Chemistry and Physics of Lipids 165 (2012) 454–461

Contents lists available at SciVerse ScienceDirect

Chemistry and Physics of Lipids

journal homepage: www.elsevier.com/locate/chemphyslip

Development, characterization and *in vivo* assessment of effective lipidic nanoparticles for dermal delivery of fluconazole against cutaneous candidiasis

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ARTICLE INFO

Article history:
Available online 31 January 2012

Keywords:
Nanoparticulate carrier systems
Fluconazole
Topical delivery
Sustained release
Localized effect

ABSTRACT

The nanoparticulate carrier systems as solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs) have gained interest for the topical treatment of skin associated fungal infection as they facilitate the skin penetration of loaded drugs. Therefore in this study, SLNs and NLCs loaded fluconazole (FLZ) were prepared by solvent diffusion method in an aqueous system and characterized for different parameters. In addition, antifungal activity was carried out on experimentally induced cutaneous candidiasis in immunosuppressed albino rats. The results showed that SLNs and NLCs represent the respective mean particle sizes of approx. 178 and 134 nm with encapsulation efficiency of 75.7 ± 4.94% and 81.4 ± 3.89%, respectively. The skin-retention studies of FLZ from *in vitro* and *in vivo* experiments revealed significantly higher accumulation of drug in the case of NLCs formulation. The *in vivo* cumulative amount of FLZ retention from NLCs was more than 5-fold that of the plain solution, while it was 3.3-fold more in the case of an equivalent-dose application in the form of SLNs at 12 h after administration. The antifungal study also confirmed the maximum therapeutic efficacy of NLCs, as the lowest number of cfu/ml was recorded. It can be concluded from this study that NLCs provide a good skin targeting effect and may be a promising carrier for topical delivery of FLZ offering the sustained release and maintain the localized effect, resulting in an effective treatment of a life-threatening cutaneous fungal infection.

1. Introduction

Topical treatment of severe life threatening skin fungal infections with fluconazole (FLZ) has shown to be emerge as an efficient therapy and occupies a prominent position among the alternatives of treatment (Gupta et al., 2010). However, topical delivery of FLZ resulted in systemic absorption, skin irritation and therefore failed to achieve mycological eradication (Bidkar et al., 2007). Therefore, these problems create the poor patient compliance and compromising the efficacy of the therapy. Moreover, the topical administration of bioactives is however a challenging field in drug delivery with the intricacy in controlling and not determining the exact amount of drug that reach the different skin layers (Schäfer-Korting et al., 2007). In addition, the entrapped moiety and the vehicle physicochemical characteristics are also contributed for the drug differential distribution in the various skin layers (Teichmann et al., 2007). To overcome the well-known adverse effects, the use of novel drug delivery systems, which present potential to reduce such occurrences without reducing the efficacy, has been proposed (Castro et al., 2009).

Recently, several new drug carrier systems are proposed for improving the bioavailability, sustained and controlled release of drug for maintaining the localized effect as well as enhanced drug accumulation in various strata of skin through liposomes or niosomes (Gupta et al., 2010), gel formulation (Bidkar et al., 2007), lecithin-based organogel (Jadhav et al., 2009), FLZ hydrogel (Abdel-Mottaleb et al., 2009) or polymeric mucoadhesive films (Yehia et al., 2009) along with PLGA microspheres (Rivera et al., 2004). Yet, various difficulties arise with liposomes due to their stability. Moreover, no satisfactory and large scale production method exists for microspheres (Castro et al., 2009). An interesting alternative approach for FLZ encapsulation may be the use of solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs) owing to their application for several drugs for topical therapy of skin diseases.

The SLNs offered a great potential for the administration of active molecules and simultaneously improved their therapeutic efficacy with great feasibility of incorporation of lipophilic and hydrophilic drugs, enhanced their physical stability, low cost compared to liposomes and ease of scale-up and manufacturing (Shah et al., 2007). Moreover, their lipid core made from physiological lipids having high biocompatibility and biodegradability with their potential in epidermal targeting, follicular delivery and controlled release of active moiety with increased skin hydration due to greater occlusivity have been very well

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doi:10.1016/j.chemphyslip.2012.01.006

established (Gupta et al., 2011; Mandawgade and Patravale, 2008).

NLCs, the new generation of lipid nanoparticles were designed to surmount some of the problem associated with SLNs mainly limited drug loading, risk of gelation and drug leakage during storage caused by lipid polymorphism (Müller et al., 2002). NLCs consist of a mixture of especially very different lipid molecules, *i.e.*, solid lipid(s) is blended with liquid lipid(s) (Joshi and Patravale, 2008). A blend of a liquid and solid lipid forms a less perfect crystalline structure with many imperfections providing thus more space for drug accommodation. Moreover, by controlling the amount of liquid lipids added to the formulation, the NLCs remain solid form at body temperature and finally the controlled drug release can be achieved (Hu et al., 2005). Their applicability for dermatological applications was also confirmed by successful formulations of drugs for skin disease treatment (Pardeike et al., 2009).

The literature abounds with studies confirmed that both SLNs and NLCs possess numerous features that are beneficial for topical route of application. These colloidal carriers are offering the controlled release profiles for many bioactives (Souto et al., 2004; Souto and Muller, 2005). Moreover, the small size of the lipid particles ensures close contact to stratum corneum (SC) and can increase the amount of drug penetrating into skin. Hence, these carriers may become an important tool to supply the drug over prolonged period of time and to reduce systemic absorption (Wissing and Müller, 2002a; Joshi and Patravale, 2008).

The epidermal targeting can be achieved with SLNs and NLCs formulations (Teeranachaideekul et al., 2008; Stecova et al., 2007; Maia et al., 2000), as well as reducing the side effects during topical glucocorticoid treatment such as skin thinning and atrophy (Wiedersberg et al., 2008) as they are associated with deeper layers of the skin, so that systemic absorption can be eliminated. It is confirmed that both burst release and sustained release, respectively are explained by differential drug distribution profiles within the lipid carriers. Drug-enrichment in the superficial layers of nanoparticles or on its surface can result in burst release, whereas prolonged release indicates intensive drug–lipid interactions and drug localization in the core of the particles (Müller et al., 2000). Indeed, lipid nanoparticles (SLNs, NLCs) have been explored as suitable carrier systems to manipulate the penetration/permeation of drugs throughout the various strata of skins (Wiedersberg et al., 2008). The nano-sized particles and their narrow size distribution allow them for site specific skin targeting which favors more drug retention (Pople and Singh, 2006). Depending on the lipid matrix composition, different release profiles may be obtained including a prolonged/controlled release of the drug which can decrease the risk of burst effect, commonly reported by the use of conventional drug solutions. Aqueous SLNs formulations were proposed for topical delivery of various glucocorticoids *e.g.*, clobetasol propionate (Hu et al., 2006), dexamethasone (Xiang et al., 2007) or betamethasone valerate (Sivaramakrishnan et al., 2004). The previous studies demonstrated that SLNs and NLCs dispersion of ketoconazole and clotrimazole (antifungal drugs) were quite useful for skin targeting *via* topical route and notably offer localized effect (Souto et al., 2004; Souto and Muller, 2005). Most of the studies established that SLNs and NLCs act as a promising carrier for topical delivery.

In view of this, exploring the potential of SLNs and NLCs in improving the topical delivery of FLZ seems worthwhile. The main aim of our investigation was to develop and evaluate the most effective system for FLZ for successful topical delivery. Furthermore, in the present study we aimed at fabricating SLNs and NLCs and investigated the drug localization in the different skin layers, their retention efficacy and providing localized effect with maximize therapeutic efficacy for complete eradication of *Candida* organisms. The potency of a developed system for antifungal activity was evaluated by using immunosuppressed Sprague-Dawley (SD) rats.

2. Materials and methods

2.1. Materials

FLZ was obtained as a gift from Torrent Pharmaceuticals, Ltd., Ahmadabad (India). Compritol 888 ATO (CA) (CA is glyceryl behenate, a mixture of mono, di and triacylglycerols of behenic acid (C_{22})) used as solid lipid material was generously provided by Colorcon Asia Ltd. Oleic acid (OA) was chosen as liquid lipid material, obtained from Fluka. L-α-Egg phosphatidylcholine (PC), pluronic F-68 (PF68) and sephadex G-50 were purchased from Sigma Chemicals Co. St. Louis, Missouri (USA). All other reagents and solvents were either of analytical or high-performance liquid chromatography (HPLC) grades.

2.2. Preparation of drug loaded SLNs and NLCs dispersion

FLZ-loaded SLNs and NLCs were prepared by solvent diffusion method in an aqueous system as reported earlier with slight modification (Hu et al., 2005). Briefly, 100 mg selected lipid (CA/CA and OA) were dissolved completely in a 10 ml mixture of acetone and ethanol (1:1, v/v) in water bath at 70 °C. This lipid solution was poured into 100 ml of an acidic aqueous phase containing 2:1 ratio of PC and PF68 under continuous mechanical agitation (Remi Instruments, Mumbai, India) with 4000 rpm at room temperature (25–28 °C) for 5 min. The pH value of the acidic aqueous phase was adjusted to 1.20 by addition of 0.1 M hydrochloric acid to form aggregation of nanoparticles. The aggregate of nanoparticle dispersion was then centrifuged (25,000 rpm for 30 min, Hitachi CPMax-100 Japan) then re-suspended in distilled water.

2.3. Shape, size and zeta potential

Prepared nanoparticles were characterized for shape by scanning electron microscopy (SEM, Leo 435 VP 501B, Philips). The average particle size and zeta potential were determined by photon correlation spectroscopy, using the Zetasizer nano ZS90 (Malvern Instruments, Ltd., Malvern, UK).

2.4. Entrapment efficiency

The entrapment efficiency (EE%) was determined by measuring the concentration of unentrapped drug in the lipidic dispersion as reported elsewhere (Vobalaboina and Kopparam, 2004). In brief, the lipid dispersion was subjected to centrifugation for 20 min at 25,000 rpm (Hitachi CPMax-100 Japan). The amount of FLZ in supernatant was determined by HPLC method as described earlier (Wattananat and Akarawut, 2006). The analytical column C_{18} (150 mm × 4.6 mm) was protected by a C_{18} guard column (4.0 mm × 3.0 mm). The mobile phase used was a mixture of 10 mM of sodium acetate buffer (adjusted to pH 5.0 with glacial acetic acid) and methanol (65:35) and it was run at a flow rate of 1 ml/min. The detection was done at wavelength of 210 nm.

2.5. In vitro skin permeation and retention studies

After cervical dislocation of the hairless rat skin, the skin was excised surgically. The full thickness skin was used after removing underlying fat and subcutaneous tissues. *In vitro* skin permeation studies were carried out by using a Franz diffusion cell, as described by Qingzhi et al. (2009), with slight modification. The skin was mounted between the donor and receptor compartments of the diffusion cell with the SC facing upward (donar compartments). Each diffusion cell, with a diffusion area of 3.14 cm^2 and the receptor medium was filled with 25 ml of PBS (pH 7.4), thermostated

at 37 ± 1 °C and continuously stirred at 100 rpm throughout the experiment. 1 ml formulation was placed on the donor compartment. At appropriate time intervals up to 12 h, samples of 200 μl from the receptor compartment were withdrawn and replaced by an equal volume of fresh medium to keep a stable receiver volume. The experiments were repeated in triplicate. The samples from the receptor compartment were analyzed by HPLC, as mentioned in above section.

At the end of the permeation study, excess formulation was removed by wiping the test area with a cotton swab, washed 3 times with PBS, and then dried with filter paper. To determine the drug concentration in the SC, 20 tapes (CuDerm® Corporation, Adhesive Tape, New Delhi, India) were applied with constant pressure for 2 min and carefully peeled away. The tapes were pooled in a tube containing 1 ml of PBS (pH 7.4) and 1 ml of methanol. Then, the tubes were vortexed for 20 min, sonicated for 30 min and vortexed again for 1 h to extract drug from the tapes. The remaining skin was cut into small pieces to determine the amount of FLZ in the viable skin (epidermis and dermis). They were pooled in a tube containing 1 ml of PBS (pH 7.4) and 1 ml of methanol, vortexed for 20 min, and homogenized (York, Mumbai, India). The resulting solution was sonicated for 30 min, vortexed again for 30 min and centrifuged at 10,000 × *g* (Hitachi CPMax-100, Japan) for 30 min. The amount of FLZ extracted from tapes in remaining skin samples was determined by using HPLC.

2.6. In vivo studies

In vivo studies were carried out as per the guidelines compiled by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, Ministry of Culture, Government of India). All the study protocols were approved by the Animal Ethical Committee of the Dr. H.S. Gour University (Sagar, India).

2.7. FLZ localization in the different skin layers by in vivo tape stripping

Drug localization in different skin layers was determined by the method reported by Song and Kim (2006), with slight modification. First, the animals were anesthetized with an intraperitoneal injection of ketamine hydrochloride (100 mg/kg). Then, 1 ml formulation was topically applied into the test area (2 cm × 3 cm) on the dorsal skin with an approximate FLZ amount (0.6 mg/ml). After 12 h post application period, the test area of the skin was removed by cervical dislocation. Excess of formulation was removed by wiping the test area with a cotton swab, washed 3 times with PBS and then dried with filter paper. To determine the drug concentration in the SC and viable skin, a similar procedure was adopted as described previously in Section 2.5.

2.8. Skin-irritation test

The irritation potential of the FLZ formulations was evaluated by carrying out the Draize patch test on rabbits (Shah et al., 2007). The back of the animals was clipped free of hair 24 h prior to the application of the formulations and 0.5 ml of formulation was applied on the hair-free skin with uniform spreading within the area of 4 cm^2. The skin was observed for any visible change, such as erythema at 24, 48 and 72 h after the application of formulations. The mean erythemal scores were recorded (ranging from 0 to 4), depending on the degree of erythema, as follows: no erythema = 0; slight erythema (barely perceptible-light pink) = 1; moderate erythema (dark pink) = 2; moderate to severe erythema (light red) = 3; and severe erythema (extreme redness) = 4 grade.

2.9. Antifungal activity

2.9.1. Preparation of the animals

The male albino rats (SD), each weighing 150–180 g, were selected and housed in individual cages and received food and water *ad libitum*. To achieve a heavy cutaneous infection, animals were immunosuppressed by cyclophosphamide (100 mg/kg, body weight) treatment given through an intraperitoneal injection on the first 3 days during the experiment prior to fungal infection.

2.9.2. Preparation of the microorganisms

Clinical isolate of *Candida albicans* (*C. albicans*, MTCC-Code*1637) was used to infect the animals. A working culture of the *candida* was grown for 48 h at 30 °C on Sabouraud dextrose agar (SDA). The cells were then collected, washed and resuspended to a final concentration of 10^7 colony forming units/ml (cfu/ml, suspended in sterile saline) of *C. albicans* (Maebashi et al., 1995).

2.9.3. Cutaneous infection

Each animal's back was shaved with an electric clipper and an approximately 3.0 cm^2 area was marked on each animal's back. The marked area was infected with 10^7 cfu/ml suspensions by gently rubbing onto the skin with the help of a sterile, cotton-tipped swab until no more visible fluid was observed (Maebashi et al., 1995). Infection was produced under an occlusive dressing and the infected area was covered with a sterile adhesive bandage, held in place with extra-adherent tape for 48 h before treatment began (Abdel-Mottaleb et al., 2009). Control animals were infected in the same manner; however, they did not receive any FLZ formulation.

2.9.4. Treatment of the infection

Treatment began 24 h after the infection was induced and test formulation was topically applied once daily for 3 consecutive days. The experimental animals were divided into four groups each containing 6 animals. Group 1 was treated with plain drug solution, group 2 was applied topically with an equivalent dose of SLNs; group 3 was applied topically with NLCs dispersion; and group 4 served as the control. All animals were sacrificed 48 h following the last treatment and 3.0 cm^2 of skin from the infected sites was excised. The infected skin samples were collected, washed and then plated into SDA culture media and incubated for 48 h at 37 ± 1 °C, and then cfu values were recorded. For the colony counts in infected skin, an analysis of variance following Dunnet's test was applied on the log10 colony counts for each day of infected skin cultures sample from days 1 to 8 (Ning et al., 2005).

2.10. Statistical analysis

All the results are expressed as mean ± standard deviation. The treated groups were compared to control by analysis of variance (ANOVA), following Dunnet's test. The statistical analysis was carried out using Instat 2.1 software, Graph Pad Software Corp., San Diego, CA, USA. The P-value < 0.05 was considered as significant.

3. Results and discussion

3.1. Preparation and characterization of carrier system

The present investigation was aimed at developing SLNs and NLCs system, for topical/localized delivery of drug with higher skin retention. This study indicates that drug delivery can be greatly affected by compositions of carrier system as used in the SLNs and NLCs.

The SLNs and NLCs were developed by solvent-diffusion in aqueous medium. It is a suitable method to prepare small, homogenously sized lipid nanoparticulate dispersion being quick and

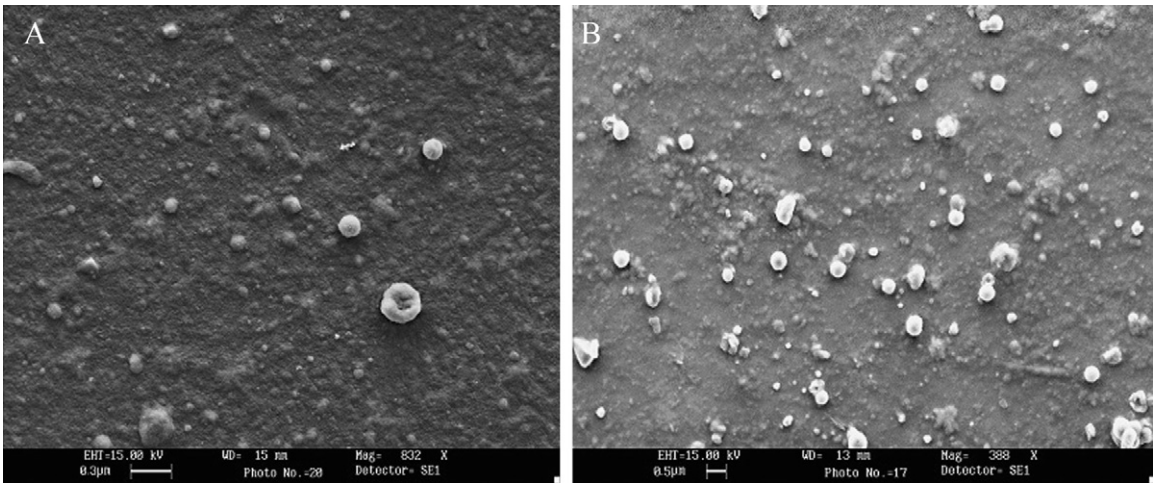

Fig. 1. SEM photographs of FLZ loaded SLNs formulation (A) and NLCs formulations (B).

Table 1
The characterization of the formulations by particle size, zeta potential and EE%.

Formulation	Lipid phase	Emulsifiers	Size (nm)	Zeta potential (mV)	EE%
SLNs	CA	PC + PF68	178.9 ± 3.8	−25 ± 3.7	75.7 ± 4.94
NLCs	CA + OA	PC + PF68	134.3 ± 5.2	−29 ± 2.4	81.4 ± 3.89

Each value represents the mean ± S.D. ($n = 3$).

possible with simple laboratory setup. CA, a glyceryl behenate was used as a core material for the SLNs. In the NLCs formulations, OA as the liquid lipid matrix was mixed with CA into which the drug was incorporated. The particulate systems were stabilized with the surfactants PC and PF68. The composition of the nanoparticle shell significantly affects the skin penetration and drug localization pattern in different skin layers (Fang et al., 2008).

Fig. 1 showed the SEM photomicrographs of drug-loaded SLNs and NLCs and these photomicrographs revealed that the particles were the smooth surfaces and almost spherical in shape. Particle sizes and surface charges of the developed SLNs and NLCs are shown in Table 1. As Table 1 enlisted, the size of SLNs was found to be 178.9 ± 3.8 nm, while in the case of NLCs, they were recorded to be 134.3 ± 5.2 nm, respectively. It can be seen that both type of formulations were in the nano-sized range, but the sizes of the SLNs were significantly larger than those of the NLCs. In order to elucidate that the crystalline lipid core of the SLNs produced a larger particle diameter compared to the amorphous core of the NLCs.

Moreover, the OA content in NLCs may be responsible for decreasing the particle size (Liu et al., 2007; Weyenberg et al., 2007). Even, though a negative zeta potential provides electrostatic repulsion to maintain the small size of these systems. As depicted in Table 1, the zeta potentials of the SLNs and NLCs were −25 and −29 mV.

3.2. Entrapment efficiency

The EE% of the developed SLNs and NLCs are shown in Table 1. It can be seen that the encapsulated moiety in the SLNs (75.7 ± 4.94%) were lower as compared to NLCs based formulations (81.4 ± 3.89%). The possible reason is that the incorporation of liquid lipids to solid lipids in NLCs could lead to massive crystal order disturbance. Therefore, the resultant matrix of lipid particles indicates greater imperfections in the crystal lattice and can afford more space to encapsulate the drug moiety, thus leading to improved drug entrapment efficiency (Jenning et al., 2000b; Jenning and Gohla, 2001; Souto et al., 2004).

3.3. In vitro skin-permeation and retention studies

To assess the influence of the lipidic nanoparticles on the permeation and accumulation of drug into the skin, *in vitro* skin permeation and retention studies were performed using hairless rat skin by Franz diffusion cells. In this study, permeation data obtained from SLNs and NLCs were compared with plain solution of the same drug concentration (*i.e.*, 0.6 mg/ml). The results revealed that the amount of permeated drug was higher in case of plain drug solution, while SLNs and NLCs based formulation represented the lesser amount of permeated drug (Fig. 2). The data indicate that lipidic nanoparticles clearly delayed the drug permeation through the skin. This may be appeared that structural composition of lipid nanoparticles was responsible for rate limiting effect in drug permeation. The cumulative amounts of FLZ from plain drug solution, SLNs and NLCs at 12 h

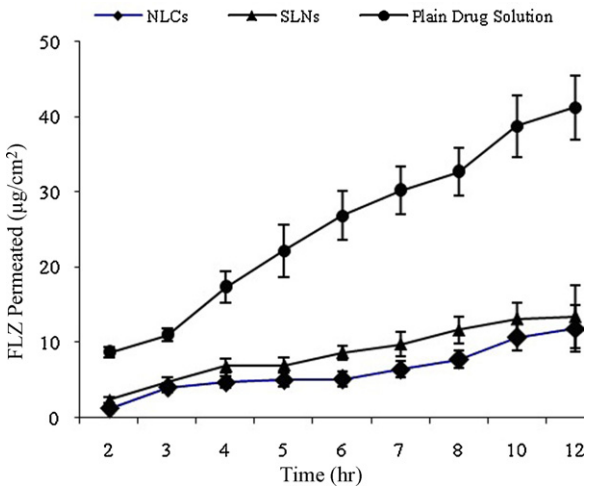

Fig. 2. *In vitro* skin permeation of FLZ for SLNs, NLCs and plain drug solution. Values are expressed as mean ± standard deviation ($n = 3$).

Table 2
Results of *in vitro* permeation and skin retention study from lipidic nanoparticulate and plain solution: amount permeated through the skin at 12 h, % FLZ accumulated into the skin at the end of the permeation experiments (12 h); and Locally Accumulation Efficiency (LAC) value: drug accumulated into SC/drug permeated through the skin ratio. The data were expressed as % dose applied per unit area. Each point represents the mean ± S.D. ($n = 3$).

Formulation code	FLZ permeated through the skin at 12 h (μg/cm^2)	Total FLZ accumulated in to skin (%)	LAC in 12 h
NLCs	11.81 ± 3.65	22.56 ± 3.27	8.3
SLNs	13.45 ± 4.11	18.34 ± 4.24	6.2
Plain drug solution	41.23 ± 4.29	10.62 ± 2.45	1.4

after dosing were 41.23 ± 4.29 μg/cm^2, 13.45 ± 4.15 μg/cm^2 and 11.81 ± 3.12 μg/cm^2, respectively. In other words, the plain solution showed higher drug permeation, in comparison to lipidic nanoparticular systems (Fig. 2). As expected, NLCs showed a decreased permeation through the skin which can be hypothesized as drug was more accumulated in the horny layer. In fact, lipidic nanoparticles have shown the peculiarity to reduce and/or suppress the permeation (transdermal delivery) through the skin while they at large enhanced the penetration (dermal delivery) especially into the upper skin layers (Jenning et al., 2000a; Alvarez-Román et al., 2004). Therefore, these findings confirms the assumption that lipid composition in nanoparticles provokes the accumulation of the embedded drug moiety into the upper skin layers, thus creating a reservoir which able to prolong the skin residence time.

The drug accumulated in the skin is represented in Table 2. Compared to the plain solution, in the case of NLCs, a 2.12-fold more significant ($P < 0.01$) accumulation was estimated, whereas in the case of SLNs, nearly 1.73-fold higher ($P > 0.05$) FLZ accumulation was observed. The increased skin delivery by SLNs and NLCs include the large surface area due to small particle sizes, an occlusive effect and a penetration enhancer effect due to presence of surfactants (Fang et al., 2008). Moreover, the higher drug retention in case of NLCs formulation may be due to the OA lipid that was also responsible for more penetration effect and may be fuse as well as mix with skin lipids to loosen their structure and increase the thickness of SC by disturbing the lamellar arrangement of the lipids (El Maghraby et al., 2008; Qingzhi et al., 2009; Zakir et al., 2010).

The percent of FLZ permeated through the skin and accumulated into the SC as well as viable skin for all formulations at the end of the experiments, has been compiled in Fig. 3. The drug retention was found to be maximal in SC invariably for all the formulations. A significantly higher SC retention of drug was obtained for NLCs (16.34%) and SLNs (13.97%) as compared to plain solution (9.74%) ($P > 0.05$). However, there was little difference in cumulative amounts of FLZ in SC between the SLNs and NLCs.

In fact in the case of viable skin, it was found that NLCs showed maximum accumulation (6.22%) among all the formulations ($P < 0.01$). Results seem to indicate that FLZ in the NLCs presented a lower permeation through rat skin (Fig. 3, Table 2). In addition, NLCs also showed a significant increase in the drug accumulation in SC and viable skin, compared to SLNs or plain solution. However, we did not significantly investigate the reason for the higher accumulation of drug in case of NLCs as compared to SLNs. The higher accumulation following SLNs and NLCs application may be ascribed to its lipidic composition as well as their small size and interaction between SLNs and NLCs in the SC, providing the deposit effect for drug in the skin, are the possible reasons why lipidic nanoparticles can increase the drug amount penetrating into the skin layers. Bouwstra et al. (2003) reported that this behavior might be expected, as SLNs and NLCs system based on lipid composition are more similar to SC lipids, in contrast to plain solution. Thus, localized delivery of FLZ from SLNs and NLCs into skin tissue is affected, leading to an effective treatment of cutaneous fungal infection.

LAC (Locally Accumulation Efficiency) value was calculated to determine the accumulation index of the drug (Sinico et al., 2005). LAC values obtained from the several formulations were listed in Table 2. The LAC value of SLNs and NLCs was higher of 6.2 and 8.3 respectively than that of the control formulation (LAC = 1.4), suggesting that the main effect of these systems was to accumulate the drug in the skin. Comparison of LAC values emphasized that in particular NLCs showed a 1.3-fold higher LAC than SLNs. This parameter would suggest the higher accumulation of the drug into the skin in the case of NLCs due to the lipid composition, presenting the local effect of this system.

3.4. In vivo drug localization in the skin layers

The amount of FLZ accumulated in the various strata of skin, after 12 h post application period from different formulations, as shown in Figs. 4 and 5. It was reported earlier that 20 strips are necessary for the complete removal of SC layers (Kim et al., 1997). There may be some loss found during the extraction process from

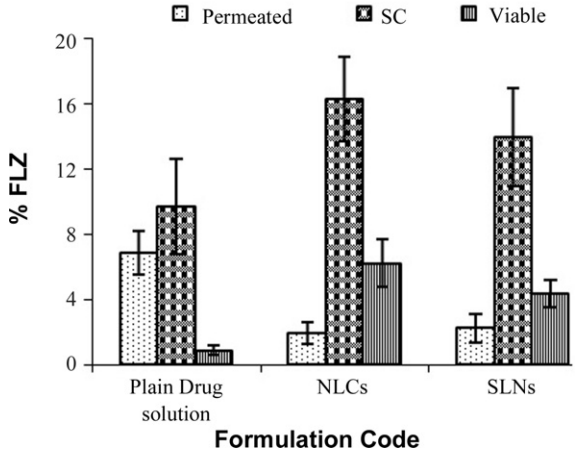

Fig. 3. Percent FLZ for SLNs, NLCs and plain drug solution for *in vitro* permeation and skin-retention studies. Values are expressed as mean ± standard deviation ($n = 3$).

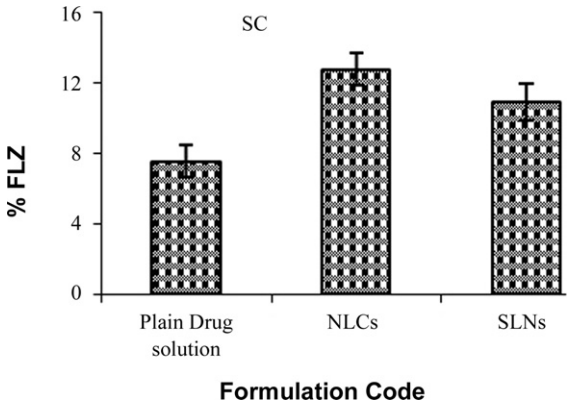

Fig. 4. Percent FLZ for SLNs, NLCs and plain drug solution in SC for *in vivo* studies. Values are expressed as mean ± standard deviation ($n = 3$).

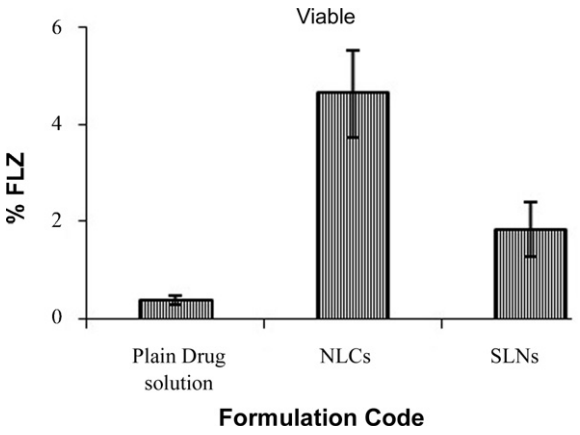

Fig. 5. Percent FLZ for SLNs, NLCs and plain drug solution in viable skin for *in vivo* studies. Values are expressed as mean ± standard deviation ($n = 3$).

tissue, showing incomplete recovery. The findings suggested that *in vivo* localization of drug creates somewhat different pictures, as seen in the *in vitro* situation. This may be owing to the disruption of nanoparticles due to interaction with skin components, such as lipase activity and other hydrolyzed product, in viable skin, which might affect the low magnitude of intradermal retention of drug *in vivo*, compared to those recorded in *in vitro* studies (Song and Kim, 2006; Bouwstra et al., 2003). The absorption of FLZ following an application of different formulations through SC (Fig. 4) decreased in the following sequence: NLCs > SLNs > plain drug solution. The data showed 1.7-fold ($P < 0.01$) and 1.5-fold higher ($P < 0.01$) retention in the case of NLCs and SLNs systems, as compared to plain solution. On the other hand, the amount of drug recovered from viable skin following NLCs application followed the same order as recorded in the case of SC; however, the amount accumulated was significantly low, as compared to SC. The data presented in Fig. 5 showed that the amount of drug in the viable skin from NLCs was 36% of the amount measured in the SC. Apart from this, it was also observed that, following SLNs and drug solution application, only 17% and 5% was extracted from viable layers of skin that recovered from SC. Compared to the plain solution, NLCs showed 11.9-fold, while SLNs showed 4.7-fold, higher accumulation of drug in viable skin. This result was in agreement

Table 3
Mean erythemal scores and PII observed for various FLZ formulations obtained at the end of 24, 48 and 72 h ($n = 3$).

Formulation code	Erythemal scores			
	24 h	48 h	72 h	PII
NLCs	0	0	0	0
SLNs	0	0	0	0
Plain drug solution	1	2	2	1.67
Control (no formulation)	0	0	0	0

with the report where drug following NLCs application showed a higher degree of retention in viable skin (Fang et al., 2008). Thus, the developed FLZ loaded NLCs formulations in the present study have the potential to deliver the effective amount of drug in a sustained, controlled fashion, enabling the maintenance of its localized depot and, therefore, prolonged residence time in viable skin for the treatment of cutaneous candidiasis.

3.5. Skin-irritation test

Conventional therapy is associated with noticeable skin irritation, which strongly restricts its applicability and acceptability by the patients. Ideally, the FLZ delivery system should be able to reduce irritation. The skin-irritation studies indicated that SLNs and NLCs exhibited considerably no irritation, as compared to plain solution, even after 24 h of application (Table 3). The primary irritation index (PII) was found to be 0.00 for SLNs and NLCs, showing no irritation. Therefore, the developed lipid nanoparticles formulation resulted in no erythema on the abraded rabbit skin, as compared to the plain solution.

3.6. Antifungal activity

The antifungal activity of different formulations was determined by challenging the animals with *C. albicans*. Fig. 6 convincingly suggested that *C. albicans* furnished a well established infection in all the challenged animals, with a range of 3.89–3.98 log10 cfu/ml on day 0 prior to treatment, with slight variability. Results of the studies revealed that NLCs possessed significant therapeutic efficacy, as compared to other formulations ($P < 0.05$). After 8 consecutive days of therapeutic treatment, in the control group *C. albicans* organisms

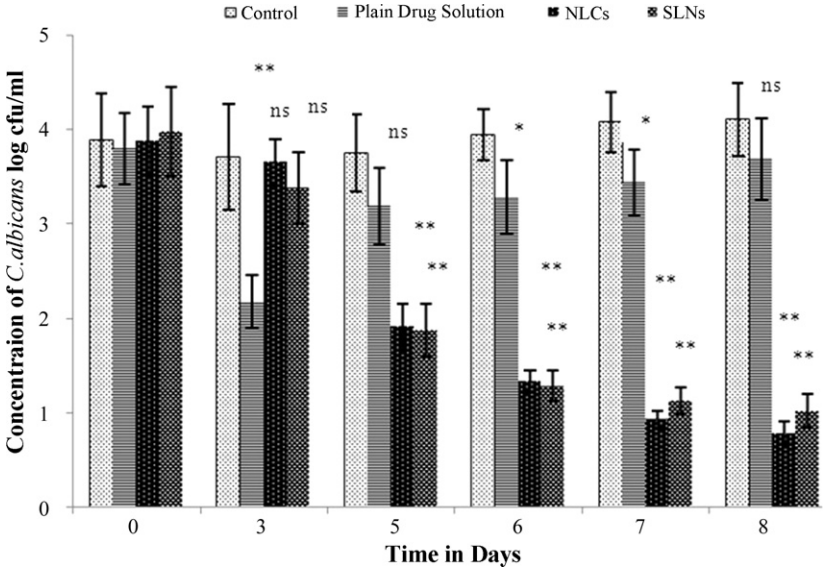

Fig. 6. Antifungal efficacy of different FLZ formulations *vs.* time in days. Values are expressed as mean ± standard deviation ($n = 6$). Statistical significance was considered between control *vs.* plain drug solution, SLNs, and NLCs, at: $^{*}P < 0.05$; $^{**}P < 0.01$; ns, nonsignificant.

were still detectable, having the signal for appropriate challenge inoculums. After the treatment with plain solution, out of 6 animals 5 remained infected. However, SLNs and NLCs based treated group showed significantly higher therapeutic efficacy with 5 of 6 animals were successfully treated respectively as negative culture was seen. As it can be clearly stated that the animals treated with lipidic nanoparticles, demonstrate low fungal burden in skin, with a colony count significantly less abundant than those treated with plain solution.

As compared to the control group, it established the presence of challenging organisms at the surface of the epithelium with desquamation of superficial layers throughout the experiment. It was observed that initially (on day 3), plain solution reduced the maximum number of cfu of *C. albicans* infection in skin however, this effect did not maintain throughout the experiment. In contrast to this, SLNs and NLCs constantly reduced the number of cfu of the infecting organism and appreciated the longer term reduction of fungal infection in skin. The higher therapeutic efficacy in the case of NLCs formulation may be expected due to penetration into the skin, followed by drug carrier accumulation in different strata of skin and resulting in to reservoir effect with higher level of localization. Moreover, the higher payload, their lipidic nature and structural integrity of nanoparticles might be the contributory factor for more prolonged and significantly enhanced their antifungal activity (Sivaramakrishnan et al., 2004; Fang et al., 2008). These findings also confirmed that lipid nanoparticles provoke the accumulation of the embedded drug into the upper skin layers, and creating a reservoir able to prolong the skin residence time. In particular, the more pronounced sustain nature is associated with these formulation may be dependent on the depletion of drug in the SC layer. This fact might be lead to the formation of reservoir that able to support the sustained drug release toward deeper skin layers. This can be ascribed to the presence of analogies (*i.e.*, C_{22}) that reasonably play a role on the SLNs or NLCs/SC interaction and, consequently, on the adhesion and reservoir formation. Moreover, the presence of other fatty acids may also enhanced SLNs/NLCs components to mix with the SC and to retard the active permeation (Bouwstra et al., 2003). Since drug localization in the particles seems also to play a significant role in skin targeting. Therefore, developed system can produce an enhancing effect on skin penetration that penetrate deeply into the SC or may fuse and mix with skin lipids to loosen their structure (El Maghraby et al., 2008; Qingzhi et al., 2009). Hence, the SLNs and NLCs, presents remarkable benefits over other formulations *viz*., provide long-term therapeutic concentrations at the site of infection for eradication of the fungal burden, improve the skin tolerability and endow with effective topical delivery of FLZ.

4. Conclusion

Cutaneous candidiasis related skin fungal infections most often reoccurs and is rarely cured; hence patients receive therapy over a long time. FLZ loaded SLNs and NLCs presented suitable average particle size, zeta potential, entrapment efficiency and *in vitro* skin permeation and retention to be compatible with topical application. Moreover, the NLCs offered enhanced drug retention and higher level of localization with sustained and controlled release of FLZ. From the above preliminary studies it has been concluded that NLCs have shown a good ability to increase drug accumulation in the various skin layers without any transdermal delivery with creation of depot effect and maximal therapeutic antifungal efficacy may be possible mechanisms for the enhancement thus improving cutaneous drug accumulation. The results indicated that the NLCs prepared in this study can serve as potential carriers for the topical delivery of therapeutic molecules for the treatment cutaneous fungal infection thus, it holds clinical applicability.

Acknowledgements

The authors are thankful to Torrent Pharmaceuticals Ltd. (India) and Colorcon Asia Ltd. for providing the gift sample of FLZ and CA, respectively. The Authors duly acknowledge the IMTECH (Chandigarh, India) for providing the fungal strain. The authors are also thankful to AIIMS (New Delhi, India) for carrying out the SEM of the formulations. Financial support provided by the AICTE in the form of a Junior Research Fellowship (JRF) to Madhu Gupta is duly acknowledged.

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