lateral diffusion. The thickness of the nail pieces were measured at the center using digital calipers (Mitutoyo). The nail

pieces were then hydrated at 100 % RH overnight prior to start of experiment (Figure A, Appendix A). The hydrated nail pieces were placed in the neoprene nail adapters with orifice diameter of 5 mm. The adapters were mounted between the donor and receiver compartment so that the ventral nail surface faces the receiving medium. The receiver compartment was filled with pH 7.4 PBS (3 mL) containing 0.1 %w/v gentamicin sulfate and the assembly was maintained at 32 ± 1 ºC throughout the duration of the experiment. The nails were equilibrated with the receiving medium for 60 min prior to the application of the first patch. Each sample was tested in triplicate. Penlac® nail lacquer was used as the positive control. The patches with diameter of 5 mm were cut. The release liner was removed and the drug matrix was placed in contact with the nail. The new patch was applied every 24 hr for 30 days. Throughout the duration of the study, the Franz cells were dosed with a new patch daily. The control cells were dosed with Penlac® daily for seven days. On the 7th day, alcohol was used to remove all the layers of the nail lacquer and a new layer applied daily for the next seven days. This process of lacquer application was based on the instructions in the package insert of Penlac® nail lacquer. On the days 11, 16, 21, 26 and 30, 0.5 mL of the sample was withdrawn from the receiver compartment and replaced with equal volume of new receiving medium. The samples were analyzed for CPO using the HPLC method described in Chapter 2. The drug concentrations within the nail were also quantified at the end of 30 days as described in Chapter 2. The concentration of CPO within the nail was reported as µg CPO/mg nail.

4.4.7.2.ii. Results and Discussion

The average thicknesses of the toenails used for evaluation of I1 film and the positive control were 0.623 ± 0.057 mm and 0.703 ± 0.065 mm, respectively. The transungual permeation profile showed similar transungual permeation of CPO from both I1 film and the positive control (Penlac® nail lacquer). There was no significant improvement in the amount of CPO permeating the human toenails. The concentration of CPO within the nails was greater in presence of Penlac® nail lacquer with significantly higher concentration in the periphery of the toenails (p-value < 0.05 obtained using ANOVA (Microsoft Excel 2007)) (Figure 4.8). The transdermal permeation, the skin penetration, the transungual permeation and accumulation of CPO within the toenails in presence of the silicone PSA film (I1) was inadequate to meet the criteria for successful topical formulation. Penlac nail lacquer was more efficient than the patch system when tested on the human cadaver skin and the human cadaver toenails.

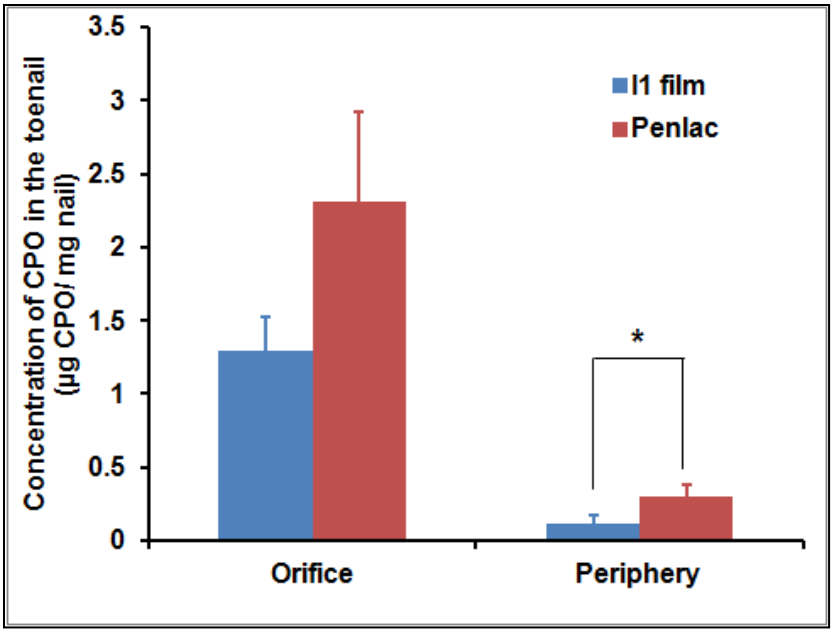


Figure 4.8: Concentration of CPO within the toenails after treatment with I1 film and Penlac® nail lacquer for 30 days.

4.4.8. Strategy moving forward

Extensive screening of the PSAs were performed to increase the drug release and to improve the ease of fabrication. All the screening studies were performed with 1 % of CPO and TU. It was planned that the data from these preliminary screening studies will be used to build a design space and different percentages of CPO, TU and plasticizers will be evaluated using Design of Experiments (DOE). However, the screening studies did not demonstrate any remarkable improvement in drug release for the patches regardless of addition of co-solvents and hydrophobic plasticizers. It was also noted that the addition of higher levels of TU in the PSA was not compatible due to crystallization of TU in the dry films (Figures 4.3, 4.4, 4.5). It was observed that the DIA design of the

patches had a limited potential for modification of the levels of the matrix components without a significant drop in the physical characteristics (tack) of the patch. Therefore, a modification of the patch design was considered to be important to obtain an efficient transungual patch.

4.5. Drug-in-hydrophilic matrix design

The design of the transungual patch was modified to overcome the major challenges in incorporation of the penetration enhancers TU and PG in the patch and to impart the flexibility to increase the concentrations of TU and CPO as deemed necessary. Unlike the initial part of this chapter, the experimental methods and the results for the modified design are discussed separately.

4.5.1. Experimental Methods

4.5.1.1 Drug in hydrophilic matrix design

The novel patch design had a drug in matrix design with a peripheral layer of pressure sensitive adhesive as shown in figure 4.9. Relative to the DIA design shown in figure 4.1, the modified design had the drug and the PEs dissolved in a hydrophilic polymer matrix using hydroxy propyl methylcellulose or polyethylene oxide polymers. The components of the new design are backing membrane (BM), pressure sensitive adhesive (PSA), CPO

and PEs dissolved/dispersed in a hydrophilic carrier polymer [$(CPO+PE)_{HP}$] and release liner (RL). The selection of PSA for this patch design is discussed in Appendix B.

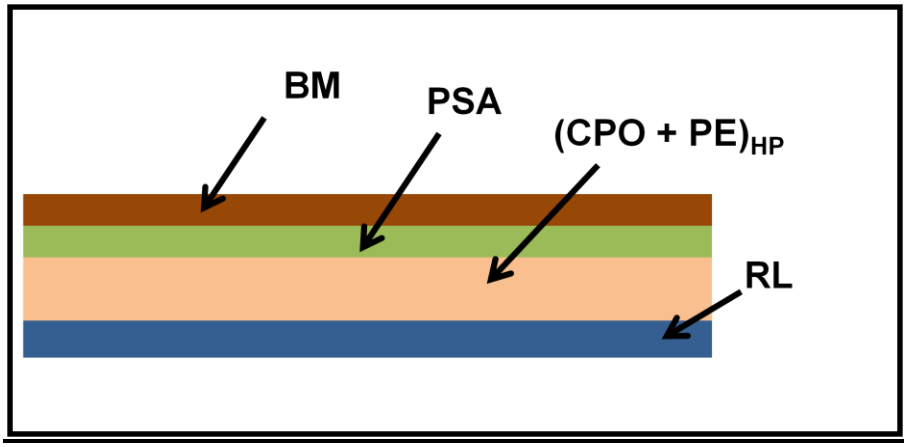


Figure 4.9: Modified drug-in-hydrophilic matrix type patch design

4.5.1.2 Determination of solubility of TU in solvent system

A solvent evaporation method was used for the film fabrication. The solvent system used was acetone:water (9:1 v/v). The addition of TU at > 1% in the PSAs lead to its crystallization in the dry PSA film. Solubility of TU in the PSAs was increased by addition of cosolvents in fabrication of the DIA design of patches. Therefore, prior to start of fabrication of the patches with the novel design, the solubility of TU at different concentrations was determined in acetone:water (9:1 v/v). TU at 1 mg, 10 mg and 100 mg was dissolved in 1 mL of acetone:water (9:1 v/v).

4.5.1.3. Preparation of drug in hydrophilic matrix

The ability of the hydrophilic polymers to form flexible films and the capability of the matrix to incorporate different drug loads was studied using the formulae in table 4.8. The quantities of the components of the matrix were calculated as percentage of dry polymer weight of 0.2 g. For the preparation of drug loaded matrix CPO, TU and the polymer were weighed into a glass vial. Six mL of acetone:water (1:1) was added to the vial and vortexed for 2 min. PG was weighed into the glass vial and vortexed for 5 min. The resulting mixture was a suspension of the polymer. The mixture was poured into a disposable petri-dish (60 mm X 15 mm) and air dried overnight (Step 3 in figure 4.10). This method of preparation of the hydrophilic matrix is a modification of the literature method (Chandak A.R. and Verma, P.R.P. 2008).

4.5.1.4. Patch fabrication

The acrylate PSA (87-4287) (15 g) was cast on the release liner (3M™ Scotchpak™ 9742) (15 cm X 15 cm) and air dried overnight. The backing membrane (3M™ CoTran™ 9707) (15 cm X 15 cm) was laid on the dry acrylate film (Step 1 in figure 4.10). Discs (60 mm diameter) were cut out from the acrylate films (Step 2 in figure 4.10). Step 3 of the patch fabrication process is described in preparation of hydrophilic matrix section. The release liner was removed from the acrylate disc (Step 4 in figure 4.10). The adhesive side of the acrylate film was laid onto the polymer matrix in the petri-dish (Step 5). This helped to separate the matrix from the petri-dish. The release liner was replaced

on top of the acrylate disc (Step 6) to form the complete patch (Step 7). The method of fabrication is shown schematically in figure 4.10.

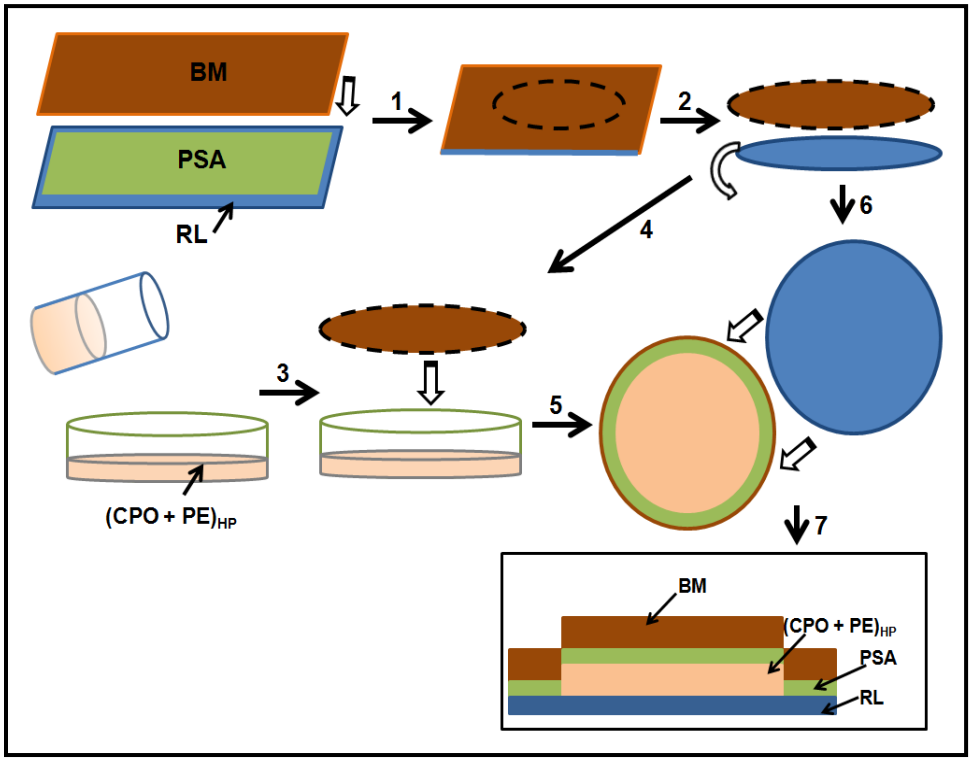


Figure 4.10: Fabrication of novel transungual patch

Table 4.8: Hydrophilic polymers screened to be drug carriers in the patch

Polymer	**No.**	**% CPO**	**% TU**	**% PG**
HPMC K15M	J1	1	1	150
	J2	5	1	150
	J3	10	1	150
HPMC K100M	K1	1	1	150
	K2	5	1	150
	K3	10	1	150
Polyox WSR-301	L1	1	1	150
	L2	5	1	150
	L3	10	1	150
Polyox WSRN-80	M1	1	1	150
	M2	5	1	150
	M3	10	1	150
Polyox WSRN-750	N1	1	1	150
	N2	5	1	150
	N3	10	1	150
Polyox WSR-303	O1	1	1	150
	O2	5	1	150
	O3	10	1	150

4.5.1.5. *In vitro* drug release

The percent drug release was determined from each of the hydrophilic polymer films and the complete patches using Franz diffusion cells. Square films/patches with 4 cm^2 area were cut from the films. For the initial screening, only the hydrophilic polymer matrix was used for drug release. These films were devoid of the acrylate layer. During the release study of the film, the hydrophilic polymer film was placed in contact with the skin and kept occluded throughout the duration of the experiment using parafilm. Since it was a screening experiment only one film per sample was studied (n = 1).

In the release study of the patch, the thickness of the patch was measured using digital micrometers (Marathon). The average thicknesses of the release liner, acrylate layer and the backing membrane were subtracted from the total patch thickness to calculate the thickness of the polymer matrix. In the case of the patch system the release liner was peeled off and the release surface was placed facing the donor compartment. The receiver compartment was filled with 3 mL of 7.4 PBS. Samples (1.0 mL) were withdrawn from the receiver compartment at 1, 2, 4, 8, 12 and 24 hr and replaced with equal volume of pH 7.4 PBS. Three patches per sample were evaluated for drug release (n = 3). The cumulative percent release for CPO from the films and the patches were calculated. The drug release kinetic models for zero order, first order and square root of time release were fit to the drug release data from the transungual patches.

4.5.1.6. *In vitro* skin permeation and penetration

The *in vitro* skin permeation studies were performed using Franz diffusion cells with internal volume of 3 mL and orifice of 9 mm diameter. The cadaver skin was thawed to

room temperature. The skin was then placed in pH 7.4 PBS for 20 minutes. The skin pieces were placed between the donor and the receiver compartment of the Franz cell. The receiver compartment was filled with 3 mL of pH 7.4 PBS and the skin was allowed to equilibrate for 60 min prior to application of the patch. The release liner was removed and the patch was placed on the skin with the release surface in contact with the skin. Samples (0.5 mL) were withdrawn at 1, 2, 4, 8, 12 and 24 hr and replaced with equal volume of fresh buffer to maintain the skin conditions. The cumulative amount of CPO ($\mu g/cm^2$) permeating unit area of the skin was calculated and plotted against time to determine the flux ($\mu g/cm^2/hr$) and lag-time (hr). The steady state flux was calculated from the slope of the linear portion of the plot. Penlac nail lacquer was used as the positive control. The CPO levels in the epidermis and dermis were determined by using the heat separation and extraction methods described in chapter 2.

4.5.1.7. *In vitro transungual permeation*

A method similar to the methods described in Chapter 2 and for the I1 film was used. The cadaver toenails (Anatomy Gifts Registry) were thawed to room temperature. The nails were washed with nanopure water three times and dried using tissue papers. The toenails were cut into 7 mm X 7 mm pieces in order to minimize the lateral diffusion. The thickness of the nail pieces were measured at the center using digital calipers. The nail pieces were then hydrated at 100 % RH overnight prior to start of experiment. The hydrated nail pieces were placed in the neoprene nail adapters with orifice diameter of 5 mm. The adapters were mounted between the donor and receiver compartment so that the ventral nail surface faces the receiving medium. The receiver compartment was filled

with pH 7.4 PBS (3 mL) containing 0.1 %w/v gentamicin sulfate and the assembly was maintained at 32 ±1 ºC throughout the duration of the experiment. The nails were equilibrated with the receiving medium for 60 min prior to the application of the first patch. Each sample was tested in triplicate. Penlac® nail lacquer was used as the positive control. The patches with diameter of 5 mm were cut. The release liner was removed and the drug matrix was placed in contact with the nail. The study continued for 30 days. Throughout the duration of the study, the Franz cells were dosed with a new patch daily. The control cells were dosed with Penlac® daily for seven days. On the 7th day, alcohol was used to remove all the layers of the nail lacquer and a new layer applied for the next seven days. This process of lacquer application was based on the instructions in the package insert of Penlac® nail lacquer. Every 5 days, 0.5 mL of the sample was withdrawn from the receiver compartment and replaced with equal volume of new receiving medium. The samples were analyzed for CPO using the HPLC method described in Chapter 2. The drug concentrations within the nail were also quantified at the end of 30 days as described in Chapter 2. A plot of cumulative amount of CPO (µg/cm^2) permeating the human nail against time (days) was plotted to determine the lag-time (days) and permeation flux (µg/cm^2/day). The concentration of CPO within the nail was reported as µg CPO/mg nail.

4.5.1.8. Statistical analysis of data

One way analysis of variance ANOVA was used to determine difference in means (Minitab 16). The post hoc test (Dunnett's test) was used to compare data from the

patches against Penlac® nail lacquer (control). p-value <0.05 was considered as significantly different.

4.5.2. Results and Discussion

4.5.2.1 Determination of solubility of TU in the solvent system

TU was found to be completely soluble in the solvent system (acetone: water 9:1 v/v) at all the three concentrations (1 mg/mL, 10 mg/mL and 100 mg/mL). The solubility to TU in the solvent used for matrix fabrication increases the potential of the matrix to incorporate greater levels of TU (if required).

4.5.2.2. Preparation of drug in hydrophilic matrix and determination of drug release

The hydrophilic films generated using the formulae in table 4.8, were studied for drug release using Franz diffusion cell. The films prepared using HPMC K15M and HPMC K100M were very flexible at all three drug loads. These films did not exhibit any tack upon touching the matrix surface. Similar to HPMC, the Polyox grades WSR-301 and WSR-303 formed very flexible films. However, the grades WSRN-750 and WSRN-80 did not dry completely to form films. The residue in the petri-dish was a viscous gel which could not be separated intact from the petri-dish. Hence the polyox grades WSRN-750 and WSRN-80 were not studied for drug release. The initial screening was done with one patch per polymer (n=1). Hence the data in figure 4.11 does not show error bars.

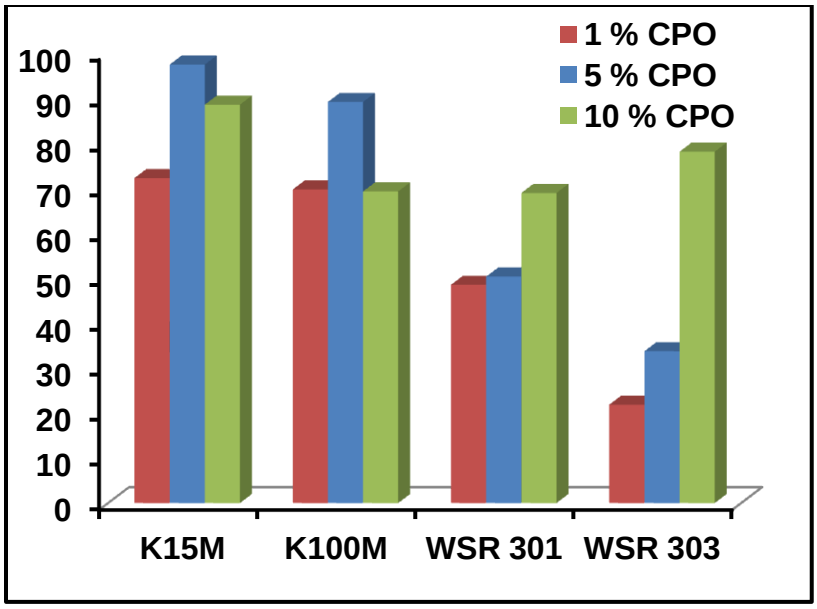


Figure 4.11: Percent CPO release after 24 hr from the patches with different drug loads (n = 1)

4.5.2.3. Patch fabrication and determination of drug release

The process of patch fabrication described in figure 4.10 was followed to prepare patches with K15M (5 % and 10 % CPO), K100M (5 % CPO), WSR-301 (10 %CPO) and WSR-303 (10 %CPO). The release of CPO from these patches was evaluated in triplicate using Franz diffusion cells.

Figure 4.12 shows the release profile for each of the five patch systems studied. The equations 4.1, 4.2 and 4.3 show the models for zero order, first order and square root of time (Gafourian,T. et al 2007). The goodness of fit was determined using the graphical residuals analysis and the adjusted R^2 values using Microsoft Excel 2007. A normal probability plot was used to determine the randomness of the residuals. The distribution

of the residuals was random for all the three models showing that all the three models fit the data well. The value of the adjusted R^2 was used to determine the best model.

$Q_t = kt$ ……..(4.1)

$Ln\ (100-Q_t) = lnQ_0 - kt$ ……..(4.2)

$Q_t = kt^{1/2}$ ..……(4.3)

In equation 4.1 to 4.3, Q_t is the percent of CPO released at time t and k is coefficient in the equations. In equation 4.2, Q_0 is the initial percentage of CPO, which is equal to 100. In all the three models, the residuals were random and the probability plot showed that the residuals were close to a straight line for the five patches. Hence the adjusted R^2 value was used to select the best fit model. The adjusted correlation coefficients shown in bold case in table 4.9 show the best fit models for the corresponding data.

The patches prepared using HPMC showed CPO release of not less than 80 % in 24 hr (figure 4.12). These patches showed promise as immediate release formulations and were therefore evaluated for skin permeation and penetration of CPO.

Table 4.9: The correlation coefficient (R^2) and adjusted R^2 for each of the three release models.

Patch	Zero Order		First Order		Higuchi	
	R^2	Adj R^2	R^2	Adj R^2	R^2	Adj R^2
WSR-303 10 % CPO	0.9198	0.8931	0.9767	0.9650	0.9863	**0.9794**
WSR-301 10 % CPO	0.9829	0.9771	0.9922	**0.9884**	0.9789	0.9683
K100M 5% CPO	0.9885	**0.9827**	0.9783	0.9674	0.9568	0.9353
K15M 10% CPO	0.9536	0.9304	0.9907	**0.9861**	0.9883	0.9825
K15M 5% CPO	0.8664	0.7996	0.9149	**0.8724**	0.8256	0.7384

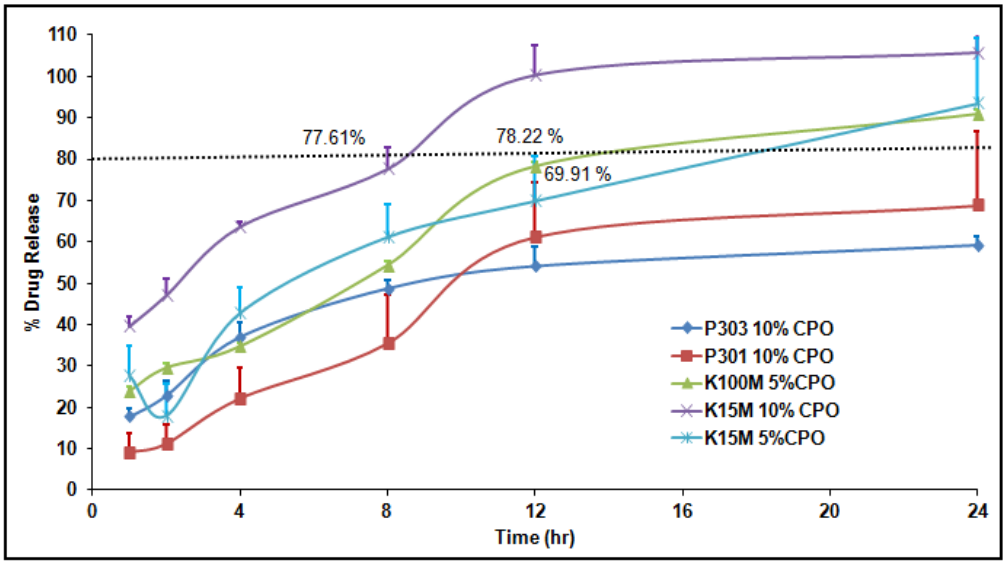


Figure 4.12: Percent CPO release from CPO loaded films using the selected hydrophilic polymers with acrylate lining (n=3)

4.5.2.4. *In vitro* skin permeation and penetration

Penlac® nail lacquer had a skin permeation of 29.64 ± 7.54 µg/cm^2 of CPO at end of 24 hr. The permeation parameters of the acrylate lined transungual patches are shown in table 4.10. The patches containing Polyox WRN-301 and HPMC K15M showed the highest lag times for permeation. The Q_{24hr} for the K15M 10% CPO and K15M 5% CPO patches are similar to that for Penlac®. The permeation of CPO from Penlac followed the non-steady state permeation and therefore the lag-time and steady state flux could not be calculated. Ethyl acetate and isopropyl alcohol are the solvents in Penlac nail lacquer (Penlac® nail lacquer package insert). The initial concentration of CPO in the lacquer is 8 % and as the solvents evaporate the concentration of CPO is 34.8 % (Gupta,A.K. et al 2005). This change in the concentration gradient leads to higher driving force for permeation of CPO across the skin at the first few time points. Ethyl acetate is also reported to a skin permeation enhancer (H. Trommer and R.H.H. Neubert, 2006). The enhancer effect coupled with increased concentration gradient in Penlac® could lead to enhancement of CPO permeation across the skin. The permeation data coupled with the penetration data helps to determine the efficiency of the transungual patch formulation in topical delivery of CPO into the skin.

Table 4.10: Skin permeation parameters for the transungual patches

Formulation	Skin permeation flux ($\mu g/cm^2/hr$)	Lag-time hr	$Q_{24\ hr}$
P303 10% CPO, 1% TU	0.56 ± 0.15	3.0 ± 1.4	9.26 ± 5.68
P301 10% CPO, 1% TU	0.29 ± 0.11	4.3 ± 1.7	5.15 ± 3.02
K100M 5% CPO, 1%TU	0.86 ± 0.62	2.5 ± 1.8	18.68 ± 12.95
K15M 10% CPO, 1%TU	1.41 ± 1.09	4.1 ± 0.3	29.67 ± 23.04
K15M 5% CPO, 1%TU	1.22 ± 0.63	3.8 ± 0.9	26.07 ± 13.05

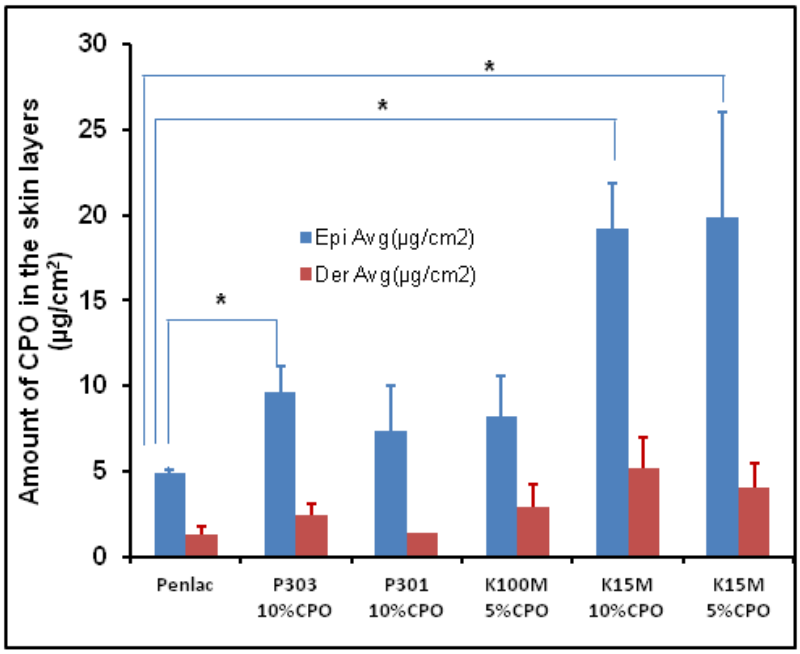


Figure 4.13: Penetration of CPO into the epidermis and the dermis using the novel patch system

The figure 4.13 shows the accumulation of CPO within the epidermis and the dermis after application of the transungual patches for 24 hr. The amounts of CPO within the unit area of epidermis after treatment with HPMC K15M 10%, HMPC K15M 5 % CPO and WSR-303 10 % CPO patches were found to be significantly greater than that in presence of Penlac® (p-value < 0.05). The patches containing HPMC K15M showed the highest levels of CPO in the epidermis without corresponding increase in permeation. Therefore it was decided to evaluate the two HPMC K15M patches for the *in vitro* nail permeation.

4.5.2.5. *In vitro* transungual permeation

Since K15M with 5 % CPO and 10 % CPO and 1 % TU each showed favorable accumulation of CPO within the epidermis, they were studied for transungual permeation. The cumulative amount of CPO permeating at end of 29 days ($Q_{29\ days}$) was 1.25 ± 1.05 µg/cm^2, 2.43 ± 2.21 µg/cm^2 and 0.77 ± 0.70 µg/cm^2 for K15M 10 % CPO 1 %TU, K15M 5 % CPO 1 % TU and Penlac® respectively. The level of CPO in the receiver compartments for each of the three samples was not significantly different. Figure 4.14 shows the concentration of CPO within the orifice and the periphery of the toenails for each of the three samples. These concentrations were not significantly greater than Penlac® (control). The transungual permeation of CPO in presence of the patches was comparable with than in presence of Penlac®. It was therefore concluded that concentration of TU has to be increased in the patch. It was proposed to increase the

concentration of TU to 10 % w/w of the polymer weight in the patch and to maintain the drug concentration at 10 % w/w.

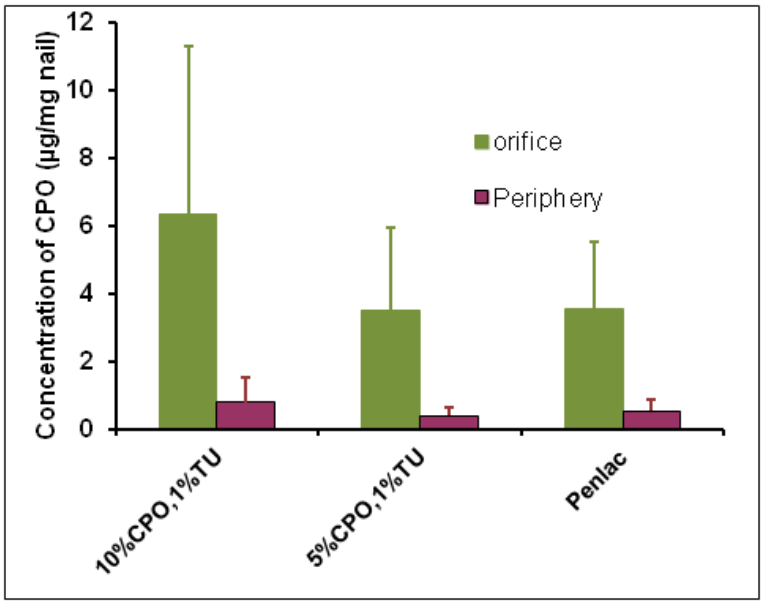


Figure 4.14: Selection of CPO concentration for the prototype patch

4.6. Conclusion

The pressure sensitive adhesives used for transdermal patch development could not be used for fabrication of a transungual patch for immediate release of the drug to meet the CPO release criterion of not less than 80 % release in 24 hr. The incorporation of penetration enhancers such as TU into these PSAs posed an additional challenge in the fabrication of the patch. The addition of higher levels of CPO, TU and PG in the drug-in-adhesive design patch gave insufficient CPO release, crystallization of TU and phase

separation of PG, regardless of addition of hydrophobic plasticizers like triacetin (TAC) and triethyl citrate (TEC). The modified design with a separate hydrophilic polymer matrix gave significant improvements in the physical appearance and functional properties (increased CPO release and delivery into the target tissues) of the patch. The patch was pliable and the design allowed the increase in concentration of the matrix components, (CPO, TU and PG) for enhanced epidermal and transungual delivery of CPO. The separate acrylate PSA layer as a peripheral adhesive helped to maintain adequate tack regardless of the composition of the hydrophilic matrix. The unique design also helped to attain the goals of increased transungual penetration and permeation and increased accumulation of drug within the epidermis while minimizing transdermal permeation. The modified design also helped to attain the critical properties of a successful topical formulation. This is the first report of a transungual patch system incorporating the skin and nail penetration enhancers (PG and TU respectively) with the drug CPO.

CHAPTER 5

PROTOTYPE PATCH FORMULATION AND ITS CHARACTERIZATION

5.1. Abbreviation

CP: Ciclopirox

CPO: Ciclopirox olamine

DSC: Differential Scanning Calorimetry

FTIR: Fourier Transform Infrared Spectroscopy

OLM: Optical Light Microscopy

PG: Propylene glycol

pH 7.4 PBS: 10 mM pH 7.4 Phosphate Buffered Saline

PLM: Polarized light microscopy

SEM: Scanning Electron Microscope

TU: Thiourea

USP: U.S. Pharmacopoeia

XRD: X-Ray Diffraction

5.2. Introduction

The composition of a prototype transungual patch was modified based on the screening data discussed in modified design of patch in Chapter 4. The patch with a concentration 10 % w/w each of CPO and TU was studied as the prototype transungual patch. The characterization of a novel transungual formulation for passive delivery of CPO into the nail and the nail folds is discussed. The prototype was characterized for its functional characteristics by determining *in vitro* drug release, *in vitro* skin permeation, *in vitro* skin penetration, *in vitro* transungual permeation and drug penetration in human nails. The absence of crystallization in the hydrophilic matrix was studied using SEM, PLM, OLM, DSC, XRD. Any potential drug-polymer interaction within the formulation was studied using FTIR.

5.3. Materials

Ciclopirox olamine (CPO) was purchased from Haorui Pharma-chem Inc., Edison, New Jersey, USA. Potassium phosphate monobasic, sodium hydroxide, gentamicin sulfate, dimethyl sulfate and triethyl amine were bought from Sigma Aldrich. Thiourea and sodium phosphate dibasic were obtained from Acros Organics. Hydroxypropyl methylcellulose (HPMC K15M) was purchased from the Dow Chemical Company (Michigan) The backing membrane (3M™ CoTran™ 9707) and the release liner (3M™ Scotchpak™ 9742) were obtained from 3M (St.Paul, MN) Franz diffusion cells and neoprene nail adapters were purchased from PermeGear. Human cadaver toe nails were purchased from Anatomy Gifts Registry (Hanover, Maryland). The human cadaver skin

was obtained from Allosource (Cincinnati, Ohio). Stainless steel patch holders were purchased from Agilent. Nanopure water was used for preparation of 10mM pH 7.4 phosphate buffered saline. HPLC grade Acetonitrile from Fischer scientific was used in preparation of the mobile phase. All the chemicals were used as received without any further purification.

5.4. Experimental Methods

5.4.1. Fabrication of prototype transungual patch

The general method of patch fabrication described in figure 4.10 was used to prepare the prototype patch. The composition of the prototype patch is shown in table 5.1. The selection of the pressure sensitive adhesive is shown in appendix B. The concentration of TU was increased to 10 %w/w in order to ensure CPO delivery across the human nail.

Table 5.1: The composition of the prototype transungual patch

Patch Layer	**Composition**
Backing Membrane	Ethylene Vinyl Acetate membrane (3M™CoTran™ 9707 Membrane)
Pressure sensitive adhesive	DURO-TAK® 87- 4287
Hydrophilic matrix	HPMC K15M, CPO (10 %w/w), TU (10 %/w/w), PG (150 %w/w)
Release liner	Fluoropolymer coated polyester film (3M™ Scotchpak™ 9742 Membrane)

5.4.2. The in vitro drug release

The novel transungual patch was characterized for drug release using the USP Apparatus 5 (Paddle over disc method). The methodology of the test was a modification of the official method reported in USP35-NF30 which is official until May 1, 2013. There is not official monograph for CPO formulated in a patch system. The transdermal drug delivery systems described in the USP were deemed most similar to the prototype patch system developed for CPO. Therefore a modification of official release tests for transdermal systems in USP 35 was used to study the release of CPO from the novel patch system. Figure 5.1 shows the components of the USP apparatus 5 used in the release study. Five hundred mL of dissolution medium (pH 7.4 PBS) was added to a standard 900 ml vessel and equilibrated to a temperature of 32 ± 0.5 ºC. The release liner of the prototype patch was removed and the patch was mounted on the stainless steel disc (Agilent). The release surface was facing the top in the patch holder (disc). The disc maintains a release area of 10 cm^2. The disc was then placed in the bottom of the vessel. The paddle was adjusted to maintain a distance of 2.5 ± 0.2 cm from the drug release surface patch holder. The paddle was started immediately at 50 rpm. At predetermined time points of 10, 20, 40, 60, 120 and 240 min, 5 mL of the dissolution medium was withdrawn and replaced with equal volume of fresh buffer. Acetonitrile (0.5 mL) was added to the samples (0.5 mL), vortexed for 5 min, and centrifuged at 10000 rpm for 10 min. This sample preparation procedure helped to remove HPMC in the sample. The supernatant was derivatized as discussed in the previous chapters. The derivatized samples were filtered through Restek® 0.22 µ syringe filters and analyzed using HPLC. The three kinetic models (zero order, first order and square root of time) were used to fit the CPO release data. The goodness of

fit was determined using the graphical residuals analysis and the adjusted R^2 values (Microsoft Excel 2007). A normal probability plot (Microsoft Excel 2007) was used to determine the randomness of the residuals and the goodness of the model fit.

5.4.3. The in vitro skin permeation

The method discussed in Chapter 4 for the *in vitro* skin permeation of CPO from the patch formulations was used to determine the skin permeation of the prototype patch. Penlac® nail lacquer was used as the positive control for comparison. At time points 2, 4, 6, 8, 12 and 24 hr, 0.5 mL of the receiving medium was sampled, derivatized and analyzed using HPLC.

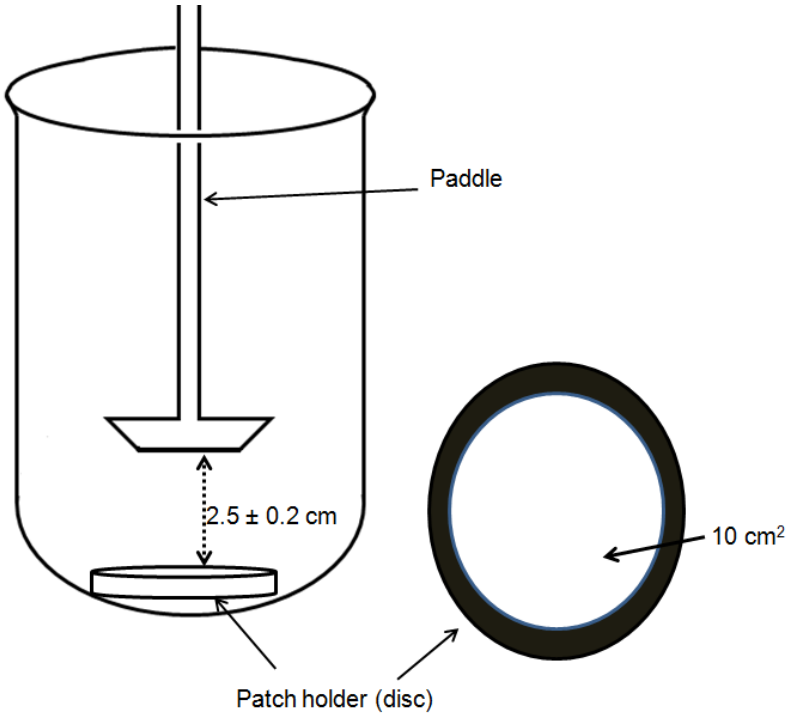


Figure 5.1: Dissolution Apparatus (USP Apparatus 5)

5.4.4. The in vitro skin penetration

The method for the determination of the *in vitro* skin penetration of CPO, discussed in Chapter 4 was followed for the prototype patch formulation. The positive control in this study was Penlac® nail lacquer. The levels of CPO in the epidermis and the dermis were quantified for the prototype patch and the control formulation. The enhancement factor for CPO accumulation in the epidermis and dermis were calculated using equation 5.1

$$\text{Enhancement factor, EF epi/der} = \frac{[\text{CPO}]\text{epi/der for the patch}}{[\text{CPO}]\text{epi/der for Penlac}} \qquad \text{.....(5.1)}$$

Where [CPO] epi/der is the concentration of CPO in the epidermis or the dermis.

5.4.5. The in vitro transungual permeation

Similar to the *in vitro* skin permeation and penetration studies, the *in vitro* transungual permeation of CPO was also studied using the same method as discussed in Chapter 4. This study was performed using human cadaver toenails obtained from Anatomy Gifts Registry. The study had duration of 32 days. Samples (0.5mL) were withdrawn from the receiver compartment on 5, 10, 15, 20, 25, and 32 days. The CPO concentration in the nail on day 32 was determined for the area under the orifice and the periphery using the method detailed in Chapter 4.The sample preparation and analysis were as discussed in Chapter 4.

5.4.6. Preparation of control patches for characterization

It was hypothesized that the drug crystallization (if present) would be higher with higher drug load in the patch. Therefore control patches namely 0P, 40P, and 80P were prepared and comparatively analyzed with the prototype patch 10P. The actual drug loads in terms of the patch weight were 0 % w/w, 3.83 % w/w, 12.99 % w/w and 22.99 % w/w for 0P, 10P, 40P and 80P patches, respectively. The composition of the hydrophilic matrix of the control patches are shown in Table 5.2

Table 5.2: Composition of the hydrophilic matrix layer in the prototype patch and the control patches

Sample name	**HPMC K15M (g)**	**CPO (% w/w)[a]**	**TU (% w/w)[a]**	**PG (% w/w)[a]**	**patch weight (g)**	**CPO weight (g)**	**CPO load (% w/w)[b]**
0P	0.2	0	10	150	0.5251	0	0
10P	0.2	10	10	150	0.5594	0.0214	3.83
40P	0.2	40	10	150	0.6205	0.0806	12.99
80P	0.2	80	10	150	0.7011	0.1612	22.99

[a] Percent of weight of polymer (HPMC K15M)

[b] Percent of weight of dry HPMC film

5.4.7. Statistical Analysis

All the experimental results were analyzed using one way analysis of variance (ANOVA) using Microsoft Excel 2007. A p-value < 0.05 was considered to be statistically significant.

5.4.8. Scanning Electron Microscopy

It was hypothesized that upon storage, drug crystallization (if present) could be seen primarily at the matrix- release liner interface. The hydrophilic matrices for the prototype patch 10P and the control patches 0P, 40P and 80P were prepared (as shown in table 5.1). Each of the matrices prepared in table 5.1 had a surface area of 21 cm^2.These matrices were stored in a petri-dish for 3 weeks prior to imaging using SEM. The matrix (polymer film) was cut at the middle of the petri dish as shown in figure 5.2 and placed on the double-sided carbon tape (Ted Pella) on the sample holder (Ted Pella). For each of 4 samples shown in table 5.2 crystal formation was studied on the surface for solvent evaporation (SS), the surface in contact with the petri dish (SP) and the edge (ES) (Figure 5.2). The samples were coated with gold using SeeVac Conductavac. A vacuum of 300 mmHg and a current of 20 mA were used for coating the samples. The samples were coated for 3 min. The gold coated samples were then studied using the Agilent 8500 FESEM.

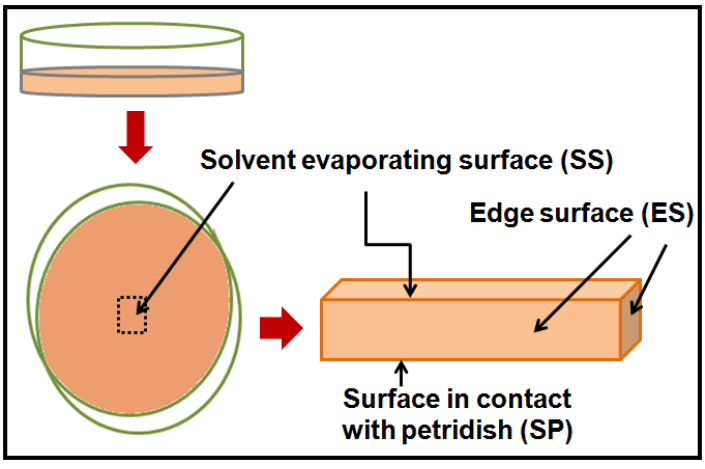


Figure 5.2: Sampling of hydrophilic matrix for SEM

5.4.9. Polarized Light Microscopy

Polarized light microscopy was used to substantiate the results obtained using SEM. An optical light microscope (Nikon Eclipse 400) equipped with a Lumenera Infinity 2 camera was modified for polarization using a pair of plane polarizing filters (Edmund Co.) called the polarizer and the analyzer. The polarizing filters were placed above and below the sample in the path of light. The assembly was used to qualitatively assess the optical properties of CPO and the polymer films. The polarizer filter was rotated to get cross polarization (90º to the analyzer) or slightly uncrossed polarizers while the analyzer filter was kept stationary. The images were analyzed using the Infinity Capture software.

5.4.10. Optical Light Microscopy

An optical microscope (Zeiss Microscope Axiostar Plus) equipped with a digital camera (SPOT Insight) was used to study the structure of the CPO crystals and the surface of the drug loaded HPMC films, The images were analyzed and stored using the SPOT Advanced software.

5.4.11. Differential Scanning Calorimetry

The thermal analysis of the hydrophilic matrices of the prototype patch, 10P and the control patches 0P,40P and 80P was performed using DSC (TA instruments Q200 MDSC, New Castle, DE) to determine the physical state of CPO in the patches. The presence of drug-polymer interaction was determined by evaluating the pure drug CPO, pure polymer and physical mixture of CPO and HPMC K15M (1:1 ratio). Approximately 2-4 mg of samples were heated from 20 to 180 ºC at a linear rate of 20 ºC/min in flat

bottomed aluminum pans hermetically sealed with a lid. Pure nitrogen gas was purged at the rate of 50 mL/min. The data was analyzed using TA Universal analysis software (Version 4.4A).

5.4.12. X-Ray Diffraction

In order to confirm the physical state of CPO in the prototype film (10P), the control films (0P and 80P), the pure drug, the pure polymer and drug-polymer physical mixture (1:1 and 3:1 ratio) were analyzed using X-Ray Diffractometer (Rigaku Miniflex II Desktop XRD) with Cu-Kα radiation anode tube and the MDI Jade 8 software. The electric current and voltage were 15 mA and 30 kV respectively. The samples were scanned over a 2θ range of 10 º to 50 º at the scanning rate of 10 º /min with a 0.1º step size.

5.4.13. Fourier Transform Infrared Spectroscopy

FTIR with the ATR crystal (Model: Spectrum 100, PerkinElmer) was used to study the interaction (if any) between the polymer and the drug in the prototype and control films. Pure drug (CPO), pure polymer (HPMC K15M) and physical mixture of drug and polymer (1:1) were also analyzed separately. A total of 64 scans were performed for each sample between 4000 cm^{-1} to 650 cm^{-1} with a resolution of 4 cm^{-1}. In case of the polymer films, the release surface was kept in contact with the ATR crystal to ascertain a higher concentration of the drug near the crystal if there is drug crystallization. The analysis of the spectra was performed using the software Perkin Elmer Spectrum (Version 10.00.00).

5.5. Results and Discussion

5.5.1. Fabrication of the prototype transungual patch

The transungual patch containing 150 %w/w of PG and 10 %w/w each of CPO and TU was prepared. The unique design of the patch offered the following advantages

- Higher concentration of solubilized form of CPO
- Presence of PEs selected for the two target tissues – the nail and the nail folds
- Increased hydration of the nail plate due to the hydrophilic nature of HPMC.
- Separate adhesive layer to improve the adhesiveness to the skin

The design of the patch is such that upon application, the peripheral acrylate PSA will adhere to the skin (2-5 mm around the nail folds) and hold the patch in place throughout the duration of application (24 hr). The nail folds and the nail plate will remain in contact with the hydrophilic matrix for a period of 24 hr (figure 5.3). The surface pH of the prototype pH was between pH 5 and 6 (Table A.1, Appendix A) which is within the broad range of skin pH of 4.0 to 7 reported in the literature (Lambers, H. et al, 2006). This reduces the likelihood of skin irritation due to the patch matrix. Hydrophilic polymers have been reported in formulation of transungual delivery systems. A hydrophilic chitosan derivative has been utilized in the development of a water soluble nail lacquer formulation for ciclopirox and amorolfine (Monti, D. *et al* 2005 and Monti, D. et al 2010). The hydrophilic layer of the bilayered nail lacquer system containing terbinafine hydrochloride consists of HPMC E15 and penetration enhancer PEG 400 (Shivakumar, N.H., et al 2010). A hot melt extrusion approach has been reported for formation of hydroxypropyl cellulose (HPC) films containing ketoconazole

(Mididoddi,P.K. and Repka,M.A., 2007). However, there are no reports in the literature of a formulation design similar to the prototype patch containing penetration enhancers for transungual and epidermal delivery as discussed in the current project.

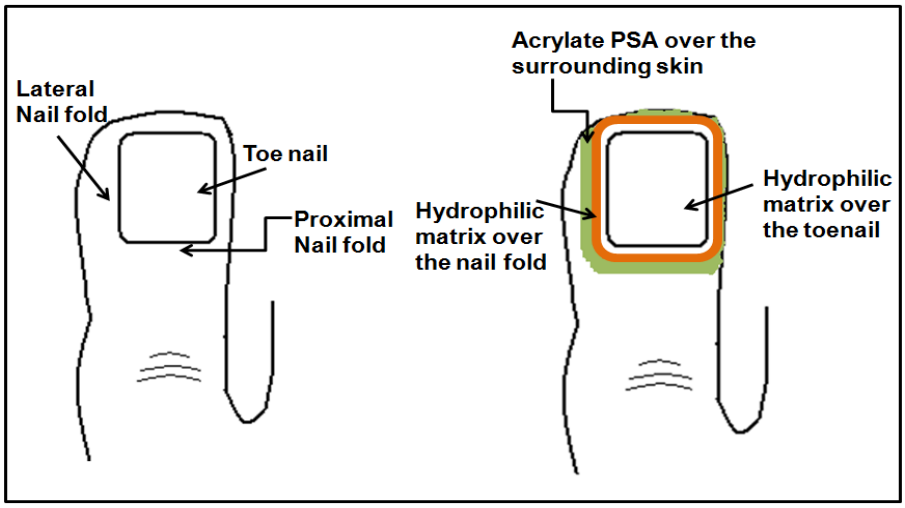


Figure 5.3: Appearance of the patch upon application to the toenail

5.5.2. The in vitro drug release

The novel transungual patch is an immediate release formulation, designed for application once a day. The acceptance criterion for drug release was not less than 80 % CPO released within 24 hr. Four individual patches were studied for CPO release using USP Apparatus 5. Since there is no official monograph in USP 35-NF 30 for a patch formulation containing CPO, a modification of the general method for drug release from transdermal drug delivery systems was used. The acceptance table in USP 35-NF 30 for drug release of transdermal drug delivery systems requires that none of the six individual patch lies outside the accepted level. At 2 hr and 4 hr the percent CPO released are 84.84

± 1.74 % and 91.99 ± 2.89 %, respectively (Figure 5.4). The plot shows an initial burst release of CPO which is evident at 10 min with about 44 % of drug release. The burst release is due to the formation of channels within the matrix, when the hydrophilic polymer (HPMC) was exposed to the dissolution medium and thereby releasing the hydrophilic drug (Chandak, A.R. and Verma, P.R.P., 2008). A similar burst release of Methotrexate from the transdermal patches consisting HPMC K4M, K15M and K100M has been reported (Chandak, A.R. and Verma, P.R.P., 2008). Figure 5.4 shows that the patch formulation does not hinder the release of CPO and >80 % drug release is attained at 2 hr for all the four patches tested. This ensures that the drug release is not the rate limiting step in delivery of CPO to the target tissues. The rate of release is faster than the release of CPO from the transungual patch with 10 % CPO and 1% TU in figure 4.12 of Chapter 4. This could be due to the different methods used for the drug release study. In chapter 4, the drug release was evaluated using Franz diffusion cells. The rate could be higher due to the increased turbulence in the USP Apparatus 5. Nevertheless both the release methods show that the formulation containing 10 % CPO in HPMC K15M is an immediate release formulation and meets the acceptance criteria of not less than 80% release in 24 hr. The release kinetics models of zero order, first order and square root of time were fitted to the release data. The model equations 4.1, 4.2 and 4.3 discussed in Chapter 4 were used. The distribution of the residuals was random for all the three models showing that all the three models fit the data well. Therefore, the value of the adjusted R^2 was used to determine the best model. Table 5.3 shows the correlation coefficient (R^2) and the adjusted R^2 value for the three models. It is clear from the values in table 5.3 that the release of CPO from the prototype patch follows a first order process.

This is similar to the results in Table 4.9 where the first order model fits the data for the transungual patch K15M 10% CPO, 1%TU studied using the Franz diffusion cell. This similarity in the results indicates that the drug release method did not significantly affect the outcome of the release studies except that the first order release rate constant (1.1736 hr^{-1}) is 8.2 fold higher than the first order release rate constant (0.1431 hr^{-1}) obtained using the Franz diffusion cells.

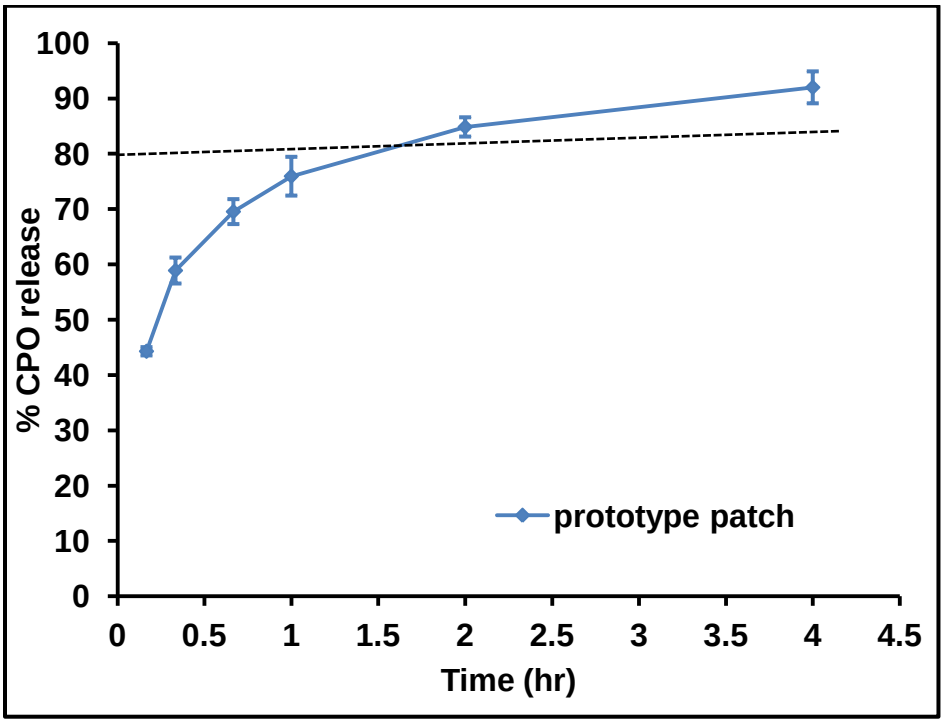


Figure 5.4: CPO release from the prototype patch using USP Apparatus 5

Table 5.3: Goodness of fit of the three kinetic models

Kinetic Model	R^2	Adjusted R^2
Zero order	0.9083	0.8625
First order	0.9890	**0.9834**
Square root of time (Higuchi)	0.9617	0.9425

5.5.3. The in vitro skin permeation

The prototype patch formulation had a similar permeation profile to the patch (K15M 10 % CPO and 1 % TU) studied in Chapter 4. Penlac® nail lacquer was used as the positive control. The steady state flux for the prototype patch was calculated from the slope of the linear portion of the plot in figure 5.5. Similar analysis of the Penlac profile could not be undertaken because it follows a non-steady state permeation.

In case of non steady state permeation the concentration gradient cannot be considered constant (Brodin,B. et al 2009). Non steady state permeation is seen when the lipophillic molecule permeates the membrane readily and there is a drop in the drug concentration in the donor compartment with a simultaneous increase in the drug concentration in the receiver compartment (Brodin,B. et al 2009). After application of the lacquer formulation on the skin, the solvents evaporate and the concentration of CP in the film increases from 8 % to 34.8 % (Baran,R. et al 1998 and Bohn,M. and Kraemer,K. 2000). Ethyl acetate is one of the solvents in the nail lacquer. It is also reported to act as a penetration enhancer on the skin (Paudel,K.S. et al 2010 and Trommer,H. and Neubert,R.H.H. 2006). It is speculated that there is increased permeation of CP after the application of lacquer, due to

the action of ethyl acetate and the high concentration gradient across the skin membrane. This leads to ready permeation of CP and a possible decline in concentration of CP in the donor compartment resulting in the non-steady state permeation of CP. Therefore the cumulative amounts of CPO permeating the skin at end of 24 hr (Q_{24hr}) after application of the prototype patch and Penlac® nail lacquer were compared. The values of Q_{24hr} were not significantly different for the prototype patch and Penlac® at 97.39 ± 26.96 µg/cm^2 and 112.52 ± 29.48 µg/cm^{2,} respectively (p-value > 0.05). It is important to note that the Q_{24hr} for both formulations is similar but the process of delivering the drug across the skin is different.

The lag-time and the steady state flux for the transdermal permeation of CPO from a previous study using hydro-alcoholic vehicle containing hydroxypropyl chitosan through excised hairless mouse skin was 1.51 ± 0.20 hr and 127.45 ± 46.29 µg/cm^2/hr respectively (Monti, D. et al 2005 and Monti, D. et al 1995). This previous study aimed at increasing the transdermal permeation of CPO. The results from the prototype patch show a lower steady state permeation flux of 5.45 ± 1.74 µg/cm^2/hr and a longer lag-time of 6.04 ± 1.75 hr. The prototype patch was designed to minimize the transdermal permeation and enhance delivery to the epidermis of the skin. Also, the membrane used in the study was hairless mouse skin which tends to over predict human skin permeation. This could explain the increased transungual permeation of CPO in the study over the prototype patch.

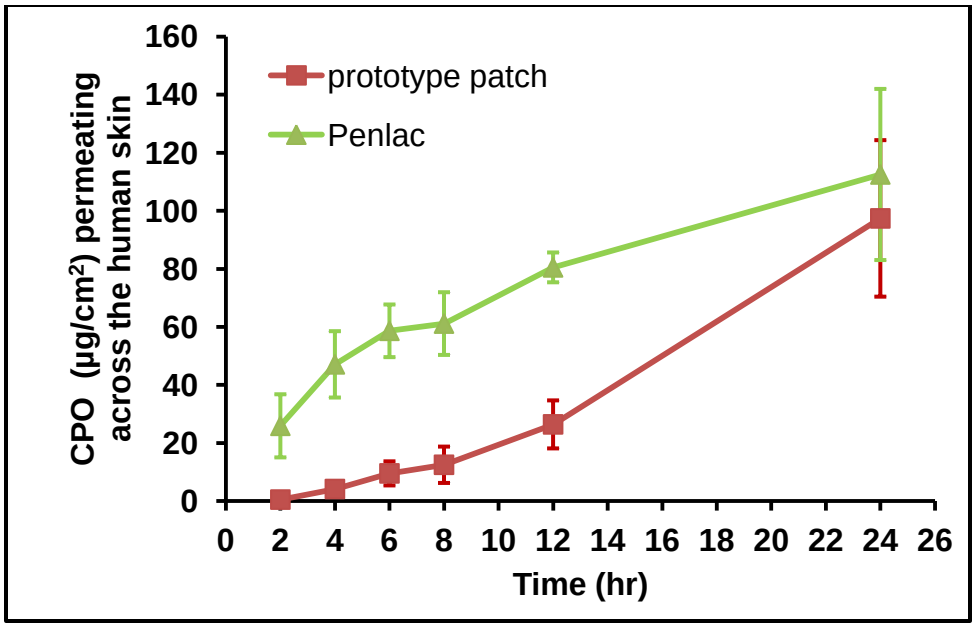


Figure 5.5: In vitro skin permeation of CPO from the prototype patch and Penlac® nail lacquer

5.5.4. The in vitro skin penetration

The thicknesses of the epidermis and the dermis were used to calculate the volume of each of the layers. The volume (cm^3 or mL) was calculated as a product of area of drug exposure and thickness of the skin layers. Figure 5.6 shows the concentration of CPO within the epidermis and dermis after the skin permeation study for 24 hr. The concentration of CPO within the epidermis was significantly higher after treatment with the prototype patch compared to that of Penlac® nail lacquer (p-value <0.05). However, the drug concentrations within the dermis were not significantly different. The enhancement factor, EF_{epi} (Equation 5.1) for the concentration of CPO attained in the epidermis in presence of the prototype patch was 2.8. Additionally, there was selective accumulation of CPO within the epidermis which had a 3.2 fold higher concentration of

CPO than in the dermis upon application of the prototype patch. This shows that the selective accumulation of CPO within the epidermis seen during the preformulation screening of PG in figure 2.12 of Chapter 2 was retained in the prototype patch formulation.

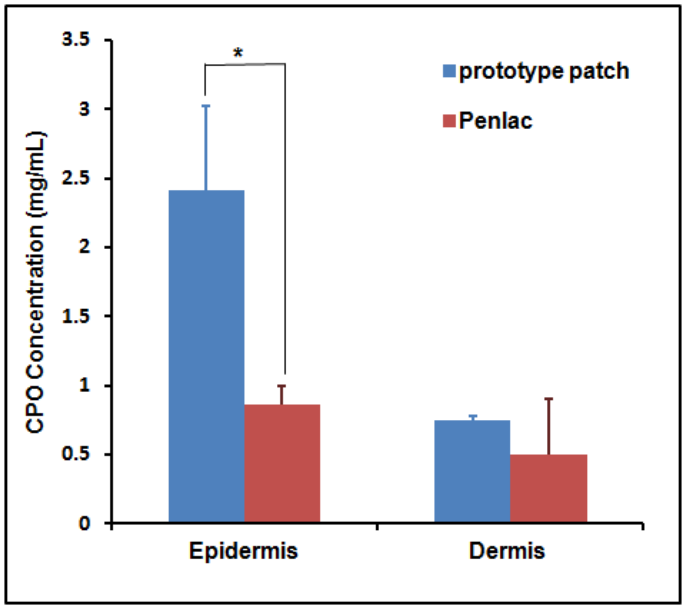


Figure 5.6: Concentration of CPO within the epidermis and dermis upon treatment with the prototype patch and Penlac® nail lacquer

In the case of Penlac®, the accumulation of CPO within the epidermis was 1.7 fold higher than the dermis. The EF_{der} for the patch formulation was only 1.5. This again shows the selective accumulation of the drug in the epidermis. The patch formulation consists of 150 %w/w of propylene glycol. The use of this high concentration of PG is integral part of the fabrication process to get flexible films using HPMC K15M. The high level of PG in the patch helps to keep the drug and PEs in solution and impart flexibility to the

HPMC matrix. At the concentration of PG used in the patch, we would expect to see the similar CPO effect on skin penetration as in the preformulation screening discussed in Chapter 2.

5.5.5. The in vitro transungual permeation

The transungual permeation was performed using human cadaver toenails. Figure 5.7 shows the *in vitro* transungual permeation of CPO across human toenails. The permeation flux attained after application of the patch formulation is 4.2 fold higher than that for Penlac® nail lacquer after application of both formulations for 32 days (Table 5.4). The cumulative amount of CPO permeating the human toenails on day 32 (79.69 ± 12.12 $\mu g/cm^2$) is comparable to the amount of CP permeating the porcine hoof from acrylate adhesive matrix at end of 4 weeks (Myoung, Y. and Choi, H., 2003). Porcine hoof has a lower density of keratin fibers and is therefore more permeable than human nails (Monti,D. et al 2011). The studies performed using porcine hoof therefore overestimate the drug permeation. The prototype patch is therefore better than the reported patches containing ciclopirox (free acid) at the delivery of CPO across the human nail.

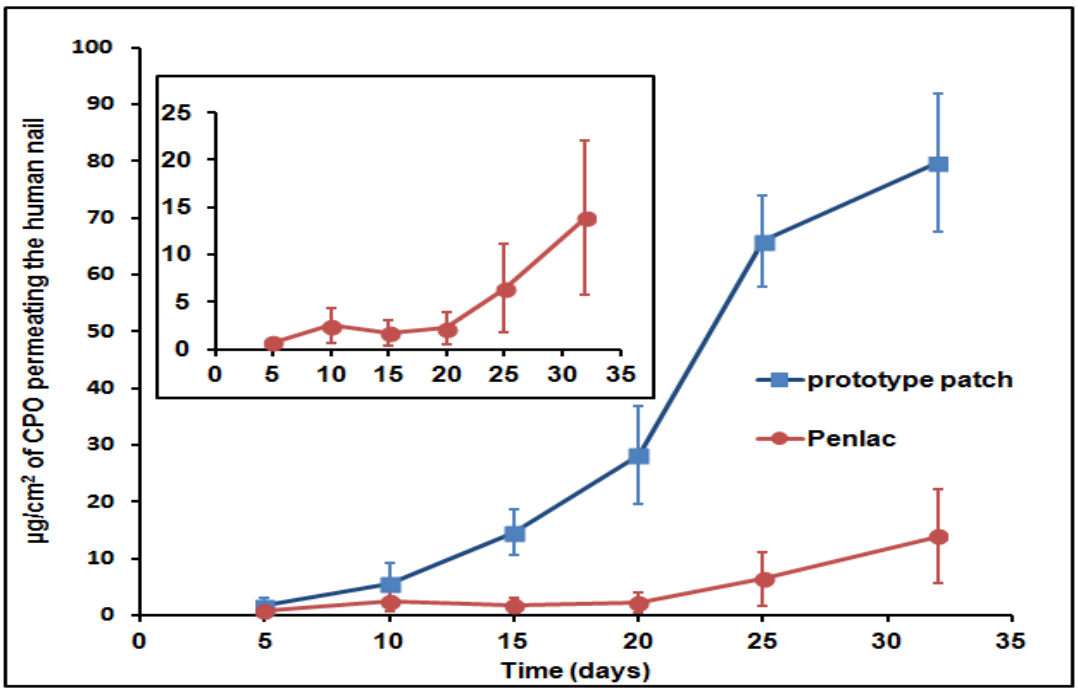


Figure 5.7: In vitro transungual permeation of CPO through human toenail

Table 5 4: Transungual permeation parameters

Formulation	Permeation Flux (µg/cm²/day)	Lag-time (Day)	$C_{32\ days}$ (µg/mL)
Prototype Patch	4.1 ± 0.59	11.45 ± 1.25	4.08 ± 0.67
Penlac®	0.98 ± 0.56	18.36 ± 1.20	0.79 ± 0.46

The MIC was attained in the receiver compartment, for the patch formulation at 15-20 days of the experiment. However, the MIC was attained only on day 32 for Penlac® nail lacquer. The drug concentration in the orifice and the periphery is shown in figure 5.8. The concentration of CPO attained in both the orifice and the peripheral layers of the toenails were significantly greater than that for Penlac® (p-value < 0.05). The thicknesses

of the toenails (0.86 ± 0.053 mm) were used to determine the volume of the nail. This volume was used to calculate the drug concentration within the nail. The CPO concentration within the orifice part of the nail is 14.09 ± 3.67 mg/mL and 5.22 ± 1.7 mg/mL for the patch and Penlac®, respectively. The concentration of CPO in the nail for both formulations is significantly higher than the Nail-MFC of 16 – 32 µg/mL reported for seven strains of *T.rubrum,* in the literature (Schaller,M. *et al* 2009).

The drug content of CPO in the patch and the lacquer formulations (Appendix C) are much higher than the highest incubation concentration (84.5 µg/mg nail) used in the drug keratin binding study in Chapter 3. Since there is transungual permeation of CPO from both formulations, it can be concluded that both formulations deliver sufficient CPO to saturate all the binding sites in nail keratin.

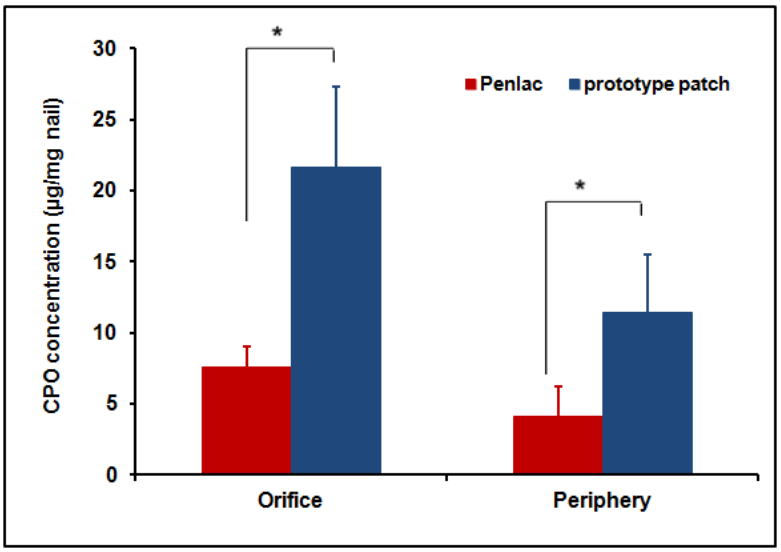


Figure 5.8: Concentration of CPO in the human toenail (orifice and periphery) after the in vitro nail permeation study

5.5.6. Scanning Electron Microscopy

Scanning Electron Microscope was used to detect the presence of any crystals in the hydrophilic matrices of the prototype patch (10P) and the control patches (0P, 40P and 80P). The aim was to study the three surfaces – solvent evaporating surface (SS), surface in contact with the petri-dish (SP) and the edge surface (ES) of the samples cut from the films (figure 5.2). The rationale for evaluating the crystallization at these three surfaces is described below.

1. During the preparation of the matrix, the polymer mixture is poured into the petri-dish and the solvent (acetone: water 9:1 v/v) is allowed to evaporate overnight. The solvent evaporates from the top surface; therefore there is a movement of solvent from the bottom of the petri-dish towards the surface of the film. This migration of the solvent can cause movement of drug towards the surface and lead to crystallization after complete evaporation of the solvent at the SS.
2. It was hypothesized that when the films are stored for a few weeks in the desiccator, there is complete loss of water and if the drug comes out of solution, it will form crystals at interfaces (e.g. film – petri-dish interface) at the lower surface of the film which is in contact with the petri-dish (SP).
3. Furthermore, if there is high crystallization in the entire matrix, there would be crystals within the matrix and they would be evident in the edge surface (ES).

Figure 5.9 shows the SEM images for the surfaces for each of the four films tested. The solvent evaporating surfaces (SS) of 0P and 10P had similar patterns showing the HPMC fibers on the top (marked with arrows).Similar patterns were obtained for 40P and 80P.

Crystals of the drug CPO were not detected on any of the surfaces evaluated. The surfaces of the films in contact with the petri-dish (SP) appeared smoother than the SS surface and were also devoid of CPO crystals. If the particle is positively or neutrally buoyant compared to the matrix, it will appear on the surface of the film, whereas if the crystals are negatively buoyant they sink into the polymer matrix (Mididoddi,P.K. and Repka,M.A., 2007). These particles can be better visualized in the edge surface. There were no crystals detected within the matrix as can be seen in the edge surface (ES) of the films. The overall appearance of all the three surfaces SS,SP and ES for the matrices for the prototype patch (10P) and the control patches (0P, 40P and 80P) were similar without any detectable presence of CPO crystals. These SEM images showed that the drug was in a non crystalline state within the films at different drug loads.

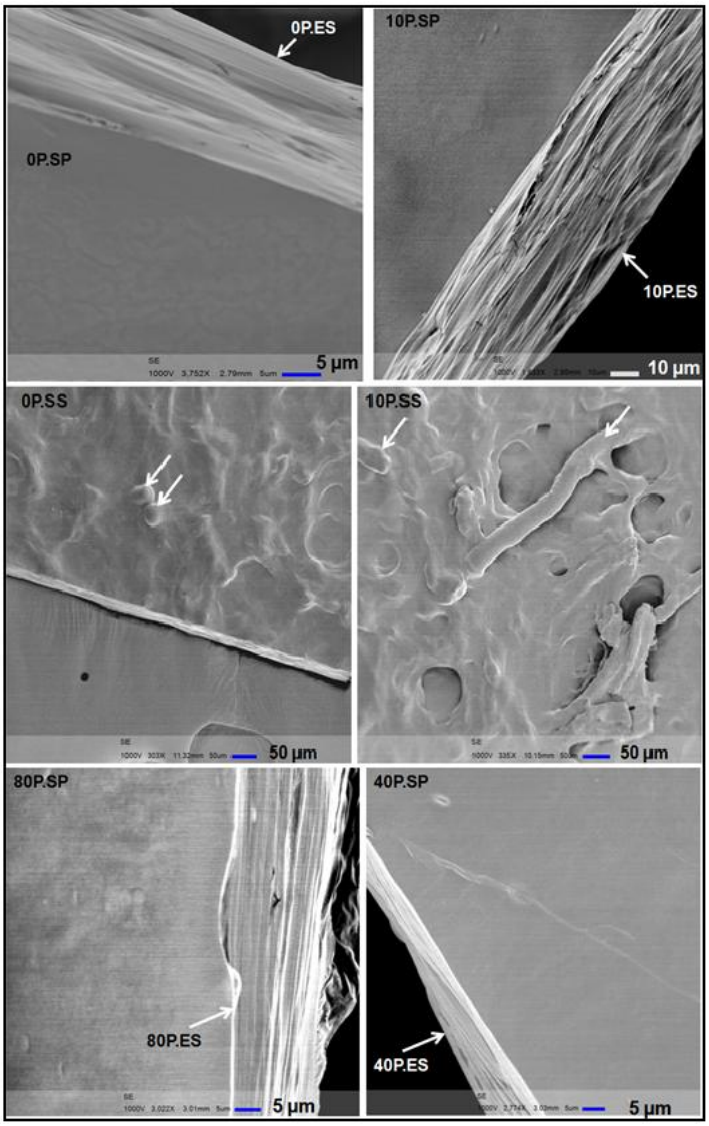


Figure 5.9: SEM images of solvent evaporating surface (SS), Surface in contact with petri-dish (SP) and the Edge surface (ES) for the hydrophilic matrices of the prototype patch 10P and the control patches (0P, 40P and 80P)

5.5.7. Polarized Light Microscopy

Polarized light microscopy helps to distinguish material based on the optical properties of the materials (Nichols,G. et al 2011). As the polarizer filter is rotated, an appropriately

oriented anisotropic material will show bright colors (Nichols,G. et al 2011). The images for the pure drug and the pure polymer were obtained at 10X. The images for the polymer films were obtained using the 4X objective. Figure 5.10 shows the birefringence for each of the samples. It was hypothesized that the crystalline drug (CPO) will show birefringence under polarized light and thus the crystals if any on the surface of the films can be determined based on the intensity of birefringence. It was expected that there would be a concentration dependent increase in the number of birefringent areas as the concentration of CPO increases from 10P to 80P in the films. Interestingly, the amorphous polymer HPMC K15M also shows birefringence under polarized light. All the films showed similar birefringence regardless of concentration of CPO. The evaluation under higher magnification showed that the birefringence in each of the films was contributed by the fibrous particles of HPMC K15M. There were no crystals of CPO detected on the SP surface of any of the films. Thus, it was a confirmation of the SEM results that there was no crystalline drug detected in the films at all the concentrations evaluated.

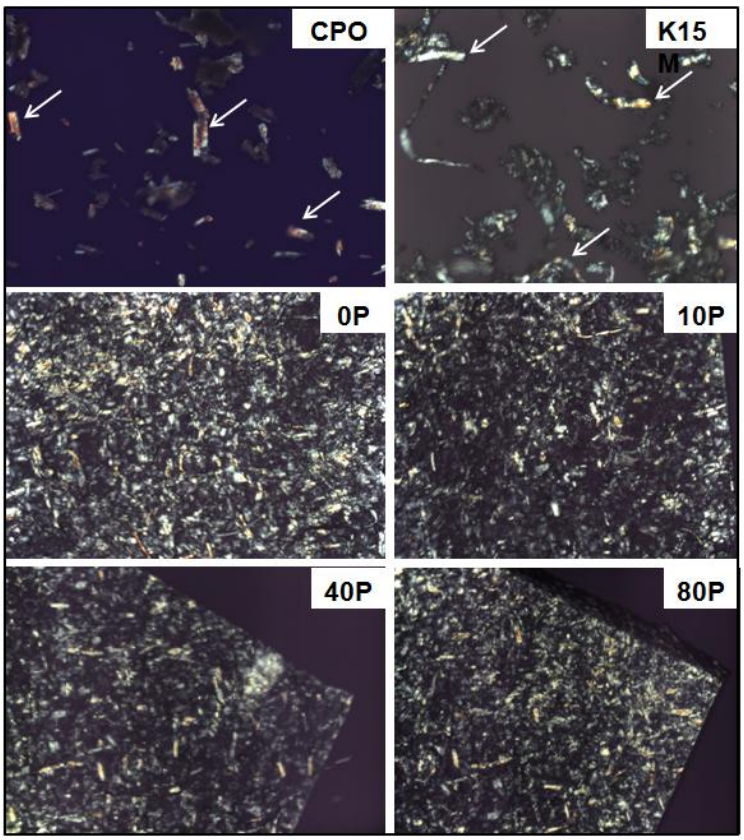


Figure 5.10: Birefringence seen in CPO, HPMC, matrices for the prototype patch (10P) and control patches (0P, 40P and 80P).

5.5.8. Optical Light Microscopy

Optical light microscopy was used to further confirm the findings in SEM. Figure 5.11 shows the micrograph obtained using 20X objective. CPO crystals appeared as plates under 20X magnification. The prototype film 10P and the control films 0P and 80P showed similar surface topography with presence of HPMC fibers (presence of fibers is clearer under 5X objective). Since HPMC is not soluble in the dispersing medium (Acetone:water (9:1 v/v)) the surface of the HPMC films shows the fiber like particles of

HPMC. Under the higher magnification, there were no detectable crystals for CPO in 10P and 80P confirming the results obtained from SEM and PLM.

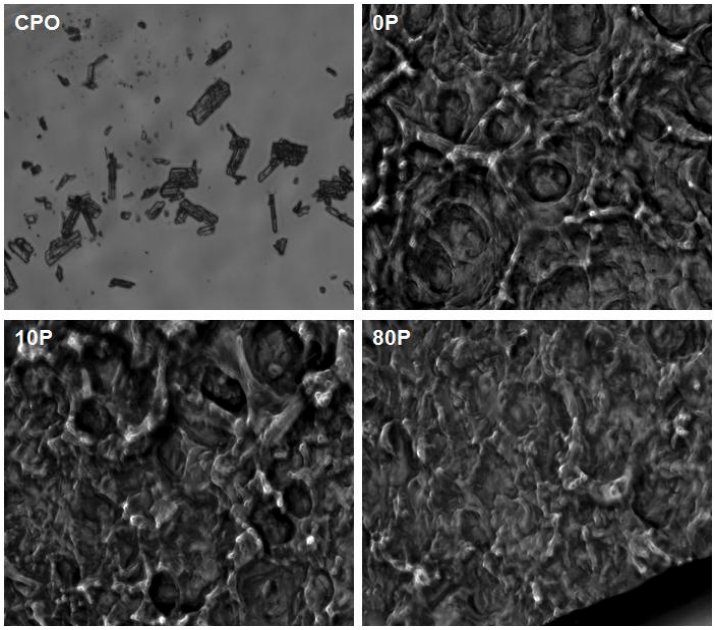


Figure 5.11: Optical Microscopy Images of CPO crystals and the HPMC films

5.5.9. Differential Scanning Calorimetry

In order to confirm the absence of crystallinity in the drug loaded films, DSC was used. Figure 5.12 shows the DSC thermogram for the samples. The DSC curve for CPO shows two endotherms. The endotherm at the onset temperature of 118 ºC is due to absorbed moisture. The pure drug shows a melting endotherm at the onset temperature of 142 ºC (Figure A.4, Appendix A). This is in agreement with the melting point of 143 ºC (140-145 ºC) reported in literature for CPO (Material Safety Data sheet and Tarawneh,R.T. et

al 2005). The physical mixture of the drug and polymer (1:1) shows the melting endotherm of the drug at the onset temperature of 145 ºC (Figure A.5, Appendix A). The physical mixture also shows an endotherm for absorbed moisture at the onset temperature of 107 ºC. The HPMC films 10P, 40P and 80P did not show any endothermic peak for CPO. The absence of the melting endotherm in the polymer films shows that CPO is present in a solid solution in the polymer matrix (Mididoddi,P.K. and Repka,M.A., 2007 and Repka,M.A. et al 2004).This indicates that there is no crystalline drug present in each of the samples regardless of the drug load in the film.

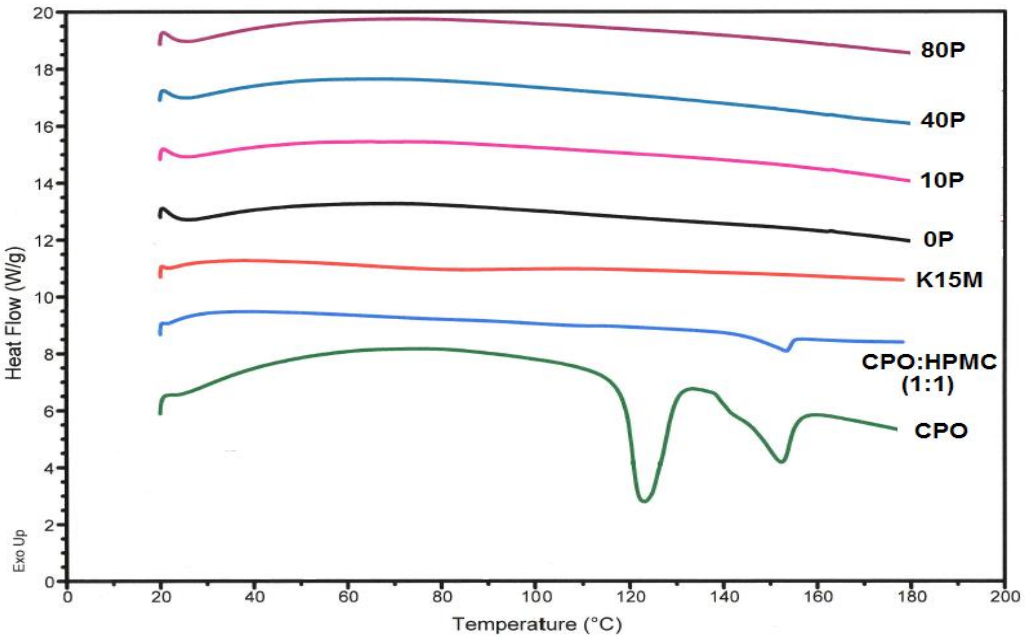


Figure 5.12: DSC Thermogram of the HPMC films with different drug loads compared to CPO and HPMC

5.5.10. X-Ray Diffraction

X-Ray Diffractometer was used to confirm the results obtained from the thermal analysis of the samples in DSC. Figure 5.13 shows the X-ray diffraction obtained for the films and the pure drug and pure polymer. The pure drug CPO shows intense peaks at 2θ values of 11º, 19º and 21º. The crystalline form of CPO can be seen at the same 2θ values in the physical mixture with HPMC K15M at two drug: polymer ratios of 1:1 and 3:1. It is in alignment with the DSC data showing absence of any drug polymer interaction in the physical mixture. Pure HPMC K15M showed the typical profile for an amorphous material with a broad peak from 2θ values of 14º to 22º. The blank film without any drug (0P) also showed a plot similar to that of pure HPMC. However, the plots of films (10P) and (80P) with 10 %w/w and 80 % w/w of CPO respectively, did not show the typical amorphous profile with broad peaks as the 0P film. Therefore it cannot be concluded with certainty that the drug in the two films 10P and 80P is amorphous. On the other hand, there are no intense peaks at the 2θ values of 11º, 19º and 21º for the two drug loaded films (Table A.2, Appendix A). Hence it was concluded that CPO was present in a non crystalline form within the drug loaded films in a dissolved state at all the drug loads investigated (Mididoddi,P.K. and Repka, M.A., 2007). This could be attributed to the high level of PG used for the fabrication of the patch. The preformulation studies show that CPO shows has a high solubility of 307.25 ± 21.71 mg/mL at 32 ºC in PG. Therefore, CPO is at a sub-saturation level in all the drug loaded films and this is the reason why no crystallinity is detected in SEM, DSC and XRD.

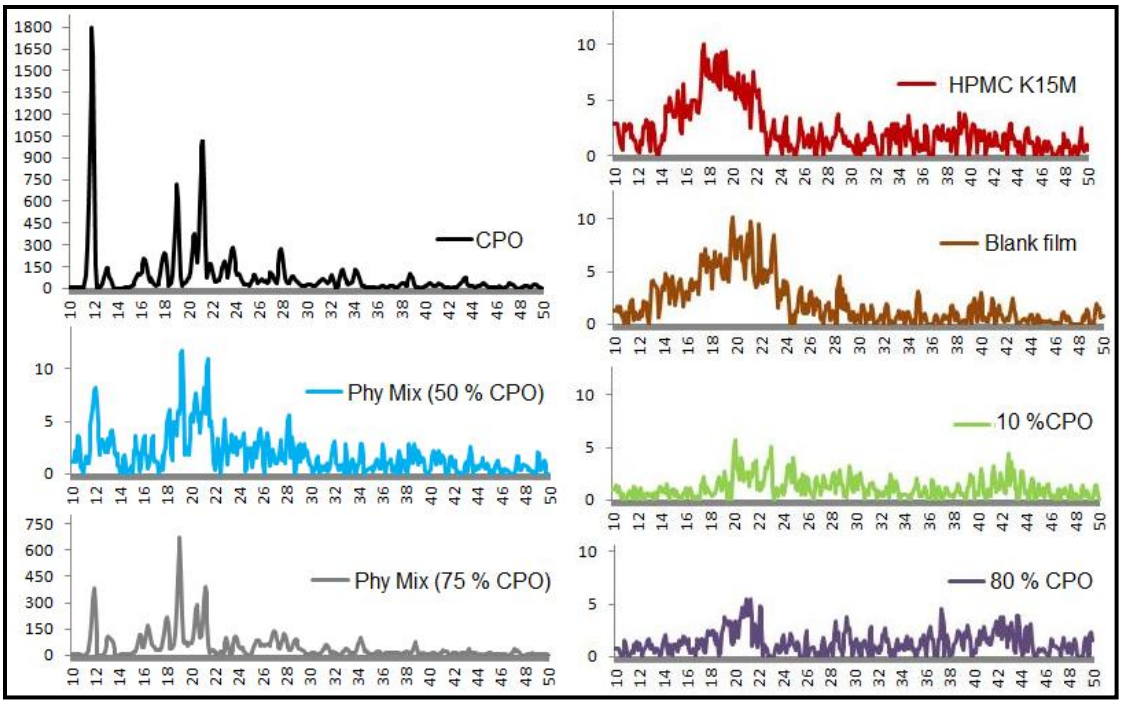


Figure 5.13: X-Ray Diffraction for CPO, HPMC, CPO-HPMC physical mixtures with 50% and 75% CPO, Blank film (0P), prototype film with 10 % w/w CPO (10P) and control film with 80 %w/w CPO (80P).

5.5.11. Fourier Transform Infrared Spectroscopy

Figure 5.14 shows the FTIR spectra for the pure drug, drug-polymer physical mixture (1:1), pure HPMC K15M, prototype film (10P) and control films (0P, 10P and 80P). The prominent peaks for CPO are the carbonyl stretching peaks at 1514 cm^{-1}, 1548 cm^{-1} and 1640 cm^{-1} (Tarawneh,R.T. et al 2005). Table 5.5 shows the peak wavenumbers similar to those reported for ciclopirox olamine and the absorption values for the samples.

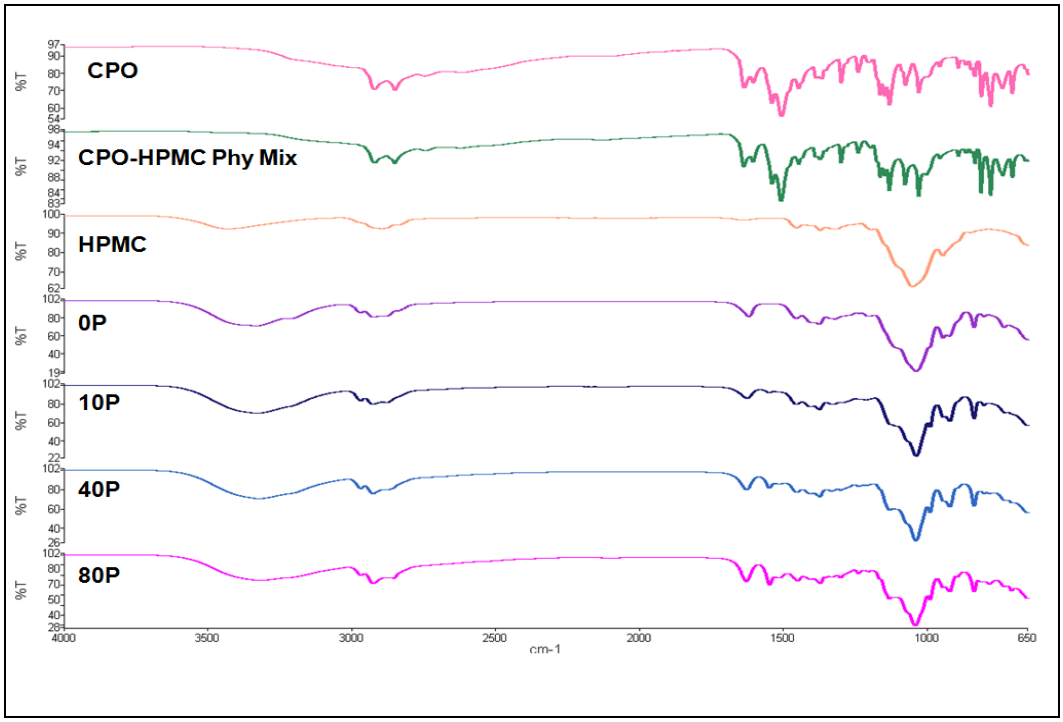


Figure 5.14: FTIR spectra of CPO, HPMC K15M, Placebo film (0P) and HPMC films with different drug loads of CPO

HPMC K15M polymer shows a peak at 1639 cm^{-1} which is similar to the peak at 1636.70 cm^{-1} for pure CPO. Hence the peak at 1639 cm^{-1} was not selected for analysis of the drug loaded films. The peaks at 704 cm^{-1}, 1507 cm^{-1} and 1540 cm^{-1} were specific for presence of CPO in the sample.

All the HPMC polymer films show the stretching band corresponding to the hydrogen bonding with water and/or intramolecular hydrogen bonding between the hydroxyl group and the oxygen in the carbonyl group in the region of 3000 to 3500 cm^{-1} (Tarawneh,R.T. et al 2005). However, this area of the spectra cannot be considered specifically for the intramolecular hydrogen bonding in the drug because the control film without CPO (0P)

also shows the peak in the region. So this peak in all the polymer films could be due to hydrogen bonding with water.

Table 5.5: Absorption (A) and wavenumbers (cm^{-1}) of peaks in the samples

Sample	***cm*$^{-1}$ (A)**	***cm*$^{-1}$ (A)**	***cm*$^{-1}$ (A)**	***cm*$^{-1}$ (A)**	***cm*$^{-1}$ (A)**
Pure CPO	704.73 (0.16)		1507.02 (0.26)	1540.54 (0.20)	1636.70 (0.15)
CPO:HPMC (1:1) Physical Mixture	707.04 (0.05)	1373.96 (0.04)	1508.46 (0.08)	1540.38 (0.06)	1638.19 (0.04)
Pure HPMC K15M	NA	1372.73 (0.04)	NA	NA	1639.4 (0.014)
0P (0 %w/w CPO)	NA	1376.11 (0.14)	NA	NA	1620.56 (0.06)
10P (10 %w/w CPO)	Not detected	1375.37 (0.13)	Not detected	1548.3 (0.032)	1629.30 (0.06)
40P (40 %w/w CPO)	705.67 (0.17)	1374.03 (0.14)	1516.6 (0.07)	1548.83 (0.09)	1628.14 (0.10)
80P (80 % w/w CPO)	707.18 (0.19)	1372.51 (0.15)	1516.0 (0.112)	1548.26 (0.15)	1629.52 (0.13)

The peaks for pure CPO at 1507 cm^{-1}, 1540 cm^{-1} and 1636 cm^{-1} match with the reported peaks for CPO, except that all the peaks show a slight shift in the wavelength compared to the literature values (Tarawneh,R.T. et al 2005)

The peak corresponding to 704 cm^{-1} of the pure drug was seen only in the drug loaded films and the physical mixture. Its intensity in the films 40P and 80P were comparable with that of the pure drug (Table 5.5). The drug specific peaks at 1507 cm^{-1} and 1540 cm^{-1} were found in all the drug loaded films and the physical mixture. The intensity of these peaks showed dependence to the concentration of CPO in the films. The peaks corresponding to 704 cm^{-1} and 1507 cm^{-1} were not detected in 10P with 10 %w/w CPO. This could be because of the very low concentration of the drug. In terms of dry weight of the film, the drug load in 10P was only 3.84 %. At such a low concentration the drug would remain undetectable because of quenching of the sample.

In case of the drug loaded polymer films (10P, 40P and 80P), the drug peak seen in pure CPO and physical mixture at 1540 cm^{-1},is seen at 1548 cm^{-1}. This shift in wave number is associated with hydrogen bonding of CPO with HPMC. The presence of the prominent drug peaks in the physical mixture sample indicates that there is no significant interaction between CPO and HPMC K15M. A peak specific to HPMC K15M at 1373 cm^{-1} is seen in all the polymer films and the physical mixture (Table 5.5). The peak specific to HPMC K15M is also retained in all the samples, further confirming the absence of any drug polymer interaction. The presence of drug and polymer specific peaks in the polymer films indicates that there is no significant interaction between the CPO and HPMC and that the drug is not in an amorphous form in the film (Cho, C. et al 2006). This corroborates the XRD result that CPO is present in a non-crystalline form in solution in the prototype patch.

5.6. Conclusion

A prototype transungual formulation containing the antifungal drug CPO (3.83 % w/w), TU (3.83 % w/w) and PG (53.63 % w/w) of the dry weight of hydrophilic matrix was successfully fabricated and characterized for its functional and physical characteristics. The patch was designed for immediate release of CPO and enhanced permeation into and across the human nail plate. The patch efficiently delivered CPO to the epidermal layer with minimal transdermal permeation in 24 hr. Thus, the patch successfully delivered CPO to the two identified target tissues of the human nail (14.09 ± 3.67 mg/mL) and the nail folds (2.42 ± 0.60 mg/mL) at concentrations greater than the reported minimum inhibitory concentrations (0.04 to 1.0 µg/mL) of CPO. The functional characteristics (*in vitro* drug release, *in vitro* epidermal delivery of CPO and *in vitro* transungual permeation of CPO) of the patch were determined and it was observed that the patch performed more efficiently as a topical delivery system in comparison to a commercial topical formulation (Penlac® nail lacquer). There were no crystals in the hydrophilic matrix upon visual inspection. The absence of drug crystals in the prototype patch was confirmed using SEM, PLM, DSC and XRD. The FTIR data confirmed the absence of any significant drug-polymer interaction in the prototype patch. These data suggest that a transungual formulation containing 0.95 mg/cm^2 of CPO for once daily application was successfully developed. The patch formulation met the target product profile of an immediate release formulation and showed efficient delivery of CPO to the target tissues at concentrations greater than the reported MIC for CPO.

CHAPTER 6

CONCLUSIONS

Topical treatment of onychomycosis is challenging because of the compact nature of the nail barrier and limited drug permeability across the nail. This limits the use of topical formulations for drugs that have a very low MIC and then they may only be useful to treat mild to moderate onychomycosis. There is an unmet need to develop an efficient topical formulation to extend its utility to treat moderate to severe stages of the disease. Ciclopirox olamine is an antifungal drug with unique mechanism of action where it chelates the multivalent cations in the fugal cell and thereby impairs the microbial metabolism. Relative to the azoles and the allylamines, it shows and favorable physicochemical properties of low molecular weight, hydrophilicity and greater aqueous solubility. The free acid form of the drug, ciclopirox is commercially available as an FDA approved nail lacquer formulation (Penlac®). Ciclopirox olamine is reported to have greater aqueous solubility than the free acid and therefore it was hypothesized that it will penetrate the hydrophilic nail plate better than the free acid ciclopirox. The design and development of a transungual patch as a reformulation strategy to improve topical delivery of ciclopirox olamine into the human nail and surrounding skin (nail folds) is discussed.

This is the first attempt at delivering ciclopirox olamine into the nail plate and the nail folds using penetration enhancers for the two structurally different membranes. It was hypothesized that treatment and prevention of relapse of OM can be achieved by delivering the drug to both of these tissues simultaneously. A preformulation strategy was

successfully developed to select the penetration enhancers specific for the topical delivery of CPO into the nail plate and the nail folds. It would have been ideal to have one enhancer or a combination of enhancers that is effective on both tissues. However, the results of the preformulation study revealed that thiourea was a penetration enhancer for transungual delivery of ciclopirox olamine. The analysis plots showed that 5 % w/w thiourea increased drug solubility and disrupted the barrier properties of the nail. The *in vitro* skin permeation and penetration data showed that 100 % propylene glycol resulted in accumulation of the drug in the epidermis while minimizing its transdermal permeation. However thiourea and propylene glycol did not enhance CPO delivery into the skin and the nail respectively. The combination of these two enhancers was also ineffective in sufficient delivery of CPO into the two membranes. Thus it was important to incorporate the two enhancers in the formulation and retain their effects on the nail plate and the nail folds.

The drug-membrane interaction studies revealed that ciclopirox olamine partitioned favorably into the human nail from the saturated drug solution in presence of 5 % w/w thiourea. The drug partitioned into the infected nails to a greater extent than the healthy nails and lesser amount of drug diffused out of the infected nail. This showed that studies performed using healthy nails will still show the trend of drug permeation and that the lag-time of transungual permeation can be expected to be longer in infected toenails. The partitioning of CPO into the human nail is beneficial in topical delivery because the drug released from the formulation will readily diffuse into the nail plate. The drug also showed extensive binding to keratin. Owing to this binding, it is assumed that the drug within the nail plate will not permeate out of the nail efficiently until all the binding sites

are saturated in the membrane. Since only the unbound drug is active against the causative organisms and available for diffusion, it is important to saturate all the binding sites in the nail plate by delivering sufficiently high concentration of CPO into the nail plate. It is imperative that the topical formulation is an appropriately designed immediate release formulation to deliver high levels of the drug into and across the nail. The concentration gradient is the driving force for diffusion of drug molecules across the nail plate. The *in vitro* transungual permeation of ciclopirox olamine is severely reduced due to the competing process of lateral diffusion in the nail plate. This is the first study to quantify the lateral diffusion and its effect on *in vitro* transungual permeation. It was noted that use of nails with surface area $\geq$ 7 times the area of drug application on the nail, during the *in vitro* permeation studies reduced the permeation flux and prolonged the lag-time. This was mainly ascribed to the extensive lateral diffusion seen in a larger piece of the nail. A recommendation on the size of the nail relative to the orifice diameter of the nail adapter was made to avoid any erroneous elimination of a formulation or penetration enhancer. The recommended size of the toenails was used in all subsequent transungual studies. Similar to the nail keratin, CPO was also found to bind extensively to the epidermis. The drug binding to the epidermis along with the mechanism of action of propylene glycol (solubilizing the drug, increased penetration of drug by solvent drag and forming drug depots in skin) facilitated the accumulation of ciclopirox olamine in the epidermis.

Pressure sensitive adhesives belonging to the polyisobutylene, polyacrylate and silicone classes were screened for CPO release from the drug-in-adhesive type of transungual patch. In spite of the addition of hydrophobic plasticizers (Triacetin and Triethyl citrate),

the incorporation of the penetration enhancers (thiourea and propylene glycol) in the adhesive matrix caused physical instability (phase separation and crystallization) and no improvement in drug release. This problem was solved by changing the patch design by incorporating CPO and the penetration enhancers into a hydrophilic matrix using a hydrophilic polymer. The drug and the penetration enhancers could be easily incorporated into the hydrophilic polymer (HPMC) matrix. The novel design gave the flexibility of modifying the concentration of the drug and the PEs to obtain the desired release (not less than 80 % in 24 hr), transungual permeation and skin penetration.

The prototype patch formulation composed of 10 % w/w CPO, 10 % w/w TU and 150 % PG in HPMC K15M matrix, was designed as an immediate release formulation for daily application. The developed patch system shows significantly higher flux for transungual permeation with higher drug accumulation in the nail plate and the epidermis compared to the positive control (Penlac® nail lacquer).The patch formulation is advantageous over Penlac® nail lacuer because: 1) Ease of application, 2) Solubilized form of the drug 3) Hydration of the nail due to the hydrophilic matrix and 4) Use of effective concentrations of two penetration enhancers. The theoretical drug load and efficiency calculations suggested that the patch formulation may be further optimized to lower the dosing frequency and the drug load. However, this has to be tested further. The prototype patch formulation was characterized for the physical state of the drug by analyzing the CPO loaded HPMC matrix using , PLM, OLM, DSC, XRD and FTIR. All the analytical methods showed that the drug was in a non-crystalline state in the hydrophilic matrix. The developed patch (composed of 10 % w/w CPO, 10 % w/w TU and 150 % w/w PG in HPMC K15M matrix) did not hinder the drug release and showed enhanced *in vitro*

transungual permeation and penetration into the human toenails. Thus, all the three specific aims of the study were successfully met and the final formulation has a potential to be more efficient in CPO delivery into the nail plate and the nail folds than the commercial formulation in an *in vivo* study.

FUTURE DIRECTIONS

The following are some of the areas that can be explored to further the project:

1) **Development of a dissolution method relevant for the patch system:** The *in vitro* release of CPO from the patch was studied using side-by-side diffusion cells in Chapter 4 and USP apparatus 5 in Chapter 5. Both the methods show first order release kinetics for the transungual patch. However, there was extensive swelling of HPMC in the side-by-side cell and in case of the USP apparatus; there was erosion of the polymer matrix due to the turbulence of the paddle. Thus both methods do not adequately mimic the *in vivo* condition for CPO release from the transungual patch. Therefore there is a need to develop a relevant dissolution method to study CPO release from the transungual patch.

2) **Antimicrobial testing to confirm the attainment of MIC:** The CPO concentrations attained in the receiver compartment during the transungual permeation experiments were compared against the literature reports of MIC for CPO to determine the patch efficiency. However, the samples withdrawn at specific time points during the transungual permeation study can be evaluated using microdilution method against various strains of dermatophytes to determine the MIC and the time to reach MIC in the receiver compartment.

3) **Hypo-osmosis as a strategy for transungual drug delivery:** A hypo-osmotic solution containing CPO can be used as a donor solution to deliver antifungal

drugs across the human nail plate. Water is reported to be the best plasticizer for the human nail (Khengar R. *et al* 2007). It is hypothesized that the water from the hypo-osmotic solution will hydrate the nail plate and also drag the antifungal drug into the nail plate. This could be a promising strategy for hydrophilic antifungal drugs like CPO.

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APPENDICES

A: Raw Data

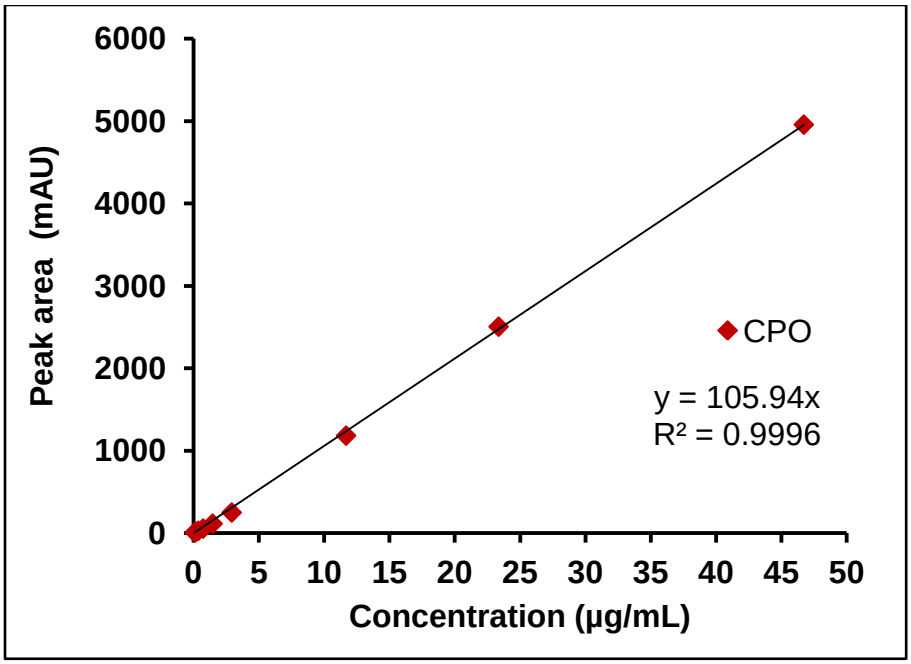

Figure A.1: Standard curve for ciclopirox olamine using HPLC

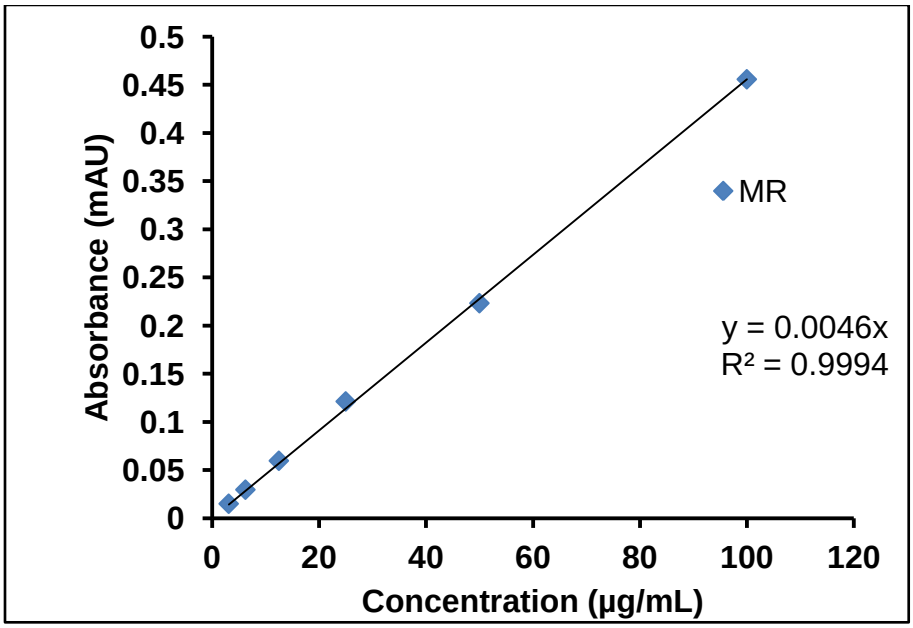

Figure A.2: Standard curve of methyl red sodium salt using UV/VIS spectroscopy

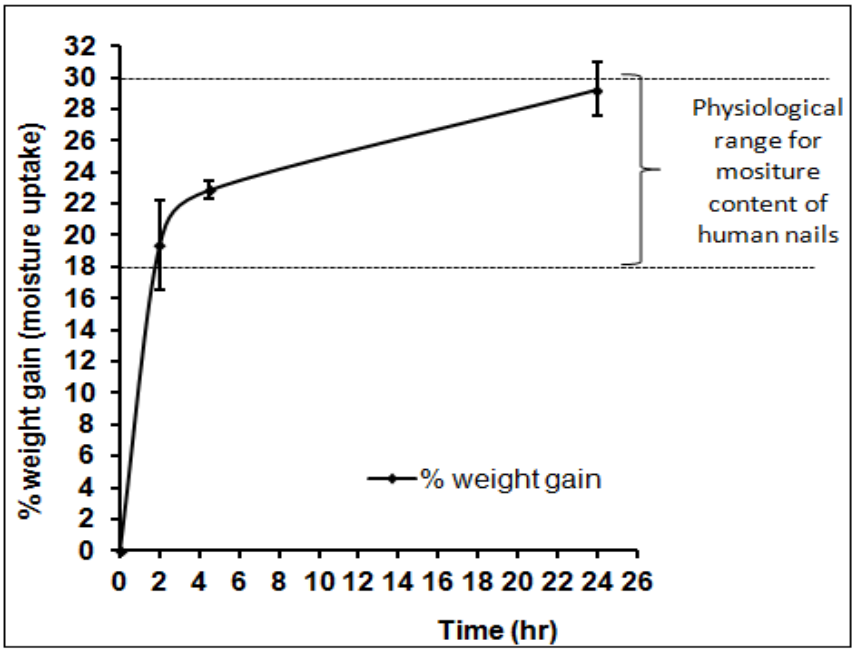

Figure A.3: Moisture uptake in cadaver toenails on exposure to 100 % RH

Table A.1: Surface pH of transungual patches

Patch	***Surface pH[a]***
0P	*5*
10P	*5-6*
40P	*5-6*
80P	*7-8*

[a]Determined using pH strips on hydrated patch surface

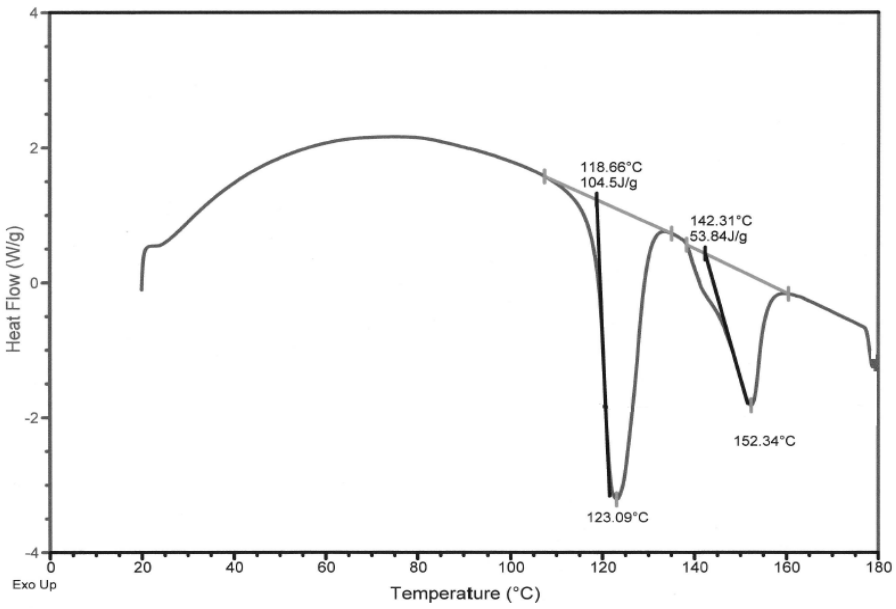

Figure A.4: DSC thermogram of CPO showing the onset temperature

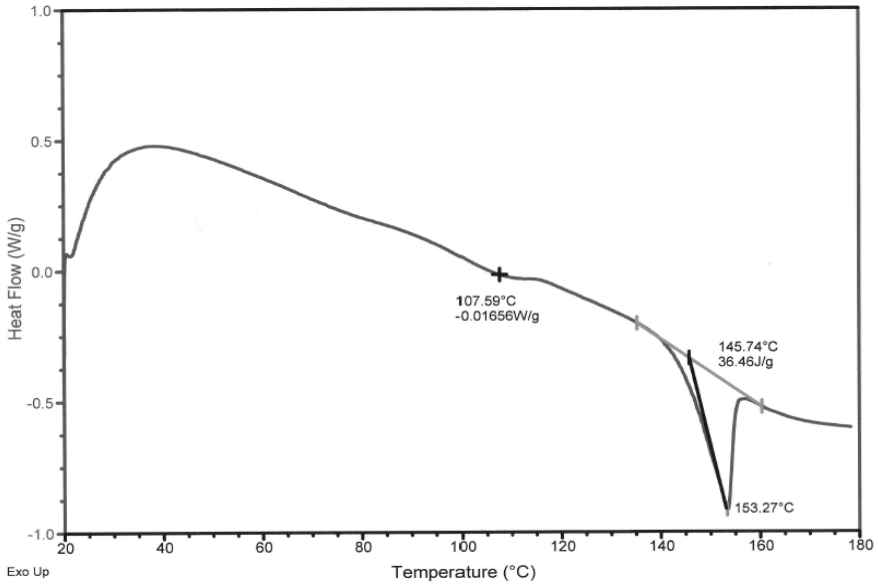

Figure A.5: DSC thermogram of CPO:HPMC (1:1) Physical mixture showing the onset temperature

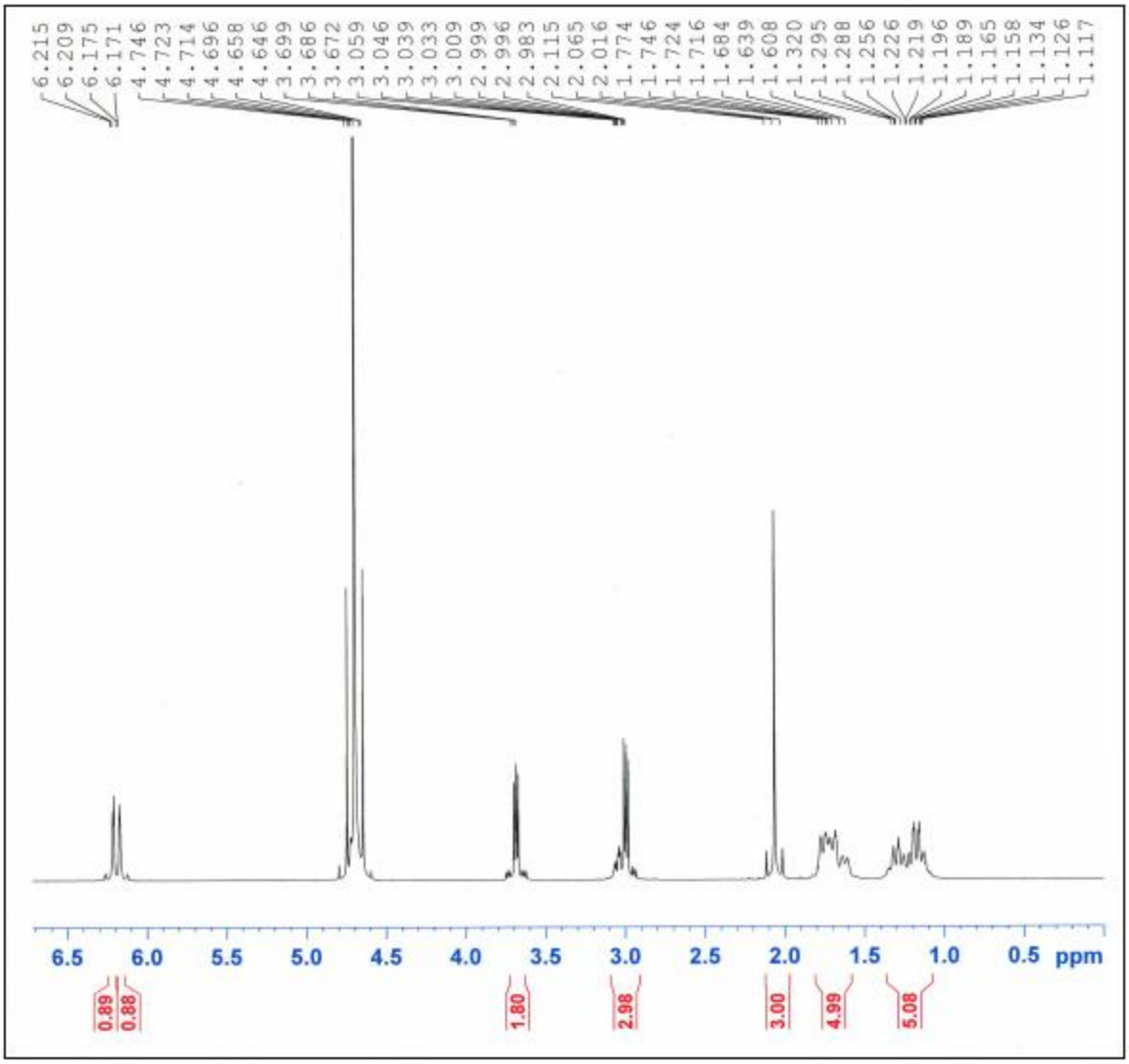

Figure A.6: ^{1}H-NMR spectrum of CPO

Table A.2: Intensities of XRD peaks for CPO at corresponding 2θ values

Sample	2θ					
	11.8º	**11.9º**	**12º**	**19.1º**	**21.3º**	**21.4º**
CPO	1396	1795	1612	720	1030	723
HPMC	1.4	0.6	0.3	9.3	6.5	5.5
CPO:HPMC 3:1 physical mixture	319	383	283	669	352	192
0P film	0.91	0.71	0.5	6.25	8.78	4.95
10P film	1.24	1.16	0.18	1.67	2.53	2.48
80P film	1.4	0.6	1.3	3.2	5.4	3.7

B: SELECTION OF PRESSURE SENSITIVE ADHESIVE FOR THE PROTOTYPE PATCH

Objective: To select the pressure sensitive adhesive with best tack for the prototype patch.

Method: Thin films of the PSAs were prepared on the release liner and the backing membrane was laid over the PSA films. The release liner was removed and the films were place on the underside of the ten-holed brass plate accessory (with 9 mm openings) (Texture technologies). The 7 mm probe was used with a 5 Kg load cell and calibrated with 2 Kg weight. The peak force (Kg) was determined using a 7 mm probe using the Texture Analyzer TA-XT2i (Texture technologies) (Figure B.1). The peak force was measured at three locations of the PSA film by moving the plate to align the hole with the probe. The sample test for adhesives in the Exponent software was used for the determination of the peak test. The test settings are shown in table B.1.

Results: Peak force is a measure of the resistance to withdrawal of the probe from the sample (Exponent software). Peak force is a measure of adhesion or tack of the sample where an increase in peak force, corresponds to an increase in tack. The typical plots obtained for the PSAs are shown in figure B. 2. Table B.2 gives the mean (± standard deviation) of the peak force values for the PSAs. DURO-TAK® 87- 4287 was selected as the PSA for the novel prototype patch because it showed the highest peak force value. Since the patch design has the adhesive in the periphery to hold the patch in place, it is important to use a PSA with high tack.

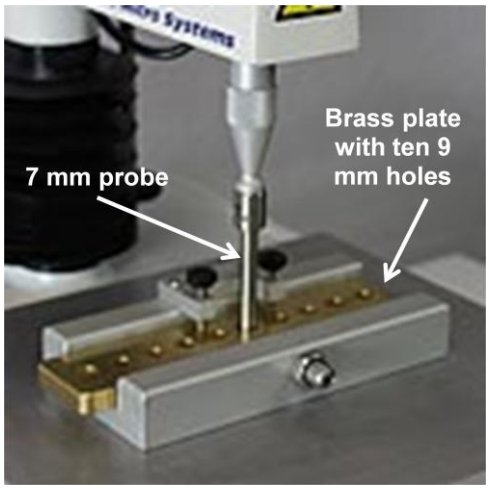

Figure B.1: Multi-holed brass plate and 7 mm probe accessory

(Texture Technologies)

Table B.1: Adhesive Test settings

Parameter	**Value**
Pre-test speed	0.1 mm/s
Test speed	0.05 mm/s
Post-test speed	5.0 mm/s
Force	100 g
Dwell time	2 s
Distance	15 mm
Trigger type	60 g
Data acquisition rate	500 pps

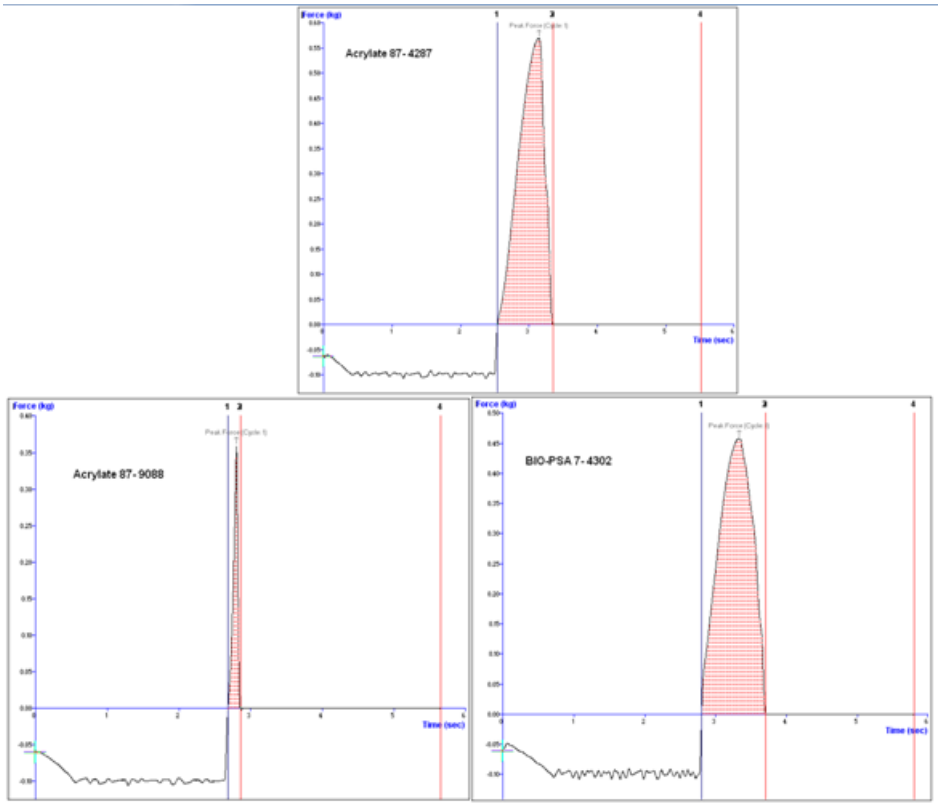

Figure B.2: Plots showing the peak force for selected PSAs

Table B.2: Peak force values for PSAs

PSA	Peak force (Kg)
DURO-TAK 87- 2287	0.385 ± 0.001
DURO-TAK 87- 4098	0.301 ± 0.008
DURO-TAK 87- 4287	0.574 ± 0.044
DURO-TAK 87- 9088	0.508 ± 0.129
DURO-TAK 87- 9301	0.253 ± 0.041
BIO-PSA 7- 4302	0.490 ± 0.095

C: CALCULATION OF THE DRUG LOAD IN THE PROTOTYPE PATCH AND PENLAC® NAIL LACQUER

Calculation of Drug Load In The Prototype Patch

The petri-dish (Greiner Bio-One) used for the fabrication of the patches has an internal area of 21 cm^2. The prototype patch contains 10 % w/w of CPO. During the fabrication of the hydrophilic matrix, 0.2 g of HPMC K15M was used and the corresponding amount of CPO was 0.02 g. Thus the matrix with area of 21cm^2 contains 0.02 g of CPO.

The theoretical drug load in the prototype patch:

$$\frac{20 \text{ mg CPO}}{21 \text{ cm}^2} = 0.95 \text{ mg/cm}^2$$

Thus, the daily topical dose of prototype patch used in the present study is 0.95 mg/cm^2.

Calculation of Drug Load in Penlac® Nail Lacquer

The typical topical dose is considered to be 2-10 mg/cm^2 or 2-10 $\mu L/cm^2$. Therefore assuming the daily topical dose used for application of the nail lacquer is 10 $\mu L/cm^2$. Penlac® nail lacquer contains 8 % w/w Ciclopirox in solution.

Hence, the drug load in the nail laquer is 8 % w/w of 10 mg/cm^2 = 0.8 mg/cm^2. Thus, the daily topical dose of Penlac used in the present study is 0.8 mg/cm^2.

D: EFFICIENCY OF THE PROTOTYPE PATCH AND THE APPROACHES TO IMPROVE THE EFFICIENCY

Efficiency of the prototype patch

During the *in vitro* transungual permeation studies, the prototype patch and Penlac® nail lacquer were applied daily to the human toenail, for 31 days.

The daily dose of CPO from the prototype patch was 0.95 mg/cm^2.

Thus for 31 days, the cumulative dose was 31 X 0.95 mg/cm^2 = 29.45 mg/cm^2.

The cumulative amount of CPO in the receiver compartment at the end of the study was 79.69 µg/cm^2 and the total amount of CPO in the human toenail was 1223.12 µg/cm^2.

Thus the total percent of CPO delivered from the prototype patch in 32 days was

$$\frac{79.69\ \mu g/cm^2 + 1223.12\ \mu g/cm^2}{29.45\ mg/cm^2} \times 100 = 4.42\ \%$$

On daily application of the nail lacquer for 31 days, 13.90 µg/cm^2 and 438.76 µg/cm^2 of CPO was obtained in the receiver compartment and in the toenail respectively.

The cumulative dose on daily application of Penlac for 31 days is 31 X 0.8 mg/cm^2 = 24.8 mg/cm^2. Using the calculation similar to the prototype patch, the total percent of CPO delivered by Penlac nail lacquer is 1.83 %

Approaches to increase the efficiency of the prototype patch

The patch matrix was fabricated by pouring the polymer mixture (6 mL) into a petri-dish with internal area of 21 cm^2. Therefore the theoretical thickness of the wet polymer mixture in the petri-dish will be 2.85 mm. When a coater is used for the fabrication of the matrix, the general thickness of the wet mass will be adjusted to 0.5 – 0.6 mm. This indicates that when the thickness of wet polymer mixture is reduced to 0.5 mm using a coater, the CPO content in the patch will be reduced from 0.95 mg/cm^2 to 0.17 mg/cm^2. The thinner patch will still be able to deliver the drug efficiently due to the availability of excess drug in the patch.

The daily application of the patch for 31 days results in an *in vitro* drug delivery of only 4.42 %. Since the transungual permeation is the rate limiting step, there is significantly higher levels of CPO still available in the patch for a lower frequency of dosing. Therefore the patch has a potential to be optimized for once weekly application. These optimization approaches will reinforce the efficiency of the patch over the nail lacquer.

In case of nail lacquer, drug delivery is due to the concentration gradient developed across the nail plate. This concentration gradient is the direct result of evaporation of the solvents in the lacquer. Thus, the maximum delivery of ciclopirox from the nail lacquer is already attained during the daily application. The lacquer formulation cannot be further modified without addition of other components.