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Targeting tacrolimus to deeper layers of skin with improved safety for treatment of atopic dermatitis

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ABSTRACT

Atopic dermatitis (AD) is chronically relapsing eczematous skin disorder having significant impact worldwide. Tacrolimus is the drug-of-choice which inhibits T-cell activation resulting in suppression of inflammation. However, despite being effective, most common adverse events of tacrolimus are low-and-variable bioavailability, burning sensation and pruritus at application site, which prompt for development of novel carrier that could effectively target tacrolimus to site-of-action without producing undesirable side-effects. Tacrolimus-loaded lipid-nanoparticles (T-LN) were prepared and optimized. DSC and FT-IR have been employed to study drug-excipient incompatibility and encapsulation of drug in lipid which was further confirmed by ^{1}H NMR. *In vitro* studies revealed much higher drug release, skin penetration and enhanced skin accumulation as compared to reference Protopic®. *In vitro* and *in vivo* occlusion studies demonstrated similar occlusiveness for T-LN and reference however; T-LN showed significantly higher drug levels penetrating into deeper skin layers where dendritic cells responsible for immunopathogenesis of AD mainly reside. *In-vivo* skin retention demonstrated 3.36, 30.81 and 28.68-times higher stratum corneum, epidermal and dermal levels respectively compared to reference. Visualization of cutaneous uptake *in-vivo* using CLSM confirmed targeting to deeper skin layers and Draize test showed no skin irritation with PII 0.00. Thus T-LN displayed superior performance, effective skin targeting and improved safety as compared to reference.

1. Introduction

Atopic dermatitis (AD) is a chronic, inflammatory skin disease characterized by an itchy, erythematous and intensely pruritic rash with periods of exacerbation and remission. The disease exacerbations often require treatment with topical corticosteroids, the prolonged application of which can be associated with dermal atrophy and other side effects (Furue et al., 2003; Yamamoto and Nishioka, 2003). Therefore there is a need for an alternative treatment that is efficacious and free of the long-term side effects associated with corticosteroids.

Tacrolimus is a new powerful macrolide immunosuppressant, isolated from the fermentation broth of *Streptomyces tsukubaensis* and has shown notable efficacy as a rescue or primary immunosuppressant therapy for immune-inflammatory conditions including AD (Rubins et al., 2005). It inhibits early T-cell activation in gene transcription such as interleukins, which result from its inhibition of phosphatase (calcinuerin) in activated T-cells. It binds to cytoplasmic receptor FKBP-immunophilin and gain ability to associate with calcineurin and inhibits its phosphatase activity resulting in inhibition of T-lymphocyte activation (i.e., immunosuppression) and thus suppresses inflammation (Tocci et al., 1989). However, despite being quite effective, the most common adverse events associated with tacrolimus use are low and variable bioavailability, burning sensation and pruritus at the site of application and the potential to increase the risk of cutaneous infections by altering local cutaneous immune response. Side effects can be severe and can have harmful increased long term malignancy risk. It has a narrow therapeutic index, displays considerable variability in response and has the potential for serious drug interactions (Zahir et al., 2001; Spergel and Leung, 2006).

Presently tacrolimus is commercially available only as an ointment formulation, Protopic® for topical application for the treatment of AD and other inflammatory skin diseases and has been shown to be effective in children, with minimal systemic absorption (Ruzicka et al., 1999; Yoshida et al., 2004, Choi and Cho, 2009). Most of the studies with tacrolimus ointment have reported its low and highly variable absorption (Ruzicka et al., 1997; Boguniewicz et al., 1998; Cheer and Plosker, 2001; Kang et al., 2001; Paller et al., 2001) with tacrolimus pharmacokinetic concentrations below 1 ng/ml and sometimes the samples were below the lower limit of quantification of the assay. The ointment has found to be associated

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with high individual variation in mean disposition half-life ($t_{1/2}$) values and the apparent longer half-life values were attributed to the rate of absorption being slower than the rate of elimination. Also the skin irritation (skin burning, itching) with subsequent pain and redness is the most frequently reported adverse event by the patients with the use of tacrolimus ointment. The other adverse events include flu-like symptoms, fever, allergic reaction, headache, increased cough, asthma, and skin infection. At the same time because of the inherent property of ointment vehicles, the conventional formulation suffers from undesirable stickiness or greasiness, not water-washable with subsequent uneasy feel to the patients and having a limited ability to release medication to the skin (Kudla, 1979). These findings prompt for development of novel delivery system for treatment of AD which could effectively target the tacrolimus molecule to the site of action without producing undesirable side effects with added improved safety values.

Particulate formulations have proven to be an efficient approach to achieve better drug release profiles and to increase the bioavailability of drugs (Fang, 2006; Kaparissides et al., 2006; Guterres et al., 2007; Schäfer-Korting et al., 2007). Therefore various alternative dosage forms including incorporation of tacrolimus into particulate carriers have been suggested. However most of these particulate carriers are for systemic immunosuppression. Solid dispersion formulations of tacrolimus have been devised with enhanced solubility and improved oral bioavailability (Yamashita et al., 2003; Park et al., 2009). Significantly prolonged survival of islet allografts have been observed with a single subcutaneous administration of biodegradable microsphere-loaded tacrolimus (Wang and Uno, 2004). Tacrolimus loaded poly(lactic-co-glycolic acid) nanoparticles entrapped into pH-sensitive microspheres could achieve greater selectivity to the colon when administered orally (Lamprecht et al., 2005). Water-in-Oil-in-Water-type multiple emulsion of tacrolimus avoided the complications of systemic immunosuppression and simultaneously enhanced immunosuppressive efficacy by delivering the drug locally to the site of the target organs, liver and spleen when administered as an intravenous drug carrier (Uno et al., 1997). A significantly higher immunosuppressant activity overcoming a low and erratic bioavailability was observed with Self-Microemulsifying Drug Delivery System (SMEDDS) of Tacrolimus (Borhade et al., 2008). Liposomal formulation of tacrolimus containing endogenous phospholipids showed 40% increase in spleen concentration with increased mean residence time when compared with conventional intravenous dosage form of tacrolimus (Lee et al., 1995). Liposomal tacrolimus lotion could achieve higher concentration of tacrolimus in the skin when compared to intravenous injection of tacrolimus (Erdogan et al., 2002) however no comparison with tacrolimus ointment has been reported. Liposomes though have shown improved skin penetration they are associated with problems such as vesicle rupture due to mechanical stress, degradation of incorporated drug, size inhomogeneity, limited internal volume in addition to reproducibility, stability and scale-up issues (Adamich and Bach, 1984; Mozafari, 2005) which demands need for new preparation techniques involving topical tacrolimus for treating inflammatory skin conditions such as AD.

Lipid based nanoparticles have shown advantages such as enhanced bioavailability, physical stability, protection of incorporated labile drugs from degradation and controlled release (Mühlen et al., 1998; Jenning et al., 2000b; Maia et al., 2000; Wissing and Müller, 2003; Pople and Singh, 2006; Liu et al., 2007; Schäfer-Korting et al., 2007; Nagi et al., 2008; Bhalekar et al., 2009). Lipid nanoparticles have been termed as "nanosafe" carrier and due to the production from biodegradable and/or physiological lipids pose negligible health hazard and exhibit excellent tolerability and safety (Pardeike et al., 2009). In the present study we report tacrolimus loaded lipid nanoparticles with significantly superior skin penetration qualities, enhanced cutaneous uptake and improved drug accumulation at the target site of AD i.e. the epidermal and dermal layers where immuno-inflammatory cells mainly reside, as compared to the marketed ointment Protopic® as reference.

The present study focused on the development of tacrolimus loaded lipid nanoparticles (T-LN) by hot homogenization technique. The formulation was optimized for process and product variables and evaluated for particle size, polydispersity index (PI), drug entrapment efficiency and rheology. The drug-excipient incompatibility/interaction and encapsulation of drug in the lipid matrix were determined by DSC and FT-IR spectroscopy. ^{1}H NMR further confirmed the encapsulation and immobilization of drug in the lipid matrix. *In vitro* drug release and *in vitro* skin penetration properties have been studied using pig ear skin. The effect of skin occlusion of T-LN and marketed ointment on skin penetration has been studied both by *in vitro* and *in vivo* methods. Further the targeting and localization of drug in various layers of the skin has been studied in detail *in vivo* in rat, as the immunoinflammatory cells which are the target for tacrolimus are present mainly in the deeper layers of skin. The results were further confirmed by visualization of cutaneous uptake *in vivo* using confocal laser scanning microscopy technique. The safety of T-LN was established using Draize skin irritation study in albino rabbit. All the experimental protocols involving animal experimentation were approved by the Institutional Animal Ethics Committee.

2. Materials and methods

2.1. Materials

Tacrolimus was a gift from Panacea Biotec Ltd., Punjab (India). Glyceryl trimyristate (Dynasan 114) was obtained from Sasol (Germany). Polysorbate 80 (Tween 80) and Sorbitan monooleate (Arlacel 80) were obtained from Uniqema (Netherlands). Carbopol 980 was obtained from Lubrizol Advanced Materials, Inc. (USA). All other chemicals and solvents were of HPLC or analytical reagent grade and were purchased from S.D. Fine Chemicals, Mumbai, India.

2.2. Preparation of tacrolimus loaded lipid nanoparticles (T-LN)

T-LN was prepared using hot melt emulsification employing high pressure homogenization technique. Briefly, glyceryl trimyristate (5%, w/w) was melted and tacrolimus (0.1%, w/w) was dissolved to obtain clear solution. The dispersion medium (i.e., distilled water with hydrophilic surfactant) was heated to 65 °C, 10 °C above the temperature of the lipid melt. The hot lipid phase was emulsified in the dispersion medium by high speed stirring using Ultra-turrax T 25 (IKA-Werke, Germany). This dispersion was then subjected to high pressure homogenization using APV 2000 (Invensys, Denmark) homogenizer. The obtained nanodispersion was allowed to cool to room temperature forming lipid nanoparticles by recrystallization of the dispersed lipid.

2.2.1. Optimization of surfactant concentration and homogenization parameters

Response surface optimization (Shivakumar et al., 2007; Yadav et al., 2008) was applied to determine the critical concentration of surfactants using 3^2 factorial design. The effect of two independent variables hydrophilic surfactant concentration (X1) and hydrophobic surfactant concentration (X2) was determined on stability of the lipid dispersion in terms of sedimentation volume (SV) as response variable. Three combinations of surfactants Brij 721–Brij 72; Poloxamer 188–Plurol Oleique and Polysorbate 80–Sorbitan monooleate were investigated to determine the optimum surfactant combination. For each of the surfactant combination, 9 treatment

experiments were designed with each factor being tested at 3 designated levels −1, 0 and +1. Response surface methodology (RSM) plots were generated to identify the effect of significant variables. The optimization of homogenization parameters (homogenization pressure and number of cycles) was carried out by preparing batches at homogenization pressures ranging from 800 to 1200 bars at 5, 8 and 10 homogenization cycles.

2.3. Preparation of gels enriched with T-LN

The ideal topical formulation for delivery to the skin should exhibit ease of application to the skin with adequate rheological characteristics. Therefore to mimic professional use and to get desired semisolid consistency aqueous T-LN dispersions were incorporated into gels. The gel forming polymer, Carbopol 980 (0.5%, w/w) was incorporated in T-LN dispersions under continuous stirring. Triethanolamine was added to neutralize the dispersions and to obtain the desired semisolid consistency. Glycerol (5%, w/w) was incorporated in the gels as humectant.

2.4. Characterization of T-LN and gel

2.4.1. Measurement of physicochemical properties

The nanoparticulate dispersions and gels were characterized for color, odor, pH and stability. Tacrolimus, a 23-membered macrolide lactone with empirical formula $C_{44}H_{69}NO_{12}\cdot H_2O$ and molecular weight of 804.024 exhibits a strong UV absorption with λ_{max} at 210 nm. The drug content in the formulation was therefore quantified using High Performance Liquid Chromatography (HPLC) at 210 nm.

2.4.2. Particle size analysis

Particle size and polydispersity index (PI) of T-LN were measured by photon correlation spectroscopy (PCS) using Beckmen N5 Submicron Particle Size Analyzer (Beckmen, USA). All measurements were carried out at 20 °C under a fixed angle of 90° in disposable polystyrene cuvettes after appropriate dilution with bidistilled water. The values were the mean of three measurements.

Transmission electron microscopy (TEM) was performed to characterize the morphology and ultra structure of T-LN. One drop of nanoparticulate dispersion was placed on the copper grid coated with a thin film of carbon, dried for around 45 min, loaded in the transmission electron microscope Philips CM200 (Philips, Netherlands) and areas were scanned for observation of nanoparticles.

2.5. Entrapment efficiency (EE)

The amount of tacrolimus entrapped in the T-LN was determined after the free drug and the lipid were separated from the aqueous medium. Nanoparticle aggregation was achieved using 30% w/w aqueous tetrahydrofuran solution (Lee et al., 2007). The amount of free drug was detected in the supernatant and the amount of incorporated drug was determined as a result of the initial drug minus the free drug.

2.6. Thermal analysis

DSC measurements were carried out on DSC Q_{200} (TA instruments, USA) and an empty Standard aluminum pan was used as reference. Thermograms were obtained with a heating rate of 10.0 °C/min, over the temperature range of 30–200 °C.

2.7. Fourier Transform Infrared Spectroscopy (FT-IR) analysis

FT-IR was used to study the interaction between the drug and excipients (Viriyaroj and Ritthidej, 2006; Lee et al., 2007). The IR spectra of lipid, drug and T-LN after vacuum drying were obtained by the potassium bromide disc method using JASCO FT-IR–4100typeA instrument (Jasco, USA) with TGS detector. Polystyrene film (40 μm) with known spectra was used as the standard with scanning Wavelength Range of 4000–600 cm^{-1}.

2.8. Proton nuclear magnetic resonance spectroscopy (^1H NMR)

To investigate the arrangement of the components and the molecular environment of tacrolimus in the nanoparticles ^1H NMR experiments were performed using an Oxford NMR AS 400 spectrometer (Oxford NMR Instruments, UK) with superconducting magnet, operating at 400 MHz and 20 °C. An aliquot of aqueous nanodispersion was filled in a NMR-tube and accurately weighted quantities of deuterized water (for all aqueous samples) or chloroform-D1 (for measurement of solid lipid and bulk drug) were added for field login.

2.9. Stability of nanoparticles

The T-LN were subjected to stability studies as per ICH guidelines at four different storage conditions (viz. refrigeration, 25 ± 2 °C/60% RH ± 5% RH, 30 ± 2 °C/65% RH ± 5% RH and 40 ± 2 °C/75% RH ± 5% RH) over a period of 12 months in well-sealed glass vials. The samples were withdrawn at the end of 1, 2, 3, 6 and 12 months and studied for physical appearance, clarity, particle size and PI.

2.10. Rheological measurements

2.10.1. Viscosity

The viscoelastic properties of aqueous lipid nanodispersions were investigated using Brookfield Rheocalc® V 32 Rheometer (Brookfield, USA) LV model with CP40 spindle using cone and plate geometry. Data analysis was done with Brookfield Rheocalc® 2.010 Application Software.

The rheologic properties of the gels enriched with T-LN were studied by continuous shear investigations using Brookfield R/S-CPS Plus Rheometer (Brookfield, USA), with cone and plate geometry using spindle C25-2 DIN as the measuring system. The shear rate was increased in ascending order from 0 to 100 *D* [1/s] (up curve) and then decreased from 100 to 0 *D* [1/s] (down curve) and the resulting shear stress [Pa] was measured. The sample was equilibrated at 25 °C prior to each measurement. All measurements were made in triplicate.

2.10.2. Spreadability

The gels enriched with T-LN were characterized for spreadability which denotes the ease with which a formulation may be spread on the skin, using a wooden block and glass slide apparatus. A weight of 100 g was added to the pan and the time was noted for upper slide (movable) to separate completely from the fixed slide. Spreadability was then expressed as the ratio of time required for the upper plate to slide down (s) by weight of the sample (g) (Honary et al., 2007).

2.11. In vitro drug release studies

The Franz static diffusion cell system have been reported to be the most appropriate for characterizing skin deposition and penetration of drugs from formulations with very low drug permeation rates (Neubert and Wohlrab, 1990; Santoyo et al., 2002; Shahiwala and Misra, 2002; Souto et al., 2004; Manconi et al., 2006). *In vitro* drug release studies were performed on modified Franz diffusion cells as per the method reported by Jenning et al. using Cellulose nitrate membranes (0.1-μm pore diameter, Whatman GmbH,

Germany), soaked with isopropyl myristate to simulate lipophilic properties of the stratum corneum (SC) (Jenning et al., 2000b). Weighed quantity of gel (0.25 g) enriched with T-LN was applied to the donor compartment. At predetermined time intervals the fluid in the receptor chamber was sampled over a period of 48 h and analyzed using HPLC at 210 nm.

2.12. Skin permeation study through pig ear skin

Fresh pig ears were obtained from a local abattoir (Deonar, India). The ears were removed post-sacrifice before the carcass was exposed to the normal high-temperature cleaning procedure in order to ensure integrity of the skin barrier. Pig ear skin, after removing subcutaneous fat tissue, was mounted on the diffusion cells with the SC side facing upwards into the donor compartment and the dermal side facing downwards into the receptor compartment. The receptor medium was sampled at predetermined time intervals and analyzed for tacrolimus by HPLC. At the end of 48 h the excess formulation from the surface of the skin and the entire dosing area was collected and tacrolimus content in the skin was determined. The drug remained unabsorbed on the skin surface was also quantified.

2.13. Occlusive properties

2.13.1. In vitro occlusion test

In vitro occlusion test was carried out as per the method given by De Vringer (1997). Briefly, preweighed beakers of 50 ml capacity were filled with 25 ml of purified water, covered with standard laboratory paper filter (Whatman microfibre filters, 9.0 cm) and sealed properly. T-LN enriched gel, plain gel base (without T-LN) and reference each 250 mg were spread evenly on the filter paper surface and the beakers were stored at 32 °C and 60 ± 5% RH. At specified time intervals of 24 and 48 h the beakers were removed and their weights were recorded. Beakers covered with filter paper but without applied sample served as comparative standard. Percent water loss through the filter paper for all the beakers was quantified and occlusion factor F was calculated as per the formula given in Eq. (1).

The occlusion factor F was calculated using the following formula:

$$F = \left(\frac{A - B}{A}\right) \times 100 \quad (1)$$

where A is the water loss without sample and B is the water loss with sample.

2.13.2. In vivo skin hydration studies

The gels enriched with T-LN were applied to the shaved skin of albino rat. At the end of 24 h the animals were sacrificed and the skin was isolated, vertically sliced, and stained with hematoxylin and eosin. The slides were observed under optical microscope and thickness of SC was measured (Norlén et al., 1997). The skin hydrating potential (SHP) of the developed formulation was evaluated and compared with plain gel base and reference ointment.

2.14. In vivo skin retention studies in albino rat and quantification of drug in different skin strata

The weighed quantities of gels enriched with T-LN (250 mg) were applied on the depilated skin of albino rats. The animals were sacrificed after 24 h, the formulations were removed and the skin was collected. The SC layer from the skin was removed by stripping with an adhesive tape. The epidermis was separated from the dermis using heat separation technique (Loice et al., 2005) and the tacrolimus content in each of the three skin layers SC, epidermis and dermis was extracted and quantified.

2.15. Visualization of skin penetration in vivo using Confocal Laser Scanning Microscopy (CLSM) studies

The cutaneous uptake of T-LN was studied *in vivo* in albino rat using Confocal Laser Scanning Microscopy. The formulations labeled with nile red as the fluorescent marker were applied to the depilated skin for 24 h. At the end of exposure period the animals were humanely killed, the application area was collected and sliced (10 μm) using freeze microtome Micron HM 560 (Microm International, MI). Fluorescence pictures were taken (20× magnification) using Confocal Laser Scanning Microscope LSM 510 (Carl ZEISS, Germany) and Nile red fluorescence was recovered in the red band by exciting the specimens at 546 nm. These slices were subjected to both normal light and fluorescence microscopy. Fluorescence yield was quantified (Borgia et al., 2005) by image treatment software (Axiovision 3.1.3.1, ZEISS); the integration of pixel brightness values (arbitrary brightness values, ABU) giving the relative dye contents.

2.16. Skin irritation studies

The skin irritation studies were carried out using albino rabbits (Draize et al., 1944; OECD 404). Formalin was taken as positive control and plain gel was used as negative control in the study. The scores for erythema and edema were totaled for intact and abraded skin for all the rabbits at 24 and 72 h. Data reporting and grading of skin reactions was carried out as per OECD Guideline 404-for the testing of acute dermal irritation/corrosion and the primary irritation indices (PII) were calculated. Protopic® was evaluated as reference.

2.16.1. Data analysis

The data from various study groups were expressed as mean ± standard deviation (SD). The differences between the groups were examined using analysis of variance (ANOVA) employing Tukey multiple comparison post test. Mean differences with $p < 0.05$ were considered to be statistically significant.

3. Results and discussion

3.1. Preparation of T-LN and gels

3.1.1. Optimization of surfactant levels and homogenization parameters

T-LN were successfully prepared by melt homogenization method and optimized for surfactant combination as well as concentration. Preliminary batches were prepared and optimized for surfactant concentration using 3^2 factorial design which revealed a correlation between concentration of surfactant and SV. In order to select a suitable surfactant combination, Brij 721–Brij 72; Poloxamer 188–Plurol Oleique and Polysorbate 80–Sorbitan monooleate were used as hydrophilic and hydrophobic surfactant combinations respectively. The independent variables and their levels were selected based on the preliminary trials undertaken. The formulations were prepared at three concentration levels −1 (low), 0 (intermediate) and +1 (high) of both the surfactants. The SV values varied from 0.1 to 1. The trials revealed that lower surfactant concentrations were ineffective in producing lipid dispersions with acceptable physical stability for all the three surfactant combinations. It is evident from the RSM that as the concentration of stabilizers increase the dispersions tend to remain stable (Fig. 1A–C) and the highest SV values indicating high stability were obtained at high levels of both the surfactants. The lower concentrations of hydrophilic stabilizer were unable to produce stable lipid dispersions even with higher concentrations of hydrophobic stabilizers {−1, +1}, and the sedimentation was observed with low

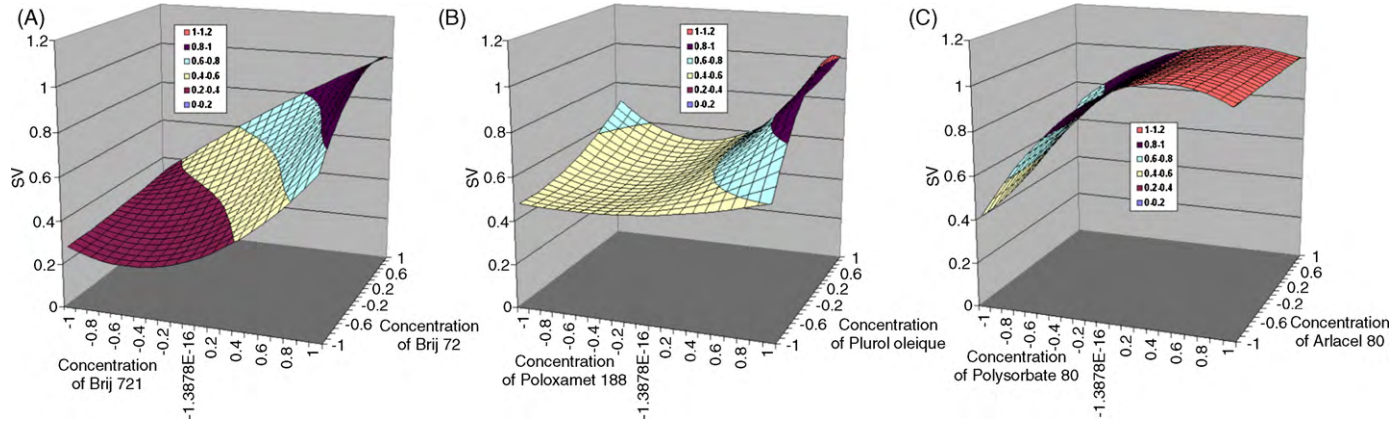

Fig. 1. Response surface plots for optimization of surfactant concentration; Brij 721–Brij 72 (A), poloxamer 188–plurol oleique (B) and polysorbate 80–sorbitan monooleate (C) as hydrophilic and hydrophobic surfactant combination.

values of sedimentation volume with all the three combinations. This could be attributed to the inadequate coating of the nanoparticles by the surfactants leading to coalescence and ultimately resulting in sedimentation of the lipid particles. The intermediate levels were ineffective in producing stable lipid dispersions in case of Brij 721–Brij 72 and Poloxamer 188–Plurol Oleique combinations and only higher levels of both the surfactants could produce stable lipid dispersions. Polysorbate 80 and Sorbitan monooleate were selected as the optimum surfactant combination for further evaluation because of their effectiveness at intermediate concentration and excellent stability provided as compared to other combinations.

The optimum homogenization parameters were determined by passing the preliminary emulsion at different homogenization pressures ranging from 800 to 1200 bars at 5, 8 and 10 homogenization cycles. The influence of homogenization pressure and cycle number on the particle size and its distribution (PI) is shown graphically in Fig. 2. The mean particle size and PI decreased with an increase in homogenization pressure and number of cycles. The decrease in particle size and PI is mainly because of the disruption of larger particles to the submicronic range due to the forces of high shear stress and cavitations acting on the lipid particles as the liquid is pushed through a narrow gap (in range of few microns) at a very high velocity. The particle size reduced when the homogenization pressure increased from 800 to 1200 bars. Similarly increasing the number of homogenization cycles from 5 to 10 resulted in decreased particle size. A similar observation has been reported by C. Schwarz et al with Dynasan 112 nanoparticles stabilized with Lipoid S75 where the number of particles >1 and >2 μm per fraction decreased with increasing homogenization pressure and cycle numbers (Schwarz et al., 1994). To establish which of the homogenization conditions, if any, were discriminatory, analysis of variance (ANOVA) was used to compare the results from different homogenization conditions. The ANOVA results indicated that there were significant differences between the particle size values that were obtained from the various homogenization conditions ($p < 0.05$) indicating the profound effect of homogenization parameters on the particle size and its distribution (PI). The optimum homogenization parameters resulting in low particle size and relatively narrow particle size distribution (PI = 0.141) were found to be 1200 bars at 10 cycles, and therefore were selected for constructing the T-LN formulation.

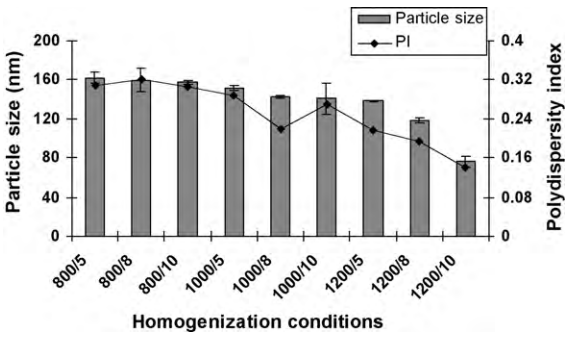

Fig. 2. Comparative profile showing effect of homogenization conditions on mean particle size and polydispersity index (PI).

3.2. Characterization of T-LN and gel

3.2.1. Physicochemical properties

The T-LN dispersions were translucent, odorless and stable after centrifugation at 2000 rpm for 30 min. Gels were white in color, odorless with pH in the range of 6.5–7. Drug assay showed 97–100% contents of stated amount.

3.2.2. Particle size and polydispersity index (PI)

PCS results proved the colloidal state of T-LN having particle size in the range of 20–150 nm with a mean particle size of 75.9 nm and a narrow size distribution with PI of 0.141. The TEM images of T-LN illustrated spherical shape of nanoparticles (Fig. 3) and confirmed the particle size to that obtained from PCS.

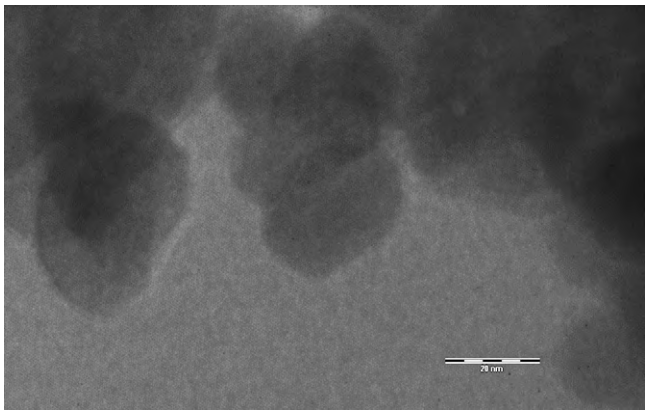

Fig. 3. The TEM imaging of T-LN.

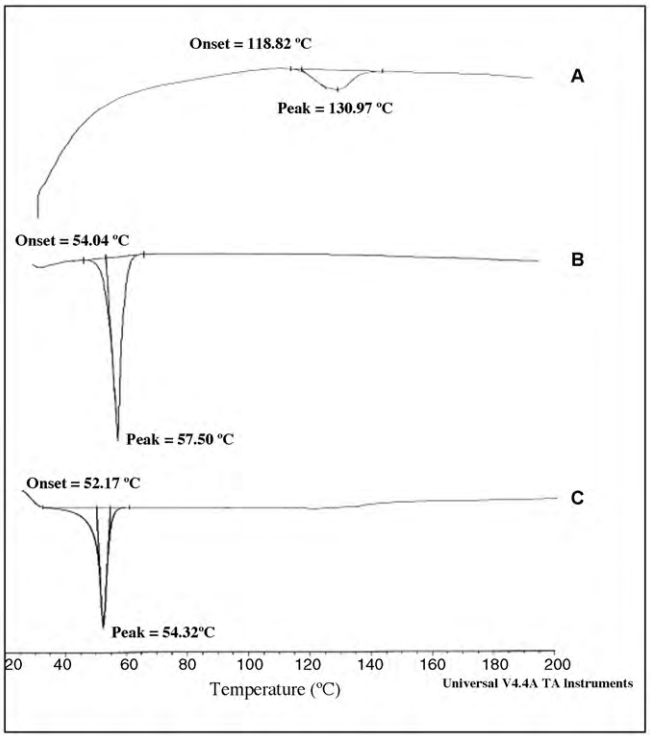

Fig. 4. DSC thermograms of pure tacrolimus (A), glyceryl trimyristate (B) and T-LN (C).

3.3. Entrapment efficiency (EE)

A high entrapment efficiency of 93.92% could be obtained with T-LN. Tacrolimus is practically insoluble in water and in hexane. The partition coefficient in *n*-octanol/water system is greater than 1000. Thus the lipophilic character of drug, good solubility in the selected lipid (data not given) and a relatively high concentration of lipid (5%, w/w) used could probably be responsible for this high EE.

Tacrolimus is a potent drug and its immuno-suppressive activity is 50–100 times higher than that of cyclosporine *in vitro* and 10–20 times *in vivo* respectively (Lauerma and Maibach, 1994). During preparation process, higher drug concentrations generally lead to supersaturation of drug in the melted lipid, causing the drug to precipitate from the superficial lipid matrix, adsorbed on the nanoparticle surface (ZurMühlen et al., 1998). Lower drug concentrations (0.1%, w/w) means more space for drug to get entrapped properly in the lipid imperfections which could probably be responsible for this high EE

3.4. Thermal analysis

DSC measurements were performed to study the crystallinity of drug as well as for the characterization of lipid matrix state for polymorphic modifications, if any. The DSC thermogram of tacrolimus showed endothermic peak corresponding to its melting point at 130.97 °C (Fig. 4A). DSC run of T-LN showed a complete absence of peak corresponding to tacrolimus indicating that tacrolimus was completely solubilized inside the lipid matrix. The bulk glyceryl trimyristate revealed peak at 57.50 °C (Fig. 4B). In comparison to the pure lipid, the DSC of T-LN showed lower onset temperature and peak temperature values (peak at 54.32 °C). Incorporation of tacrolimus inside the lipid matrix resulted in an increase in the number of defects in the lipid crystal lattice, hence causing a decrease in melting point of the lipid in the formulation

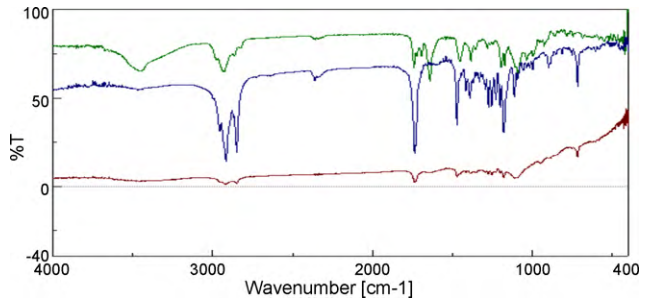

Fig. 5. Comparison of Fourier Transform Infrared Spectra; tacrolimus (upper); glyceryl trimyristate (middle) and T-LN (bottom).

(Fig. 4C). These results indicate that the lipid has changed from β modification (in the bulk) to the unstable α modification in the lipid nanoparticles. These observations are in agreement with Soukharev, who have made similar conclusions that the transformation of lipid bulk material into lipid nanoparticles leads to changes of the melting behavior of the lipid accompanied by potential occurrence of lower melting α and β_i modifications (Soukharev, 2007).

Also the melting behavior of lipid nanoparticles strongly depends on the particle size. A shift of the melting transition to lower temperatures with nanoparticles was observed, meaning that the smaller particles melt at lower temperatures. Reduction in particle size with lipid nanoparticles and subsequent increase in surface area leads to a decrease in melting enthalpy as compared to the heat flow through larger crystals. As the heat flow through larger crystals requires more time, decrease in melting point of lipid nanoparticles can be assigned to the colloidal dimensions of the nanoparticles (Hou et al., 2003; El-kamel et al., 2007; Al-Haj and Rasedee, 2009; Freitas and Muller, 1999; Hou et al., 2003).

3.5. Fourier Transform Infrared Spectroscopy (FT-IR)

Infrared (IR) spectroscopy is one of the most powerful spectroscopic tools used for identification of compounds. The combination of fundamental vibrations or rotations of various functional groups and the subtle interactions of these functional groups with other atoms of the molecule result in unique IR spectrum for each individual compound. Thus FT-IR represents potentially highly useful and sensitive technique of determining the identities of materials in multicomponent formulation (Beckette and Stenlake, 1988; Morrison and Boyd, 1992).

The FT-IR spectra of tacrolimus, glyceryl trimyristate bulk and T-LN are shown in Fig. 5. Tacrolimus bulk material (upper graph) showed the typical infrared absorption character at 3446.17, 2935.13 and 1639.2 cm^{-1} corresponding to –OH, –C–H alkanes and –C=C respectively, but the T-LN (lower graph) showed absence of these peaks. The infrared spectrum of T-LN showed superimposition to its parent lipid. Therefore it can be proposed that the matrix lipid glyceryl trimyristate successfully encapsulated the drug tacrolimus. Also no new peak was observed in the dispersion indicating no strong interaction and thus no incompatibility was observed in the T-LN formulation.

3.6. Proton nuclear magnetic resonance spectroscopy (^{1}H NMR)

Information about the environment and arrangement of the molecules could be derived from ^{1}H NMR studies (Fig. 6). Since T-LN dispersion contains no fractions of lipid, which are liquid at room temperature, only very weak and broad signals corresponding to the surfactants were detected in the T-LN dispersion as ^{1}H NMR gives spectra of chemical moieties which are in the solubilized

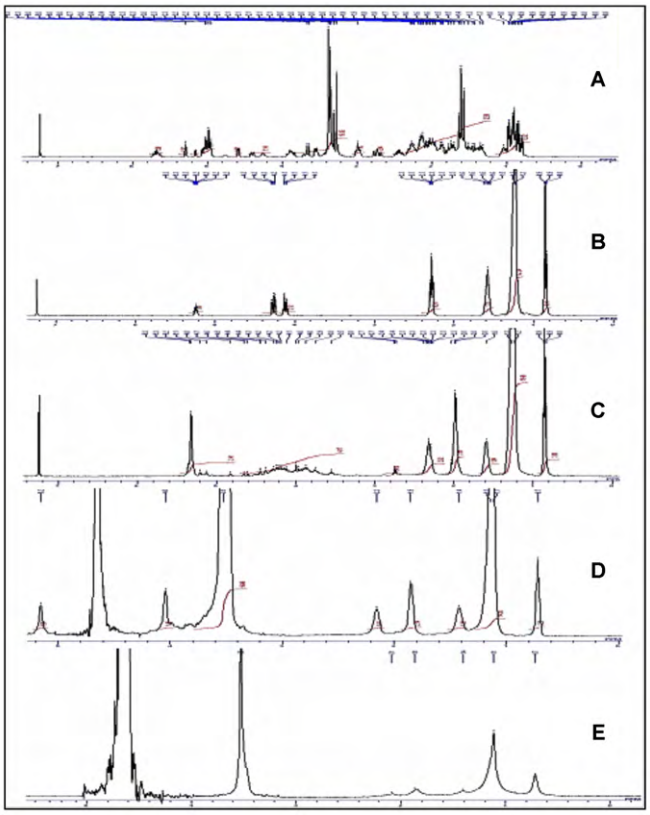

Fig. 6. Comparison of ^{1}H NMR spectra of T-LN with corresponding spectra of active ingredient tacrolimus (A), glyceryl trimyristate (B), sorbitan monooleate (C), polysorbate 80 (D) and T-LN (E).

form. In the T-LN, only surfactants (i.e. polysorbate 80 and sorbitan monooleate) derived broad signals were observed with no signals corresponding to the glyceryl trimyristate because of it being present in the solid state exhibiting very short relaxation times. Also most of the peaks of drug are not evident in the T-LN spectra suggesting complete encapsulation and immobilization of drug in the lipid matrix. Thus, these colloidal particles are practically completely solid excluding the phenomenon of supercooled melt. We attribute this observation to the crystallization of nanoparticles as described by Jores et al. (2003). The authors have reported similar results with ^{1}H NMR study of poloxamer stabilized glyceryl behenate (GB) SLN, where only poloxamer derived but no GB-related signals were observed.

3.7. Stability of T-LN

PCS mean diameters and the PI of T-LN after stability studies as per ICH guidelines are shown in Fig. 7. The dispersions stored at refrigeration condition remained stable with almost no change in particle size without any significant increase in PI values during the study period of 12 months. A gradual increase in particle size and PI values was observed with increasing temperature at accelerated storage conditions suggesting the particle size growth to be a function of storage temperature. On storage at 25 °C/60% RH, the particle size increased from 75.9 nm to 88.03, 127.9 and 296.5 nm and the PI changed from 0.141 to 0.321, 0.327 and 0.33 at the end of 3, 6 and 12 months respectively. However the particle size remained lower than 300 nm at 25 °C/60% RH during the study period. In contrast, a significant increase in particle size and PI was observed for T-LN stored at 30 °C/65% RH and 40 °C/75% RH. Mean particle size of T-LN stored at 30 °C/65% RH increased from 75.9 to 209 nm and PI from 0.141 to 0.45 at the end of 6 months and to 528 nm and 1.029 at the end of 12 months respectively. T-LN stored at 40 °C/75% RH showed drastic increase in particle size from 75.9 to 237.7 nm at the end of 3 months and reached micron range (1.045 μm) at the end of 6 months. ANOVA showed that there were significant differences between the particle size values obtained at various time points ($p < 0.05$) at 30/65% RH and 40 °C/75% RH with variation among the groups being significantly greater than expected by chance. Similar storage temperature dependent increase in particle size was observed by Murthy et al during their study of the stability of Olanzapine-loaded glyceryl tristearate SLN (Murthy et al., 2007).

3.8. Rheology

3.8.1. Viscosity

3.8.1.1. Rheological measurements of T-LN dispersions. The degree of dispersion and particle–particle association can be clearly seen from the rheology profiles. The lipid dispersions revealed very low viscosity of 0.39 cp with a yield value of practically zero (0.64). T-LN dispersions showed Newtonian flow behavior as implied by a straight line passing through the origin of shear rate versus shear stress (Fig. 8A). The viscosity was found to decrease with increasing shear stress (Fig. 8B). Yield value is representative of the force of flocculation. It is present because of the contacts between adjacent particles (brought about by van der Waals forces), which must be broken down before flow can occur. T-LN displayed very low yield value indicating that the nanoparticles in the dispersion are not flocculated demonstrating good stability. The low viscosity can also be contributed to the small particle size (75.9 nm) of lipid particles in the dispersion.

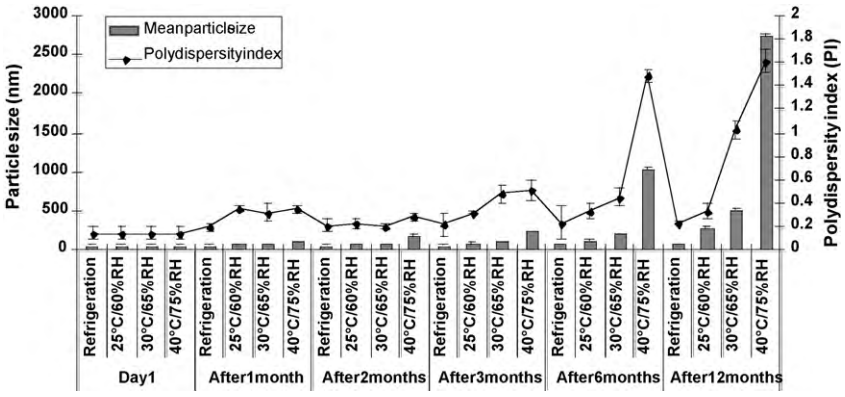

Fig. 7. Stability data of T-LN as per ICH guidelines indicating particle size (nm) and PI Data represent Mean ± SD, $n = 3$.

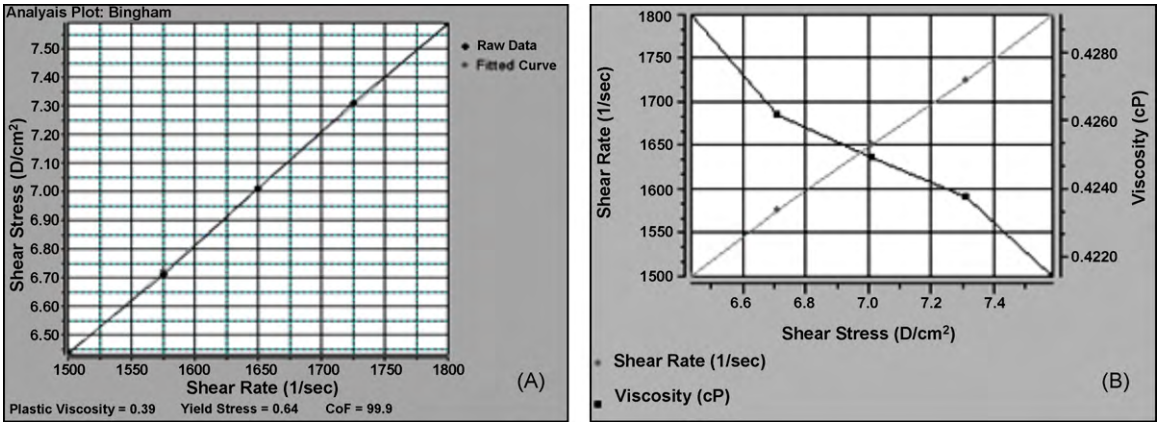

Fig. 8. Rheogram of T-LN dispersions showing correlation between shear stress and shear rate (A) and viscosity (B).

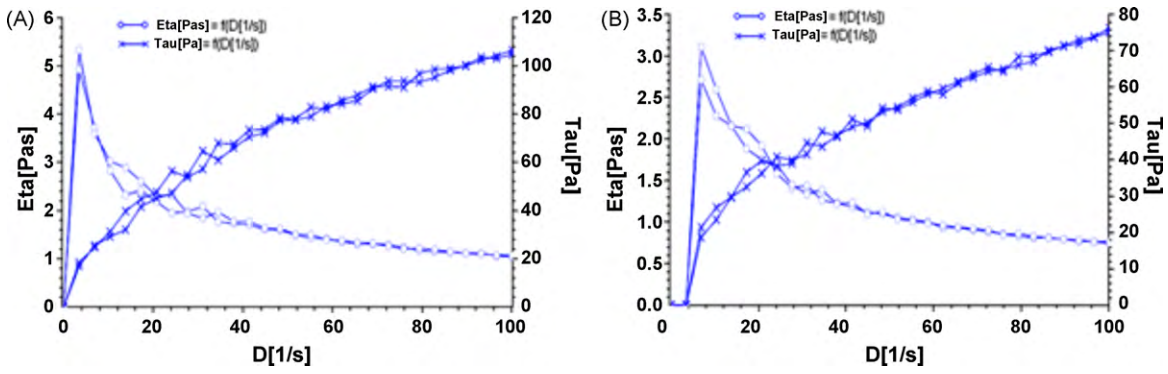

Fig. 9. Flow curves for gel enriched with T-LN (A) and plain gel base (B).

3.8.1.2. Rheological measurements of T-LN gels. Particle size is known to be an important rheological variable of dispersed systems. The greater mean size and the widest size distribution will preferentially show more viscous properties as compared to the formulations with lower particle size. The rheogram obtained by plotting shear rate D [1/s] versus shear stress τ [Pa] revealed no significant hysterises effects and up curve–down curve practically coincided under the considered experimental conditions (Fig. 9). The gel began to flow only after a shearing stress, corresponding to the yield value was exceeded, after which the viscosity decreased with increasing rate of shear indicating non-Newtonian system with pseudoplastic flow behavior. Due to smaller particle size of T-LN its incorporation resulted in only marginal increase in the viscosity of plain gel base (Table 1). By comparing the flow curves and apparent viscosities it was observed that T-LN based gels showed appropriate consistency intended for topical application and sufficient viscosity to adhere, self-immobilize and for easy application on the skin.

Table 1
Rheological measurements of gels enriched with T-LN.

Formulations	Spreadability (s/g)	Viscosity η [Pa s][a]
Gels enriched with T-LN	4.03 ± 0.472	1.052 ± 0.0153
Plain gel base	3.88 ± 0.396	0.7095 ± 0.0565

Values are given as mean ± standard deviation.
[a] Viscosity values at shear rate D [1/s] of 100 and 25 °C.

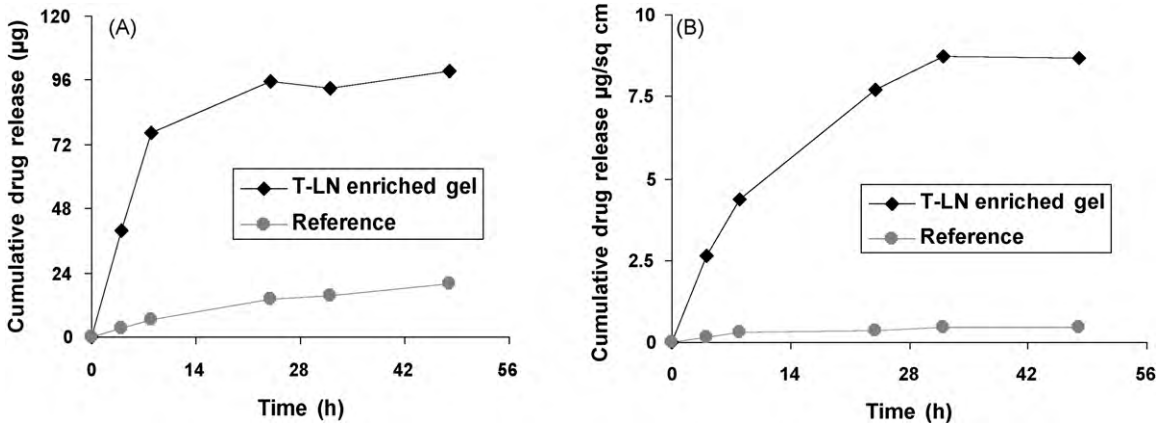

Fig. 10. *In vitro* drug release (A) and skin permeation (B) profiles.

3.8.2. Spreadability

The potential usefulness as a topical dosage form with desired semisolid consistency was demonstrated by spreadability values indicating the ease of application on the skin. The presence of lipid nanoparticles did not affect the spreadability of gel when compared with semisolid gel base devoid of LN (Table 1). This effect may be explained by the small particle size range and low PI values of T-LN contributing to low spreadability values suggesting easy spreading on the skin and good consistency.

3.9. In vitro drug release

In vitro drug release of T-LN was compared to marketed conventional ointment product Protopic® as reference. *In vitro* drug release of T-LN showed biphasic release pattern with initial burst release up to 8 h followed by sustained release up to 48 h (Muller et al., 2000; ZurMühlen et al., 1998). The drug release from T-LN was much higher and faster as compared to the reference at all the sampling points (Fig. 10A). The release of tacrolimus from T-LN gel was 11 times higher at the end of 8 h, 6 times higher at the end of 24 h and almost 5 times higher at the end of 48 h as compared to the reference ointment. The much higher drug release at the initial time points could be because of the burst release effect. The ointment showed much lower and very slow release over the period of 48 h.

3.10. In vitro skin permeation and accumulation of tacrolimus in pig ear skin

In order to assess the skin permeation and localization of tacrolimus from T-LN, the *in vitro* permeation ability through porcine skin was investigated (Fig. 10B). Tacrolimus permeation from T-LN was dramatically higher at all the sampling points as compared to the reference. The tacrolimus permeation rate was almost 16–21 times higher when compared with the reference which could be attributed to the higher drug release. The steady state flux (J_{ss}) was significantly higher for T-LN ($175.6 \pm 6.29\ ng\,cm^{-2}\,h^{-1}$) as compared to the reference ($8.8 \pm 2.23\ ng\,cm^{-2}\,h^{-1}$). The results seem to confirm that the burst release could be responsible for enhanced penetration of drug in the skin.

The comparative skin accumulation of drug from both the formulations is given in Fig. 11. T-LN showed significantly higher accumulation of drug in the skin ($25.85 \pm 5.41\ \mu g$). On the other hand reference showed almost negligible drug accumulation of tacrolimus ($0.039 \pm 1.78\ \mu g$) in the skin. It was observed that almost 93% of the applied dose remained unabsorbed on the skin surface in case of reference ointment in contrast to 47% with T-LN.

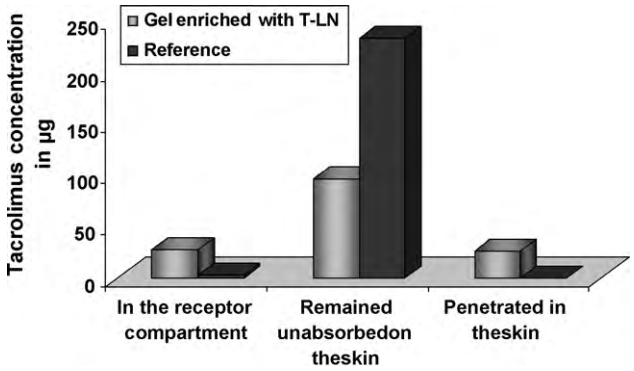

Fig. 11. Comparison of drug levels from *in vitro* skin penetration studies.

3.11. Occlusion properties

3.11.1. In vitro occlusion test

Occlusion is defined as the complete impairment of passive transepidermal water loss at the application site. The method as adapted by Vringer represents a rapid and less invasive technique to determine water loss through filter paper (simulating skin) *in vitro*. The study measures the occlusion of test formulation *in vitro* and compares with the plain gel base and reference ointment.

The beaker on which no formulation has been applied (standard) shows high water loss through the filter paper. While after application of the formulation the loss of water through the filter paper is dependent on the occlusive properties of the formulation. There was no significant difference in percent water loss through the filter paper for T-LN enriched gel and reference ointment. There was almost 1.58 times less percent water loss through the filter paper at the end of 24 h and 1.63 times less percent water loss at the end of 48 h for T-LN enriched gel as compared to the plain gel. The respective percent water loss values for reference ointment were 1.82 and 2.25 times less at the end of 24 and 48 h (Fig. 12A).

One- way ANOVA with Tukey-Kramer Multiple Comparison test showed that T-LN possessed almost similar occlusive property as that of reference ointment historically known for its occlusive properties, with no significant difference in the occlusion factors ($p > 0.05$). Incorporation of T-LN to plain gel base resulted in a significant increase in the occlusivity of plain gel ($p < 0.001$). The T-LN showed occlusion factor almost 2.69 times higher at the end of 24 h and 2.18 times higher at the end of 48 h, while reference ointment showed occlusion factor almost 3.33 and 2.70 times higher than plain gel base at the end of 24 and 48 h respectively (Fig. 12B).

T-LN was found to be superior in occlusive effect and that the addition of T-LN to a gel base enhanced gel's occlusivity without having undesirable stickiness or greasiness of conventional occlusive vehicle such as ointment. The reason for this enhanced occlusivity could be attributed to the adhesiveness of submicronic

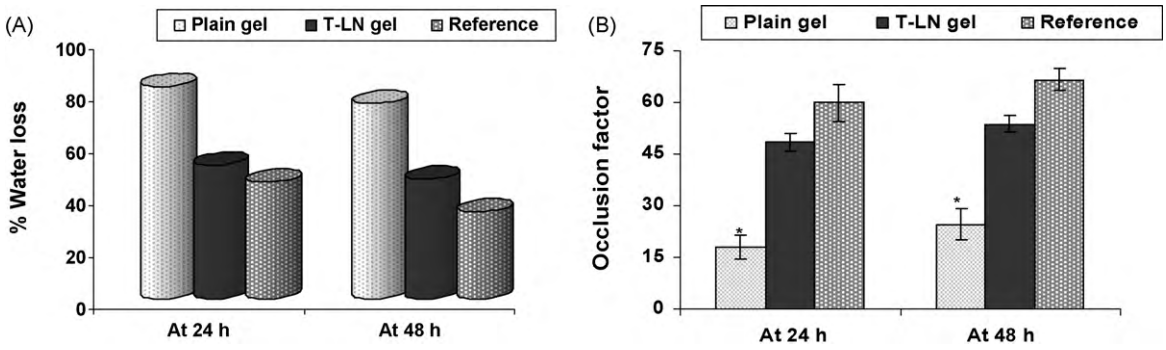

Fig. 12. Profiles showing comparative % water loss (A) and occlusion factor (B).

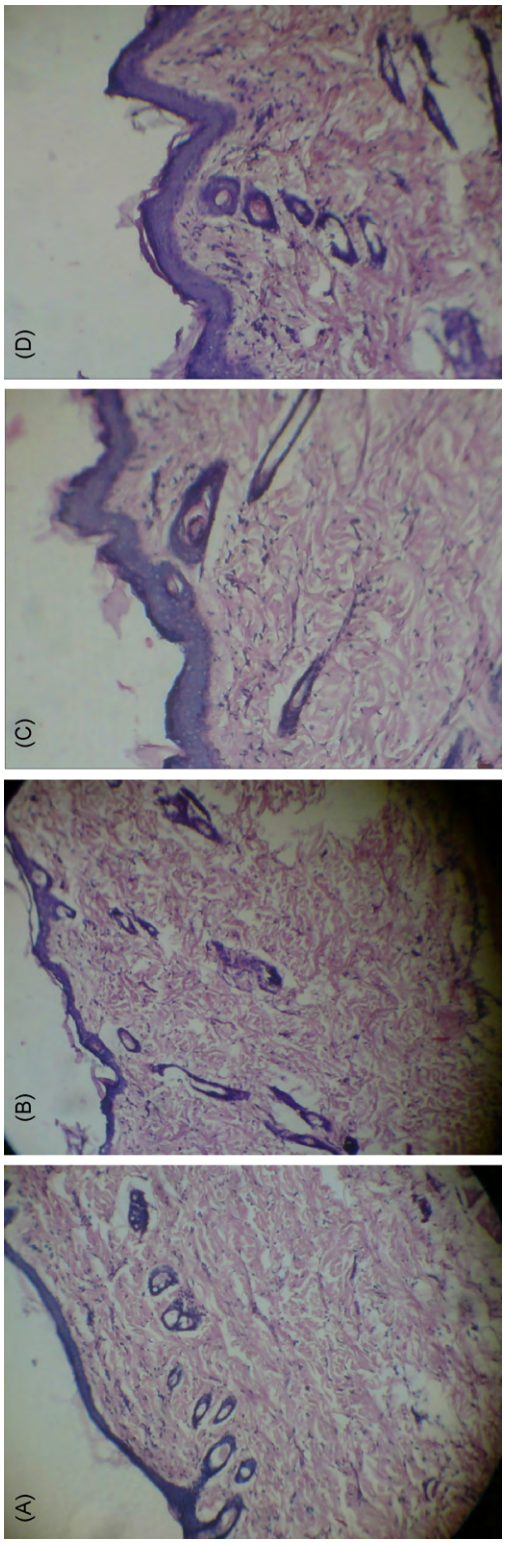

Fig. 13. Photomicrographs of rat skin showing skin hydrating potential. Untreated skin (A); skin treated with plain gel base (B); skin treated with gel enriched with T-LN (C) and skin treated with reference ointment (D).

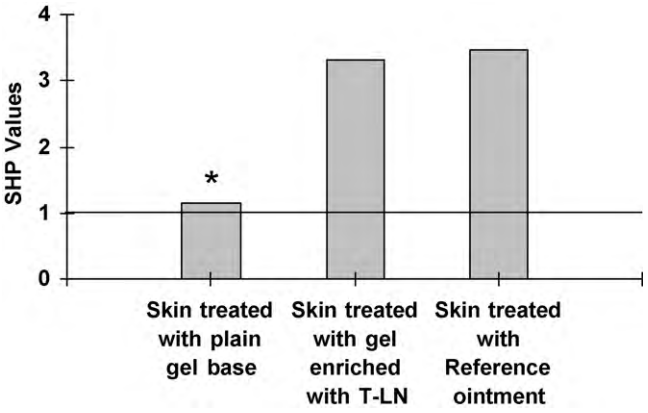

Fig. 14. Comparative skin hydrating potential (SHP) factor with respect to untreated rat skin. ($^{*}p < 0.05$). Untreated skin SHP factor (=1) is indicated by solid line.

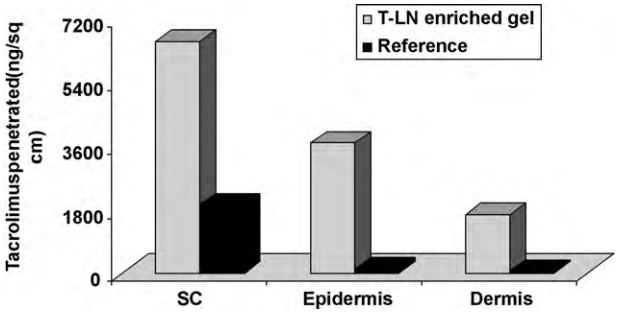

Fig. 15. *In vivo* skin retention of tacrolimus in different skin strata.

lipid particles leading to formation of coherent film on the skin surface after application (Souto et al., 2004). Skin hydration can be increased by occlusive topicals. It has been reported that lipid nanoparticles have distinct occlusive properties *in vitro* depending on their size, crystalline status and lipid concentration (Wissing et al., 2001; Wissing and Muller, 2002). This is because of the fact that after topical application, such lipid particles form an intact film due to adhesiveness thus decreasing water evaporation from the skin to the atmosphere.

3.11.2. *In vivo skin hydration*

Occlusive effects were further confirmed by *in vivo* skin hydration study in albino rats.

The upper layer in the photomicrographs (Fig. 13) represents the top layer i.e., SC of the skin. There was no significant difference in the SC thickness of rat skin treated with T-LN enriched gel and reference ointment ($p > 0.05$).

The application of plain gel base showed only slight change in the thickness of the SC (Fig. 13A and B), while application of the gel enriched with T-LN showed a significant increase in the thickness of SC, almost 2.9 fold as compared with the plain gel and 3.32 fold compared with the untreated rat skin (Fig. 13C). The reference ointment showed almost 3.03 fold increase in thickness of the SC as compared with the plain gel and 3.47 fold as compared with the untreated rat skin (Fig. 13D).

Skins hydrating potential (SHP) values are given as relative to the untreated rat skin (Fig. 14). One way ANOVA showed almost similar SHP values for gels enriched with T-LN (SHP = 3.32) as that of reference ointment (SHP = 3.47) ($p > 0.05$). Skin hydration was greatly enhanced by incorporation of T-LN to plain gel base with SHP of 1.14. This increase in SC thickness could be caused by increased water content in the SC on application of T-LN enriched gel.

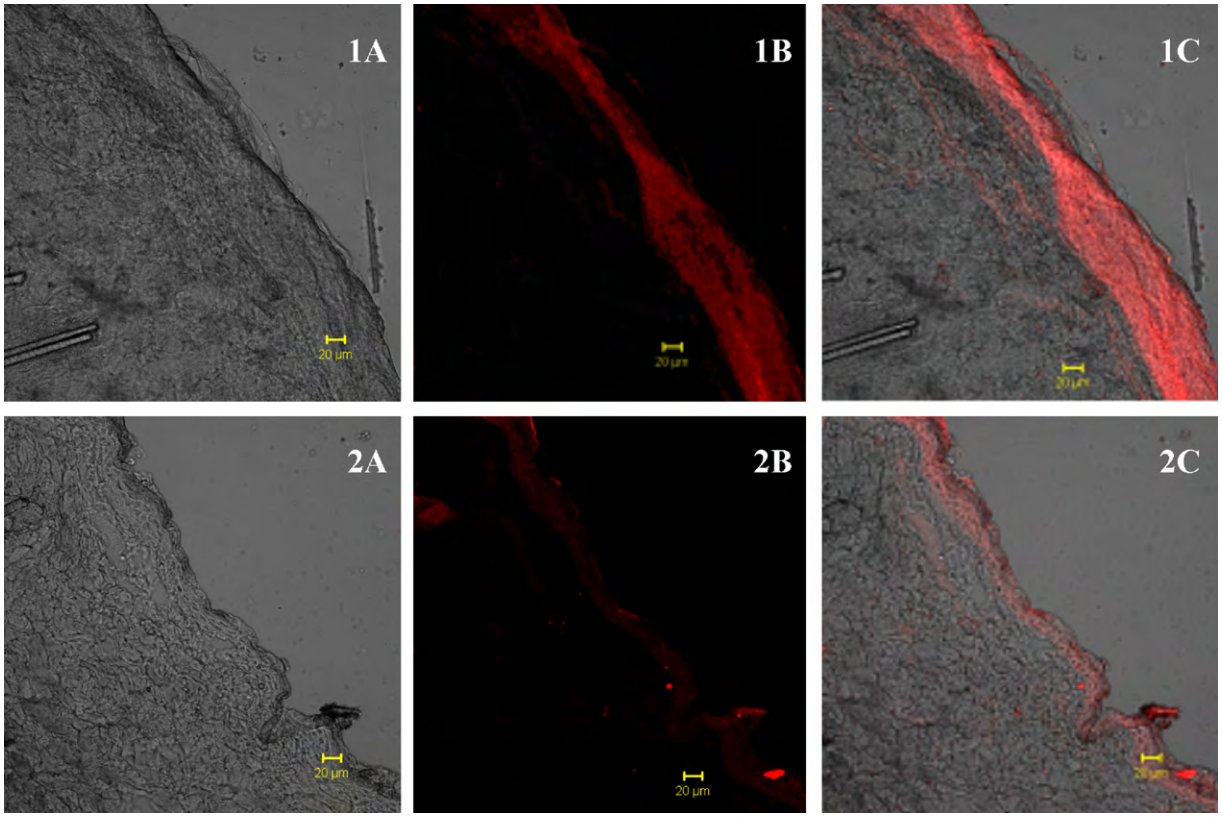

Fig. 16. Confocal laser scanning microscopy images showing cutaneous uptake and distribution of nanoparticles in the skin; bright field (A), detection of nile red labeled nanoparticles (B) and overlap of these two images (C); 1 denotes skin treated with T-LN and 2: reference.

It has been reported that occlusion favors drug penetration into the skin (Jenning et al., 2000a). In the present study both the gels enriched with T-LN and reference ointment showed highly occlusive properties. However in spite of similar occlusion factors, a significantly higher drug release and skin permeating properties with increased skin accumulation has been observed for T-LN as compared to the reference ointment. Thus earlier reported occlusion may not be the only reason for increased penetration of drug into the skin.

3.12. *In vivo skin retention studies and quantification of drug in different skin strata*

As during *in vitro* skin penetration studies T-LN demonstrated significantly high drug accumulation in skin, *in vivo* skin retention studies were performed in rats to further understand the exact localization of drug in various skin layers. Gels enriched with T-LN clearly promoted the penetration of tacrolimus as compared to the reference ($p < 0.05$). The SC levels of T-LN were 3.36 times higher while epidermal and dermal levels were 30.81 and 28.68 times higher as compared to the corresponding reference (Fig. 15). The studies revealed that with reference ointment most of the drug remained in the SC, which is not the target site for drug action while in case of T-LN sufficient drug levels could reach deeper skin layers.

The inflammatory dendritic cells responsible for immunopathogenesis of AD mainly reside deep in the skin (Ruzicka et al., 1999). As stated earlier tacrolimus inhibits cytokine and chemokine release from activated dendritic cells by binding to intracellular protein—FKBP, ultimately leading to inhibition of phosphatase activity of calcineurin which could initiate formation of various inflammatory mediators. Thus for effective treatment of AD, tacrolimus applied topically must penetrate into the deeper layers of the skin such as epidermis and mainly dermis. The present study demonstrated that T-LN targeted and reached the target site i.e. the deeper skin layers more efficiently than the reference. Thus T-LN is a better carrier for the cutaneous delivery of tacrolimus than the conventional ointment with much higher amounts of drug accumulating at the target site in the skin.

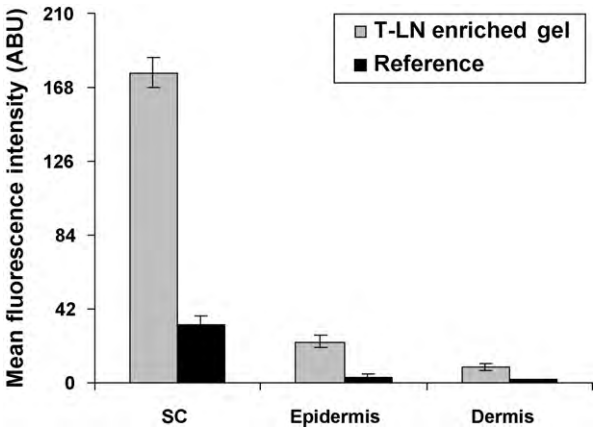

Fig. 17. Comparative cellular uptake of fluorescence by T-LN and reference using confocal laser scanning microscopy.

3.13. *Visualization of skin penetration in vivo using Confocal Laser Scanning Microscopy studies*

Fig. 16 shows the cutaneous uptake and distribution of nanoparticles in the skin. In this figure, A shows bright field, B shows the detection of nile red (Red channel), and part C is the overlay of these two images. With both the formulations, the highest fluorescence intensity was observed in SC, with fading of fluorescence towards the deeper skin layers. The visualization qualitatively indicates the trends of fluorescent staining in the various skin strata. T-LN clearly demonstrated deeper penetration of the florescent marker as compared to the reference ointment. In confirmation to the *in vivo* retention studies, CLSM also demonstrated higher penetration of fluorescent marker to various strata of skin (in the order of 5–6 times) than reference reflecting an improved capacity of T-LN to deliver the drug to the target site (Fig. 17).

3.14. *Skin irritation studies*

AD is characterized by itchy, erythematous and intensely pruritic rash ultimately leading to dry skin conditions. Tacrolimus

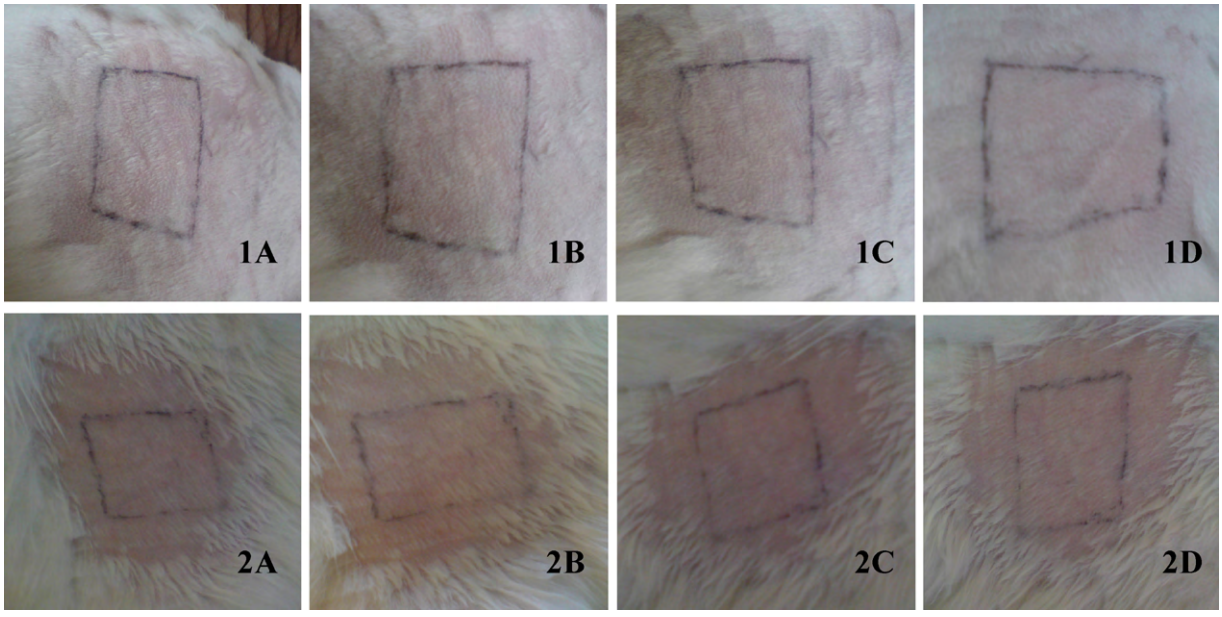

Fig. 18. Primary skin irritation photographs of rabbit skin; 1 denotes skin treated with T-LN and 2: reference; intact (A) and abraded (B) after 24 h; intact (C) and abraded (D) after 72 h.

used for treatment of AD itself is associated with skin irritation as the major side effect. The currently marketed product has shown side effects like itching and burning sensation at the site of application. Therefore the use of tacrolimus topically can be uneasy to the patients with already compromised skin conditions. Thus reducing this drug induced irritation can lessen itching, minimize the number of flares and improve the skin appearance. The entrapment of tacrolimus in lipid nanoparticles would avoid direct contact of the drug with skin, alleviating the drug related local side effects.

Skin irritation study in rabbit showed signs of irritation with reference ointment. The irritation was found to be mild on intact skin while the ointment displayed moderate skin irritation on abraded area with PII 3.33 (Grading as per OECD Guideline 404). In none of the cases, edema was seen. The itching of the treated area after the exposure might be responsible for this observation. T-LN enriched gel showed no erythema or edema on the intact and abraded rabbit skin with PII 0.0 (Fig. 18). The encapsulation of tacrolimus in lipid nanoparticles might be responsible for this improved safety. Thus T-LN would prove to be highly beneficial over conventional product with reduced skin irritation.

4. Conclusion

The tacrolimus loaded lipid nanoparticles were optimized and prepared successfully by hot high pressure homogenization technique with good entrapment efficiency and stability. Differential scanning calorimetry showed that the majority of T-LN possessed less ordered arrangements of crystals than the corresponding bulk lipid, which was favorable for increasing the drug loading capacity. From FT-IR and ^{1}H NMR studies it could be concluded that there were no incompatibility and the drug remained in the dissolved state encapsulated in the nanoparticles. Lipid nanodispersions showed very low viscosity and yield value of practically zero indicating that the nanoparticles in the dispersion are not flocculated. Rheological evaluation of gels enriched with T-LN showed appropriate consistency and viscosity to adhere and self-immobilize for easy application to the skin. In spite of similar occlusive properties and skin hydrating potential, higher drug accumulation with a significant skin targeting potential of T-LN has been demonstrated by *in vitro* skin permeation and *in vivo* skin accumulation studies as compared to reference ointment. Therefore the earlier reported occlusion may not be the only reason responsible for higher skin penetration. The targeting potential of T-LN was further confirmed *in vivo* by skin localization studies in various skin layers by direct quantification and dermal uptake studies using CLSM. T-LN formulation showed significantly improved penetration to the deeper skin layers and effectively reached the target site for AD. It showed no skin irritation with significantly improved safety over the marketed product. Thus T-LN based formulation not only showed superior performance and better safety but would be more appealing with better patient compliance specially to treat large skin areas of AD with no visible or palpable residue when applied to the skin as compared to the ointment.

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